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Primary structure-based function characterization of BRCT domain replicates in BRCA1 ☆

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Abstract

BRCA1 is a large protein that exhibits a multiplicity of functions in its apparent role in DNA repair. Certain mutations of BRCA1 are known to have exceptionally high penetrance with respect to familial breast and ovarian cancers. The structures of the N-terminus and C-terminus of the protein have been determined. The C-terminus unit consists of two α-β-α domains designated BRCT. We predicated two homologous BRCT regions in the BRCA1 internal region, and subsequently produced and purified these protein domains. Both recombinant domains show significant self-association capabilities as well as a preferential tendency to interact with each other. These results suggest a possible regulatory mechanism for BRCA1 function. We have demonstrated p53-binding activity by an additional region, and confirmed previous results showing that two regions of BRCA1 protein bind p53 in vitro. Based on sequence analysis, we predict five p53-binding sites. Our comparison of binding by wild-type and mutant domains indicates the sequence specificity of BRCA1–p53 interaction.

Keywords: Breast cancer; BRCA1; p53; Domain recognition; Psi-BLAST; Homology

Human breast cancer susceptibility gene 1 product, BRCA1, is one of the largest human proteins, containing 1863 amino acids. Although the majority of breast cancer cases occur sporadically, 5–10% of cases are caused by inherited mutations in the breast cancer susceptibility genes *BRCA1* and *BRCA2*. Mutations in *BRCA1* and *BRCA2* indicate probabilities of breast cancer by age 70 of 45–87% and 26–84%, respectively, making these the strongest predictors of breast cancer known [1–6]. Although a large number of the more than 1500 currently documented mutations of BRCA1 lead to premature stoppage of translation or a frameshift, approximately 500 mutations are unclassified missense mutations. The potential significance of the vast majority of these mutations is unknown [7,8].

BRCA1 is involved in a variety of biochemical processes. Although the BRCA1 protein has no overall compelling sequence similarity to proteins with known functions, it does contain two domains that are similar to known motifs and numerous regions that are functionally identified as binding regions for other proteins. A RING finger domain, located near the N-terminus of BRCA1, is a zinc-binding domain that mediates protein–protein and protein–DNA interactions [9,10]. Several point mutations associated with

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breast cancer are found in the BRCA1 RING domain. The BRCA1 carboxy terminal domain (BRCT) consists of a tandem duplication of approximately 95 amino acids. BRCT domains are loosely conserved in other proteins from a variety of organisms including 53BP1, RAD9, XRCC1, RAD4, Ect2, REV1, Crb2, RAP1, TdT, CPB11, NBS1, and three eukaryotic DNA ligases [11,12]. Most of these proteins are known to play roles in the checkpoints associated with DNA damage and repair. The BRCT domain in BRCA1 is the site of several reported missense and non-sense mutations associated with breast cancer, suggesting its functional relevance to tumor suppression.

In the process of characterizing the physiological roles of BRCA1, several laboratories have identified proteins that interact physically and functionally with BRCA1. Not surprisingly, many of these factors are themselves important players in cancer development. BRCA1 has been shown to interact with p53, a transcription factor stimulated by DNA damage that participates in both checkpoint-mediated cell cycle arrest and apoptosis. The transcription factor p53 is encoded by *TP53*, a tumor suppressor gene that is deleted or mutated in about 45% of human breast cancers [13–16]. The ability of two BRCA1 regions to bind p53 has been previously reported, but the critical residues involved in the interaction were not identified [17–19]. Other studies suggest that BRCA1 may play tightly regulated, convergent roles in transcription, regulating not only p53 transcriptional activity but also cell cycle progression, by promoting p53-independent expression of $p21^{WAF1/CIP1}$ [17,20,21]. Overall, it appears that BRCA proteins may play roles in the regulation of transcription, histone acetylation, DNA damage repair, checkpoint response, and possibly meiotic recombination [18].

In this report, we describe the development of recombinant expression constructs for the production of two apparent evolutionary homologues of the BRCT domain of BRCA1. Circular dichroism (CD) spectra of the homologous domains indicate they are largely unstructured, in contrast to the well-structured BRCT domain, despite an inferred common evolutionary origin. These conformational differences are consistent with differences in function. Intramolecular interactions may be among one of the novel functions of the homologous domains. This interaction capability provides a basis for possible regulation of BRCA1 functions via these domains, and suggests that many mutations observed in these domains could increase cancer risk by altering the intramolecular interaction. These homologous domains as well as the BRCT domain are able to interact with p53 in a sequence-specific manner.

Materials and methods

Predication of BRCA1 domains. Psi-BLAST [22] searches of the NCBI non-redundant database were performed on the publicly available server (http://www.ncbi.nlm.nih.gov/BLAST/), with default parameters except for "descriptions" and "alignments," which were set to 1000. Only three rounds of Psi-BLAST were used to restrict potential matches to close evolutionary orthologues [23,24]. In some searches, segments of 400 amino acids in length were used rather than the complete protein. This approach, which we have nicknamed "Sli-Psi," uses a sliding window containing a shorter segment of the protein sequence to search for homologues. Regions of BRCA1 for which BLAST was unable to find homologues were analyzed manually for similarity to its known RING domain and to the RING-like domains that were identified by Sli-Psi.

DNA cloning and protein production. The specified protein domain coding regions of *BRCA1* were PCR amplified using a full-length *BRCA1* cDNA clone (provided by B.L. Weber) as a template and the required primer pairs. The resulting PCR fragments were cloned into pMCSG7 using ligation-independent cloning methods as described [25]. The resulting constructs, designed p0312, p0096, and p0221, encode CT1 (region: 1636–1858), CT2 (1376–1618), and CT3 (288–479), respectively. CT1 includes the BRCT domain; CT2 and CT3 are BRCT homologous domains.

For protein domain production and purification, *Escherichia coli* BL21-Gold (DE3) (Stratagene) harboring a CT domain-expressing plasmid was used. CT protein domains were induced and purified using Ni–NTA affinity technology (Qiagen), as instructed by the manufacturer.

The expression vector encoding the p53 DBD domain (residues 94–312) was transformed into *E. coli* BL21-Gold (DE3). The protein was induced and purified as described [26].

To generate alanine substitution constructs, the QuikChange site-directed mutagenesis kit II (Stratagene) was used according to the manufacturer's instructions, using required primer pairs and either p0312, p0096, or p0221 as templates. The resulting constructs, after DNA sequence confirmation, were used to produce CT domain mutants as described above.

All the purified proteins were dialyzed at 4 °C against buffer (0.25 M NaCl, 1 mM PMSF, and 50 mM Tris–HCl, pH 8.0) containing 20% glycerol, 1 mM DTT, and stored at −80 °C.

Secondary structure analysis by CD spectra. CD spectra in the far UV region (185–260 nm) were recorded on a Jasco J-810 device (Jasco Inc.), using a cell with a 1-mm light path with a Jasco PFD-4255 thermo-controller (Jasco Inc.) set at 25 °C. All samples (0.2 mg/ml) were dialyzed against 10 mM sodium phosphate buffer (pH 7.0) at 4 °C, and the dialysate was used as the background sample. The measurements were repeated five times, and the readings were averaged and plotted. The deposition analysis of CD spectra was conducted by using the CONTIN program from DichroWeb (http://www.cryst.bbk.ac.uk/cdweb/html/home.html) [27,28].

Size-exclusion chromatography. Size-exclusion chromatography was performed as described [29]. The running buffer was 10 mM Tris–HCl, 100 mM NaCl, pH 7.25. The flow rate was 0.06 mL/min, and a 50 μl sample was injected into a 5 μl sample loop for sample introduction.

Solid phase binding assay. An ELISA-based assay was used to test CTx domain binding ability against p53. Recombinant CTx domain was coated on plates (Nunc-Immuno plate, Nalge Nunc International) at indicated concentrations in phosphate-buffered saline (PBS). After blocking with 1% BSA in PBS, plates were incubated for 2 h with 40 nM p53 in PBS containing 0.5% Tween 20, 0.1% BSA (PBS-TB). After washing, the plates were incubated for 2 h with an anti-p53 antibody (Pab240, Abcam) in PBS-TB. Finally, the plates were incubated for 2 h with an anti-mouse IgG-HRP (Bio-Rad) in PBS-TB. The HRP substrate used was 1-step turbo TMB (Pierce) for detection.

Results

Proposed structure map of BRCA1

We propose that BRCA1 evolved by replications of a RING-like domain and by replications of a BRCT-like domain. From N-terminus to C-terminus, the RING-like domains are designated R1 through R7, while the BRCT-like domains are termed CT1 through CT3 (Fig. 1). Although the organizational map depicted in Fig. 1

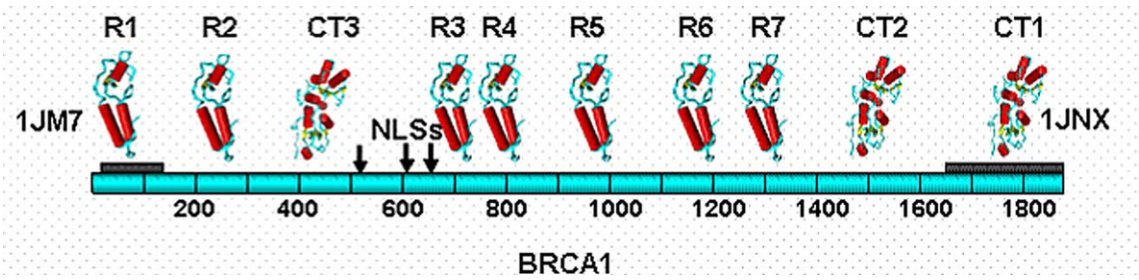

Fig. 1. Hypothetical domain map of human BRCA1. The structures of the N-terminal and C-terminal domains have been determined (PDB: 1JM7 and 1JNX, respectively). Images of these structures are used to visually indicate the approximate locations of domains that appear to have an evolutionary relationship with the terminal domains; however, no conformational prediction is implied. For clarity, an additional RING-related domain may exist in the region containing nuclear localization signals (NLSs).

designates the homologous regions with images of the known structures of the N- and C-termini, no prediction of 3D structure is implied. The perceived evolutionary relationships, however, serve as a guide for cloning and expression of the constituent units. The work presented here focuses on characterization of two predicated replicates of the BRCT domain (CT2 and CT3) with comparison to the BRCT domain (CT1).

Structure of CT protein domains

The secondary structure of the CT domains was examined by CD spectroscopy (Fig. 2). The CD spectrum of CT1 (represented by the dotted line in Fig. 2) is consistent with previously published data [30] and indicates the presence of comparable contributions of α-helix and β-strand (α-helix/β-strand/other, 43%/27%/30%). In contrast, the spectra of CT2 and CT3 (dashed line and solid line in Fig. 2, respectively) are distinct from that of CT1, suggesting that little α structure is present (α-helix/β-strand/random, 16%/28%/56%; 16%/31%/53%, CT2 and CT3, respectively). The CD spectra of CT2 and CT3 are consistent with those from disordered proteins [31–33]. Although CT2 and CT3, like CT1, bind p53 (below), the apparent distinct conformation suggests the evolution of at least one additional function, which remains to be determined.

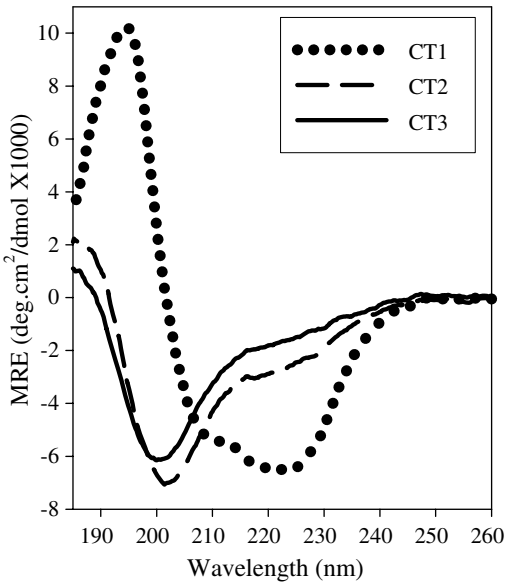

Fig. 2. Secondary structure of CT protein domains. CD spectra of CT1 (dotted line), CT2 (dashed line), and CT3 (solid line). All measurements were carried out at 25 °C in 10 mM sodium phosphate buffer (pH 7.0).

The CD spectrum of CT3 is similar to that of CT2 and is consistent with the CD data of Sturdy et al. [31] who expressed a somewhat larger region (230–534) that encompasses CT3. Thus, it would appear that CT2 and CT3 have evolved different conformations as interior region domains. This would appear to be consistent with the evolution of different functions. Whereas the CD spectrum of CT1 indicates that it is significantly α helical (43%, Fig. 2), consistent with its known structure, the computed α helical content of CT2 and CT3 indicates much lower contributions (16% and 16%, CT2 and CT3, respectively, Fig. 2). Both CT2 and CT3 appear to have substantial contributions to their conformations from β-strands (28% and 31%, CT2 and CT3, respectively, Fig. 2). Both visual inspection of Fig. 2 and the deposition analysis of the spectra indicate that the conformations of CT2 and CT3 are likely to be similar, but not identical, to each other.

Restoration of partial structure

It has been shown that certain unstructured proteins, e.g., androgen receptor [34] and glucocorticoid receptor [35], have α-helix propensity for apparently unstructured regions. We monitored secondary structural changes by CD spectra of CT2 and CT3 in the presence of the organic solvent trifluoroethanol (TFE) (Fig. 3). Addition of trifluoroethanol (TFE) apparently induces the formation of structure, dominated by α-helix formation. In 0% TFE, CT2 appeared to consist of 16% α-helix, 28% β-strand, and 56% other, while in 80% TFE the values were 60% α-helix, 10% β-strand, and 29% other. Under similar condition, CT3 shifted from 16%/31%/53% (in the absence of TFE) to 54%/13%/33% (in 80% TFE).

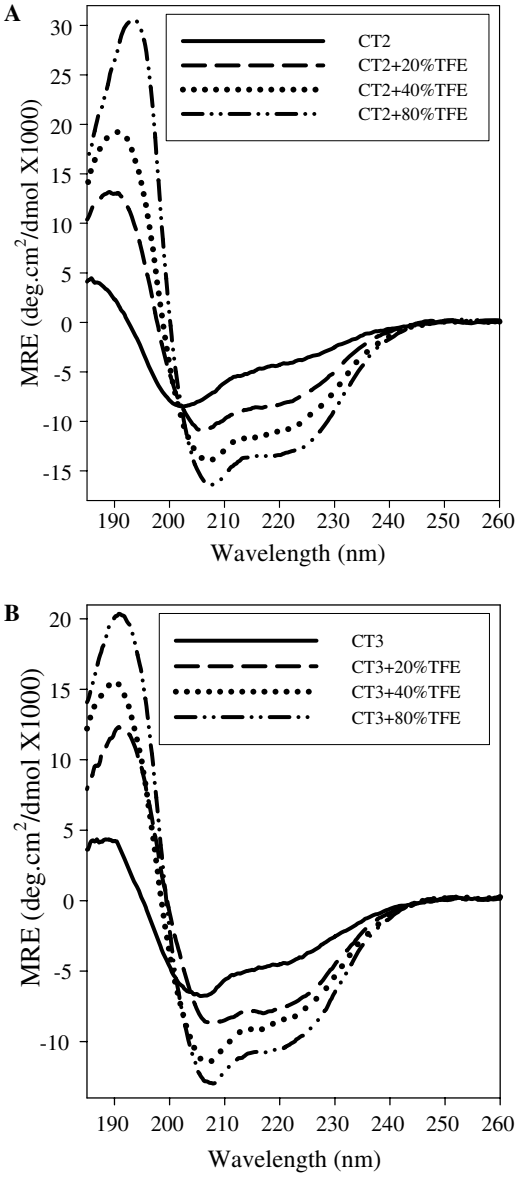

Fig. 3. Effect of TFE on the CT2 (A) and CT3 (B) structure. Protein samples were mixed with TFE at concentrations as indicated for 2 h before measurements.

The substantial increase in helical content in the presence of a denaturant, TFE, suggests the possibility that structure may be acquired in vivo upon interaction with other proteins, interaction with unknown ligands, or post-translational modification not found in the *E. coli* production systems used to generate recombinant fragments of BRCA1. It is also possible that the lack of structure is a consequence of the protein being expressed in vitro as fragments that are unable to interact with unidentified partners in other regions of the protein. For instance, the interaction we observe between CT2 and CT3 could be interpreted as supportive of this phenomenon. However, Mark et al. [32] analyzed the BRCA1 primary structure with well-established algorithms that predicted disorder. The success of the Arrowsmith laboratory [32] in generating soluble fragments by avoiding predicted regions of secondary structure, and our laboratory, as guided by regions of apparent homology to defined domains, also argues for a general lack of significant globular domain formation.

Interactions of CT domains

To further address the solution status of purified domains, we conducted size-exclusion chromatography of CT1, CT2, and CT3 (Fig. 4). CT1 (the dotted line in Fig. 4A) elutes in an asymmetric profile at an apparent molecular weight position of approximately 30 kDa, suggesting dynamic self-association of the 26 kDa (calculated: 25519.79 Da) monomer during elution [36,37]. CT2 (the solid line in Fig. 4A) elutes at a position corresponding to an oligomer of possibly octameric or higher order. CT3 (the dashed line in Fig. 4A) exhibits a bimodal distribution profile at high concentrations, with a symmetric peak and a continuum of protein ending in a peak at a position essentially corresponding to a monomer. At a tenfold dilution, the dimer position is essentially unchanged, while a larger fraction of material elutes as a monomer, suggesting slow monomer-dimer equilibrium (not shown).

Self-association of CT3 is sensitive to temperature, with an apparent approximate tenfold change in effective affinity given a decrease of 10 °C (Fig. 4B). This implies that association of the CT3 domains is accompanied by an increase in entropy. Potential sources of decreased entropy include immobilization of CT3 sidechains involved in the interaction, and restricted mobility of the N- and C-portions with respect to each other. The possibility of a temperature-dependent conformational change in CT3 cannot be eliminated at this point, although CD analysis shows little or no change from its unstructured motif (data not shown). If the temperature-dependent affinity does result from significantly increased entropy upon association, then it is likely that the dimerization at physiological temperature is much more extensive than observed at 20 °C (Fig. 4B). The weak dimerization of CT1 was not significantly diminished at 10 °C nor was a major effect on CT2 self-association observed (data not shown).

It is possible that the observed self-association of the CT domains might be a secondary consequence of an interface intended for hetero-associations with other interaction partners. Unexpectedly, we have found that CT2 and CT3 might be interaction regions located within BRCA1. As shown in Fig. 4C, CT2 and CT3 preferentially interact with each other, in lieu of the homo-interactions shown in Fig. 4A. This is evidenced by the loss of the CT2 peak that eluted at approximately 13 min in the absence of CT3 (the dashed line in Fig. 4C). Comparing CT3 only (the dotted line in Fig. 4C) and a mixture of CT2 plus CT3 (solid line in Fig. 4C), the combination of CT2 and CT3 appears to elute as a mixture of slowly equilibrating dimer and monomer.

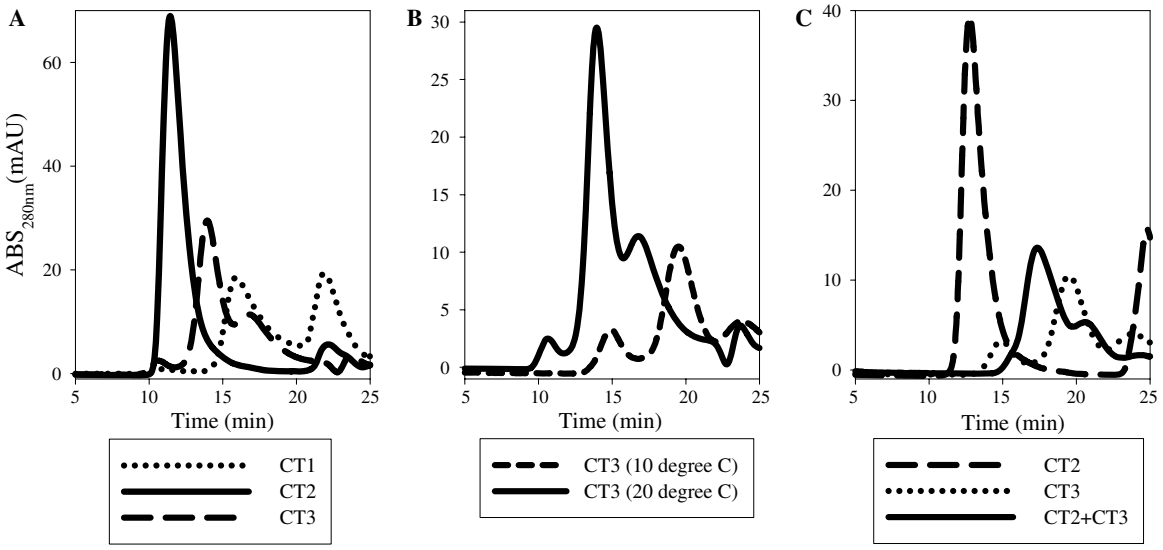

Fig. 4. Oligomeric states of CT protein domains detected by gel size-exclusion chromatography. (A) Elution profiles of CT1 (dotted line), CT2 (solid line), and CT3 (dashed line). (B) Comparison of CT3 elution at 10 °C (dashed line) and 20 °C (solid line). A small increase in elution times was noted at the lower temperature. (C) Elution profiles of CT2 (dashed line), CT3 (dotted line), and a mixture (solid line). Peak at 25 min is a contaminant.

p53-binding sites of CT domains

The proposed alignment of the amino acid sequences of CT1, CT2, and CT3 is shown in Fig. 5. Sites of documented mutations are highlighted as capital letters. Fig. 6 focuses on the alignment of the previously reported p53-binding motif [30] with predicted p53-binding sites. We hypothesize the existence of two sites in CT1, one site in CT2, and two sites in CT3. If this is correct, BRCA1 could contain at least five docking sites for p53. The binding of p53 by the CT domain and its alanine substitutions were tested by an ELISA (Fig. 7). When wild-type CT protein domain

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 283  lqhenssll--ltkdr-MnvEkaefcnkskqpglaRSq 317
1381  dcsglssqsdilttqqrdtMqHnLIkLqqemaeleavl 1418
1646  vNkrmsMVvSgltpeeFMLVykfArkhhitltnlitEe 1683

 318  hn-rwAgsketcndRRtPstekkvdlnAdplcerkewn 354
1419  eqHgsqpsnsyPsiisdSsaledlRnpeqStsekayvl 1455
1684  tThvvMkTDaeFVCeRtlkyflGiAggkwVvSy-fWvT 1720

 355  K-QklpcSenpr-----dteDvpwiTlNSsIqkvne-w 385
1456  tsqk-sseypisqNPEglsadkfevsadsSTsKnkepg 1492
1721  qSik-eRkm--------lNehdFeVrGDvVngrnHq-g 1748

 386  fsrsde--llgsddshdgeSesnaKvaDvldvlNevDE 421
1493  veRsspskcPslddRwymhScsgslqnrnYpsqeelIk 1530
1749  PkRAresqdrkiFrgLeL-ccygPfTnMptDqLewMvq 1785

 422  ysgssekidllasdpHealick----sErvhsksveSnI 456
1531  vvdVe-eqqleeSgPhDlteTsyLprqDlEgTPyLesgi 1567
1786  lCGAsvvkelssftlgtGVhPiVVVQpdawteDNgfhAi 1824

 457  edkiFGk-tyRkkAslpnlsHvtenliigaFvte-pqiiq 494
1568  -slfsddPeSDpsedrApesaRvgnipsSTsalkvpqlkv 1602
1825  -gqmceApvvtrewvldsvalyqcqeldtylipqiphshy 1863
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Fig. 5. Alignments of CT3 (residues 283–494) with CT2 (1381–1602) and CT1 (1646–1863). Residues in CT3 and CT2 identical to the corresponding residue in CT1 or to each other are in red, conservative replacements are in green. Capitalized amino acids are sites of mutation.

```
                          HNY L
p53 binding motif:        Nx   x
                          RDF V
        CT1-n      1730 NEHDFEV 1736
        CT2-n      1473 SADKFEV 1479
        CT3-n       367 TEDvPWI  373
        CT1-c      1833 vTREWVL 1839
        CT3-c       465 yRKKaSL  471
      53BP1-n      1842 NYRNYLL 1848
      53BP1-c      1965 yKHDYVs 1971
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Fig. 6. Alignments of possible BRCA1 p53-binding sites. Capital letters indicate compliance with the motif derived from the crystal complex of p53 and BRCT domain of 53BP1 [30] either by default (black), match (red), or conservative replacement (green). N- and C-terminal portions of each domain are indicated by -n and -c. Predicated binding sites are shown as italics letter.

was immobilized to plates, the quantity of bound p53 increased in a CT domain concentration-dependent manner (Figs. 7A–C, ○). In each case, binding was competitively inhibited by addition of free wild-type CT domains (not shown). To determine the effects of mutations on p53 binding, CT domains carrying alanine substitution(s) of the three central amino acids of the proposed sites were produced and purified. The mutant proteins showed CD spectrum characteristics similar to those of their wild-type protein, indicating no significant change in domain folding (data not shown). CT1 variants with a single alanine substitution ($CT1_{H1732A}$, $CT1_{D1733A}$, $CT1_{R1835A}$, and $CT1_{E1836A}$) in either CT1-n or CT1-c bound to p53 at a level comparable to that of CT1. For clarity, data for representative substitutions with D1733A or R1835A are shown (Fig. 7A, □ and ◊, respectively). In contrast, mutant CT1 ($CT1_{H1732AR1835A}$, $CT1_{H1732AE1836A}$, $CT1_{D1733AR1835A}$, and $CT1_{D1733AE1836A}$) containing substitutions involving both CT1-n and CT1-c were unable to bind p53, as

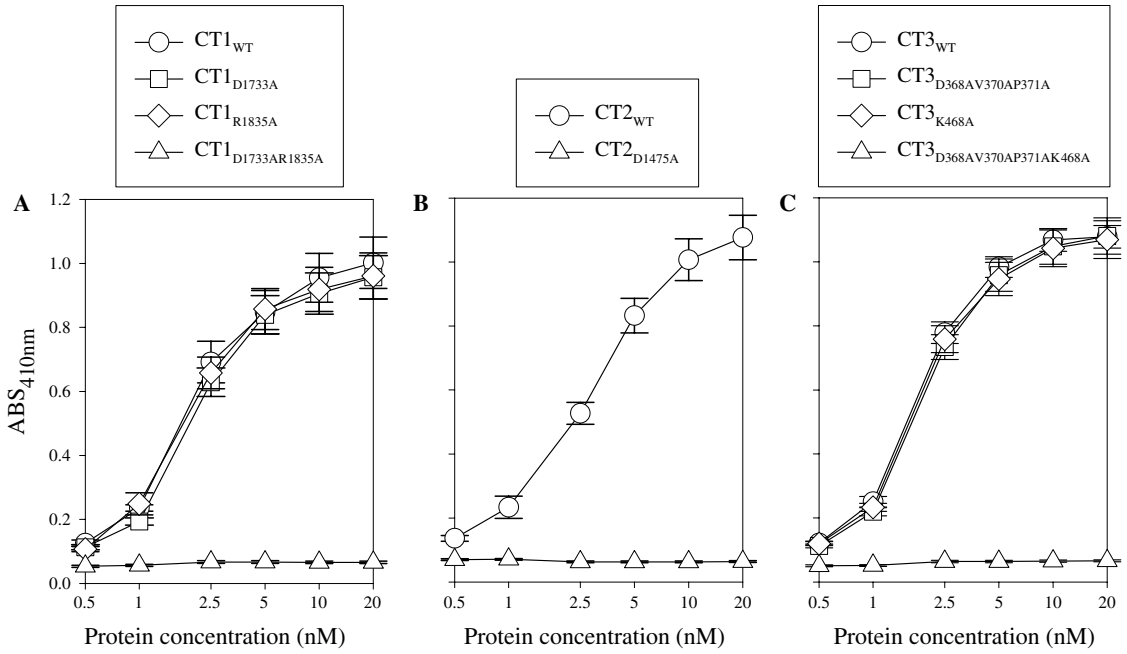

Fig. 7. ELISA showing CT domains binding ability to p53. The results from CT1, CT2, and CT3 are shown in (A–C), respectively. For simplicity, one representative mutant with alanine substitutions for each site is plotted. Means ± SDs are shown, $n = 3$ replicates.

illustrated by the $CT1_{D1733AR1835A}$ construct (Fig. 7A, △). In CT2, for which only one p53-binding site was predicted, single alanine substitutions in CT-n ($CT2_{D1475A}$, $CT2_{K1476A}$, and $CT2_{F1477A}$) abolished p53 binding (Fig. 7B, $CT2_{D1475A}$, △). CT3, like CT1, required modification of both putative binding sites to eliminate p53 binding. Mutant domain ($CT3_{D369AV370AP371A}$) with CT3-n replaced by triple alanine residues retained its p53-binding ability, as did the variants ($CT3_{K467A}$ and $CT3_{K468A}$) with single alanine substitution in CT3-c (Fig. 7C, $CT3_{D369AV370AP371A}$ and $CT3_{K468A}$, □ and ◊, respectively). However, CT3 mutant ($CT3_{D369AV370AP371AK467A}$, and $CT3_{D369AV370AP371AK468A}$) with substitutions in both CT3-n and CT3-c did not bind to p53 (Fig. 7C, $CT3_{D369AV370AP371AK468A}$, △). Therefore, site-specific mutation of the all the predicted binding sites for p53 in the recombinant BRCA1 eliminates interaction with p53. This observation strongly supports the assertion that the predicted positions in five sites of BRCA1 mediate specific interactions with p53.

Four missense mutations within p53-binding sites have been documented to date, including entries in the database of the Center for Clinical Cancer Genetics at the University of Chicago [38] as well as the 315 currently sequenced representatives of 1000 samples in the Nigerian Early Breast Cancer Cohort (Olopade et al., unpublished data). In CT1, N1730S should not drastically affect p53 binding; F1734S introduces a polar residue at a conserved hydrophobic site and can be expected to impair binding; V1736A probably has little effect on binding based on the exchange of small hydrophobic sidechains. No mutations have been documented in the CT2 site. A D369N variation has been reported in CT3, a substitution that likely only moderately reduces affinity.

Discussion

The evolution of proteins by internal tandem replication of domains (partial gene duplication) is well known [39,40], although the presence of repeats is often masked by low sequence similarity [41,42] that arises from amino acid changes associated with functional diversification. We propose here that BRCA1 incorporates two additional units that share an evolutionary relationship to the BRCT domain. Additionally, as will be described elsewhere, the remainder of the protein may consist of several replicates of the N-terminal RING domain. The proposed replicates bear no statistically significant similarity to BRCT, or to each other, when compared directly by BLAST. Nevertheless, the probable existence of the BRCT-like domains and some of the proposed RING replicates was indicated by application of Psi-BLAST to protein fragments of BRCA1 and by the finding of alternative Psi-BLAST alignments of the BRCA1 sequence against itself (data not shown). The finding of potential replicate homologues at levels of marginal sequence similarity does not provide assurance of conserved function and conformation. However, such replicated domains might be considered to be conserved units of evolution (CUE). Identification of CUEs in an extended protein primary structure can provide clues, but not guarantees, to the approximate location of a possible autonomous domain that might be cloned and expressed for systematic study. This approach led to the production of the BRCT replicate domains described here. It is likely that

this strategy may have general applicability to many very large proteins that require deconstruction for detailed biomolecular studies.

Structural studies of the RING and BRCT domains of BRCA1 by NMR and crystallography have indicated a few missense BRCA1 variants that have functional implications. Several residues in the N-terminal RING domain—R7, V11, I15, I21, M18, and I89—make direct contact with BARD1. Disruptions of these amino acids abolish heterodimerization with BARD1, while mutations of C39, C61, and C64 may affect BRCA1 ubiquitination activity [43]. The BRCT tandem repeat domain includes mutations—M1652F, S1655F, R1699W, K1702A, A1708E, G1738E, P1749R, M1775R, and V1809F—that either abolish or reduce the ability to bind BACH1 phosphopeptide or affect the structural integrity of the BRCT domain [44–47]. The remaining portions of BRCA1 appear to be unstructured, as shown here and by Mark et al. [32]. Structure determinations by crystallography and NMR of these segments, in free form or in a complex with an interaction partner, may be problematic. We have demonstrated that a homologue-based strategy, even at very low levels of sequence similarity, was able to define at the amino acid level, BRCA1 sites for p53 binding in the absence of structural visualization of the complexes.

The finding of an interaction between CT2 and CT3 was unexpected. The significance is dependent upon eventual physiological data. Circumstantial evidence of physiological function might be obtained by examining the dependence of interaction upon amino acid replacements. If the CT2 and CT3 interaction is of physiological importance, then it is likely that the interaction involves conserved, well-defined segments of the proteins, although redundancy of interaction sites, as seen by the presence of multiple p53-binding sites in CT1 and CT3, is possible. Assuming the CT3-binding site in CT2 (or vice versa) is non-redundant, as is apparently the case for p53, then it should be possible to find a limited number of residue substitutions that significantly diminish or eliminate interaction. On the other hand, if the interaction is essentially an artifact, it is highly unlikely that single mutations will have a dramatic effect on the interaction.

Our data verified the location of five predicted p53-binding sites. The BRCT domain (CT1) and its CT3 homolog exhibit two sites, either one of which is sufficient for binding of p53 (Fig. 6). Binding of p53 in the apparently unstructured CT2 and CT3 regions could be less constrained than the interaction with the structured BRCT (CT1) domain. However, the BRCT p53-binding site encompassing positions 1833–1839 is of defined structure yet is in agreement with the previously defined p53-binding motif [30] at only two positions.

Fig. 6 includes the sequence of the p53-binding site identified in the structure [30] of the complex formed by p53 and the C-terminal BRCT domain of the p53-binding protein 1 (53BP1), a protein of 1972 amino acids. Sequence analysis found a second potential binding site at the C-terminus of the protein, terminating at position 1971 (Fig. 6). However, only one molecule of p53 is found to bind the 53BP1 BRCT domain [30]. Several possible explanations might reconcile the crystallographic observation with our predicted second p53-binding site. The replacement of a conserved hydrophobic residue by serine at the C-terminus of the binding site might render the putative second 53BP1 site non-functional or of significantly lower affinity, as might the location of the binding motif very near the end of the protein. Binding at one site might preclude binding at the second site. In addition, because crystallization was accomplished with p53 in only slight molar excess [30], sufficient p53 was not present to allow binding at two sites. This consideration renders the crystal structure as having no direct bearing on the question of two possible binding sites in CT1 and CT3 of human BRCA1.

BRCA1 binding of p53 at a site other than the C-terminal BRCT-domain was previously suggested by Zhang et al. [17], who reported the formation of a complex with a BRCA1 fragment spanning residues 224–500. CT3 involves residues 288–479. Sturdy et al. [31] recently reported expression of a soluble fragment ranging from position 230 to 534 but did not evaluate p53 binding. By using an intrinsic tryptophan fluorescence quenching assay, Mark et al. [32] demonstrated that p53 C-terminal regions (311–393 and 355–393) weakly interact with a BRCA1 fragment spanning residues 219–498, a segment that encompasses CT3 (288–479). They did not test p53 binding against all of the expressed BRCA1 fragments that they produced, although two fragments (1316–1538, 1443–1649) span the region of CT2 (1376–1618) [32]. Both Sturdy et al. [31] and Mark et al. [32] did not address the binding ability of the p53 DBD domain against BRCA1 fragments. On the other hand, the high affinity between the BRCA1 BRCT domain and the p53 DBD domain was not observed when tested by chromatography and centrifugation as reported by Ekblad et al. [48]. CT3 contains no precise match to the p53-binding motif [30]. However, the N-terminal portion (CT3-n) has a neutral threonine rather than a neutral asparagine at position 1, an acidic polar (Asp) rather than a basic polar (Arg/His) at position 3, a hydrophobic proline rather than a hydrophobic phenylalanine at position 5, and a hydrophobic isoleucine rather than a hydrophobic leucine or valine at position 7. To our knowledge, no other regions of BRCA1 have been recognized as binding p53. Thus, the prediction from a primary structure analysis of p53 interactions is novel. The CT2-n site adds serine at position 1 and lysine at position 4. Thus, the p53-binding motif can be currently described as: (N/S/T)-X-(H/R/D/K)-(N/D/K/E)-(Y/F/P)-X-(L/V/I) with residues identified in this study underlined.

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