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Insights into the molecular basis of human hereditary breast cancer from studies of the BRCA1 BRCT domain

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Abstract

The C-terminal, BRCT repeats of BRCA1 are essential for the tumor suppressor function of this protein. Here we review structural and functional studies of this domain. Both repeats adopt similar folds and pack in an intimate, head-to-tail manner. The domain binds phosphorylated targets such as the DNA damage-associated kinase BACH1, with a specificity for pSer-X-X-Phe motifs. Structural studies reveal that the N-terminal repeat is responsible for pSer binding while a groove at the interface of the two repeats recognizes the Phe. Missense variants identified in breast cancer screening programs often disrupt these interactions and these molecular defects may lead to an increased cancer risk.

The discovery of BRCA1, the first gene associated with human hereditary breast cancer, made possible the detection of BRCA1 mutations in women from families with a history of this disease [1, 2]. Thousands of women have now been screened for BRCA1 mutations, many of whom have been cataloged in the Breast Information Core Database (BIC, http://research.nhgri.nih.gov/bic/). Hundreds of distinct mutations have been uncovered in BRCA1, however, for the vast majority, there is insufficient genetic linkage data for these mutations to determine the cancer risks associated with them.

BRCA1 appears to be a key protein in the regulation of the cellular response to DNA damage (for a review, see [3]). The protein becomes associated with DNA damage foci in a manner that is modulated by its phosphorylation by damage-associated kinases. Mutations in BRCA1 have been shown to sensitize cells to a variety of DNA damaging agents, and to specifically disrupt the G2/M cell cycle checkpoint.

The goal of our research has been to use structural and biochemical methods to probe the function of the BRCA1 protein to help characterize unclassified variants in the BIC. BRCA1 is a large 1863 amino acid protein with a distinct, ~100 amino acid RING domain at its N-terminus, and a pair of ~90 amino acid repeats at the C-terminus called BRCT (**BR**CA1 **C-t**erminal) repeats. The central region of the protein between the two terminal domains bears relatively low sequence identity between mammalian BRCA1 homologs, and attempts to define structured domains within this domain both by our group (unpublished) and others [4] indicate that this portion of the protein is largely unstructured. This region is extensively phosphorylated by DNA damage-associated kinases like ATM, and may serve as a phosphorylation-dependent docking site for other proteins involved in the DNA damage response, or even for damaged DNA itself [4, 5].

Analysis of the BRCA1 mutational database indicates that both the RING and BRCT repeats are most frequently mutated in women at risk of cancer, and have focused attention on these domains. Recently, work from Rachel Klevitt's laboratory have revealed that the N-terminal RING domain of BRCA1, in complex with a similar domain in the BRCA1 partner protein, BARD1, forms a ubiquitin ligase complex, probably the major catalytic activity associated with this protein [6–10]. Our lab has focused our studies on the C-terminal BRCT domain.

BRCT repeats are found in a variety of protein associated with regulation of the DNA damage response such as 53BP1, BARD1 and MDC1 (see Pfam entry (http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF00533) for a complete list of family members, and [11] for a recent review). The BRCT repeats in BRCA1 are essential for the tumor suppressor function of the protein as protein truncation and missense variants within the BRCT have

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been shown to be associated with human breast and ovarian cancers. Approximately 100 distinct missense mutations have been detected in the BRCA1 BRCT repeats through breast and ovarian cancer screening programs. Unfortunately, there is sufficient pedigree data to classify only 8 of these variants. The variants D1692Y, C1697R, R1699W, A1708E, S1715R, P1749R, and M1775R all appear to be associated with an increased risk of breast cancer, while M1652I appears to be a benign polymorphism (reviewed in [12]).

We determined the X-ray crystal structure of the two BRCT repeats of BRCA1 ([13], Figure 1). The structure revealed that the two repeats adopt very similar folds, and pack together in a head-to-tail manner. This packing is stabilized by the packing of a single helix from the N-terminal repeat against a pair of helices from the C-terminal repeat and the inter-repeat linker. While this structure alone did not reveal the molecular function of the protein, we nevertheless could use it as a basis to predict the effects of specific mutations, with the underlying hypothesis that the function would be disrupted by mutations that disrupt the structure of the domain. For example, in one cancer associated mutation, an alanine at position 1708 is replaced by a glutamic acid. The alanine packs into a small hydrophobic pocket at the interface between the two BRCT repeats. The larger negatively charged glutamate side chain could not be accommodated within this pocket and would perturb the interaction of the two domains.

We also developed a simple assay to directly test the conformational stability of BRCA1 BRCT variants [12]. The assay is based on our finding that the stable folding of the wild type BRCT domain protects it from proteolytic degradation. We generated 25 of the 100 known BRCA1 BRCT missense variants using *in vitro* transcription/translation and assayed their proteolytic stability compared to a wild type control. Thirteen of the 25 variants tested exhibited a dramatic increase in the susceptibility to proteolysis, strongly suggesting that these mutations would be unfolded *in vivo* and may be associated with an increased cancer risk (Table 1).

Some of the mutations, however, led to a more modest increase in proteolytic susceptibility. One of these mutations, M1775R, was particularly interesting as this was one of the first characterized BRCT mutations to be linked to cancer [1, 2]. We were able to crystallize and determine the structure of this variant,

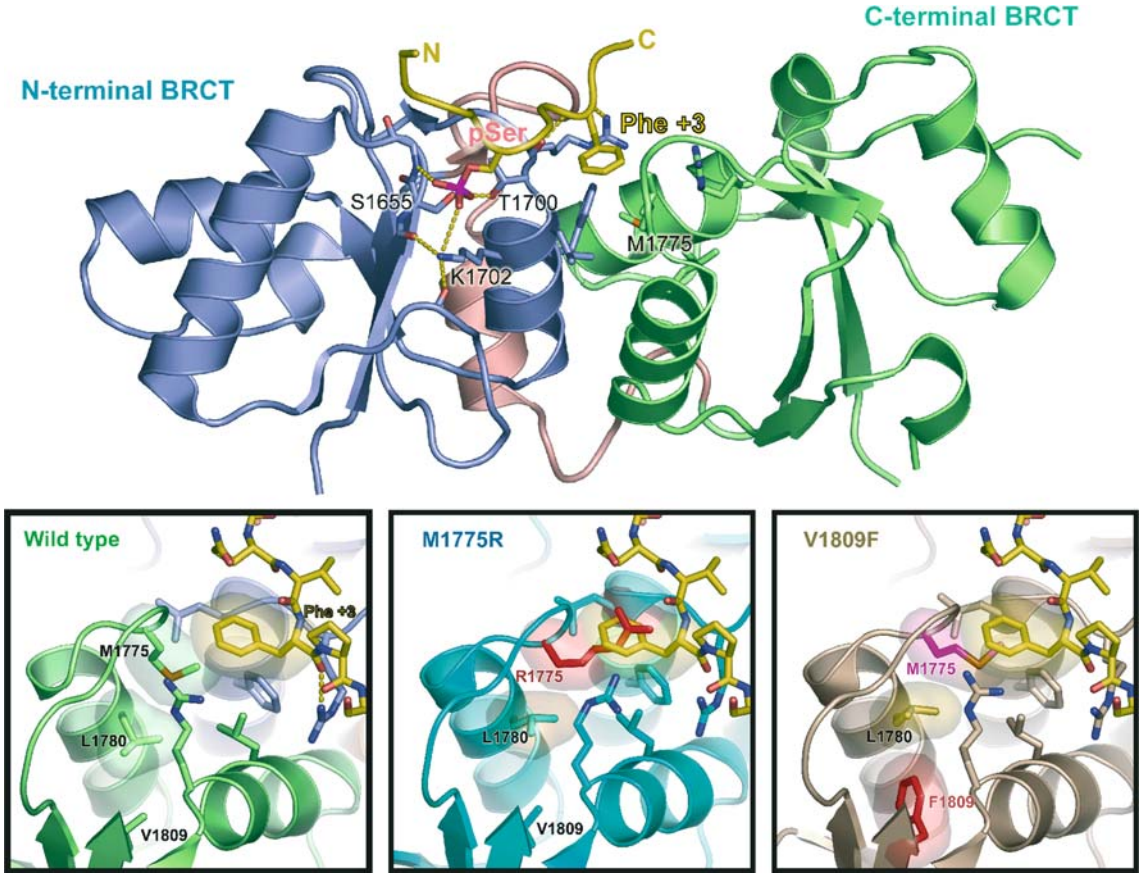

Figure 1. Structures of the BRCA1 BRCT domain and interactions with phosphopeptides. *Top panel.* Structure of the BRCA1 BRCT domain bound to a pSer-X-X-Phe peptide target. The N- and C-terminal BRCT repeats are shown in blue and green, and the inter-repeat linker is shown in orange. The bound peptide is in yellow. Residues important for peptide recognition are shown as sticks. *Bottom panels.* Details of the Phe +3 binding pocket in the wild type structure (left), the M1775R variant (center), and the V1809F variant (right). Note that the M1775R and V1809F structures were determined in the absence of a bound peptide. The yellow peptide shown in these panels is overlaid from the wild type structure.

Table 1. Structure, function and disease effects of BRCT missense mutations.

Mutant	Secondary Structure[a]	Protease Sensitivity[b]	pSer-X-X-Phe binding[c]	Disease Effects[d]
M1652I	β	-	+	-
S1655F	β	n.d.	-	?
D1692Y	C	+	+/–	+
F1695L	C	-	+	?
V1696L	C	+	-	?
C1697R	C	++	-	+
R1699W	c	+	-	+
R1699Q	c	-	-	?
A1708E	α	++	-	+
S1715R	β	++	-	+
W1718C	α	++	-	?
T1720A	α	-	+	?
G1738E	c	++	-	+
G1738R	c	++	-	?
P1749R	α	++	-	?
R1751Q	α	+	+	?
A1752P	α	++	-	?
I1766S	β	++	-	?
M1775R	c	+	-	+
M1783T	α	+	+	?
G1788V	c	++	-	?
V1804D	c	-	n.d.	?
V1809F	β	+	-	?
W1837R	α	++	-	?
W1837G	α	++	-	?
Y1853C	α	++	-	?

[a] Secondary structure is from the human BRCT domain structure [13].
[b] Protease sensitivity: (-) Wild type, no effect; (+) Intermediately destabilizing; (++) Highly destabilizing [12].
[c] Ability to specifically bind a pSer-X-X-Phe peptide compared to the non-phosphorylated peptide. (-) no specific binding; (+/–) specific binding, but below wild type levels; (+) specific binding, equivalent to wild type.
[d] Disease linkage data is from recorded entries in the BIC, [21, 22], and T.S. Frank, personal communication. (+): linked to disease, (-): not linked, (?): unknown.

which revealed a subtle rearrangement of side chains around the site of the mutation, and a perturbation of the surface features of the protein (Figure 1) [14]. Thus, we were left with the question, is the defect due to the somewhat reduced stability of this variant, or does the alteration of the protein surface perturb an important interaction surface?

Late in 2003, a major breakthrough in our understanding of BRCT function came when work from the laboratories of Junjie Chen and Mike Yaffe revealed that the tandem BRCT repeats of BRCA1 functions as a phospho-peptide binding module [15, 16]. The BRCA1 BRCT is highly selective for the sequence pSer-X-X-Phe. These interactions were shown to mediate the association of BRCA1 with the DNA helicase BACH1, which is essential for the correct functioning of the G2/M checkpoint, and with the transcriptional co-repressor, CtIP. These authors also showed that BRCT repeats from other proteins also functioned as phospho-peptide binding modules, implying that BRCT repeats might function in general as phospho-peptide binding modules in signaling events related to the DNA damage response.

To understand how the BRCA1 BRCT recognizes its specific phospho-peptide target, we determined the structure of the BRCA1 BRCT bound to a high affinity peptide derived from an *in vitro* peptide selection experiment containing the pSer-X-X-Phe motif [17]. Similar structures have also been determined by other groups [18, 19]. The structure revealed a phospho-serine binding pocket in the N-terminal BRCT repeat, and a phenylalanine binding pocket in a groove formed at the interface between the two repeats. Key residues that constitute the phospho-serine pocket include Ser 1655, Gly 1656, and Lys 1702, which all directly recognize the phosphate moiety. Thr 1700 also plays an important role in phosphate recognition, as it hydrogen bonds with Ser 1655, keeping the serine hydroxyl in a rigid orientation appropriate for phosphate recognition. This [Ser–Gly....Thr-X–Lys] motif is conserved in a variety of other BRCT repeats, suggesting that many of these proteins will also bind phospho-serine containing peptides [11, 17]. Indeed, a number of other BRCT-containing proteins, including PTIP, 53BP1, and BARD1, have now been shown to have phospho-peptide binding activity [15, 16, 20].

To directly test the phospho-serine binding capacity of our set of 25 BRCT missense variants, we assayed the ability of a pSer-X-X-Phe peptide to specifically pull down *in vitro* transcribed/translated BRCT variants, compared to a non-phosphorylated control (Table 1). Our results demonstrated that the structural integrity of the BRCT domain was required for phospho-peptide recognition, as none of the missense variants that highly destabilized the protein fold specifically bound the pSer peptide. The results confirmed the importance of the residues of the phosphate recognition pocket as mutation of any of these residues to alanine completely destroyed the ability of the protein to bind phospho-peptide [17].

The Phe at the +3 position relative to the pSer is bound in a deep groove at the interface between the two repeats (Figure 1). This interaction explains the facts that both BRCT repeats are needed for peptide binding, and loss of part of the C-terminal repeat is associated with hereditary breast cancer. BRCT domains are most commonly found as tandem repeats in other proteins involved in the DNA damage response, and sequence analysis suggests that the head-to-tail packing of the BRCT repeats in BRCA1 is conserved in other repeats [11, 13]. Thus it is likely that this secondary recognition groove at the repeat interface is conserved in other BRCT repeat proteins. However, lack of sequence conservation of residues that line the groove suggest that the peptide binding specificity of other BRCT repeat binding proteins may be different.

The importances of several of the residues that line the phenylalanine binding pocket were also tested using the pull down assay [17] (Table 1). Mutations of many of these residues were found to disrupt specific phospho-peptide interactions. For example, mutation of Arg 1699, which interacts with the backbone of the phenylalanine,

to either Trp or Gln, completely abrogated binding. The cancer-associated mutation, M1775R, also resulted in a complete loss of peptide binding. Interestingly, M1775 lies at the bottom of the inter-repeat groove and makes close van der Waals contact with the phenylalanine. Superposition of the M1775R mutant structure on the structure of the BRCT-peptide complex reveals that the substituted arginine side chain occupies the phenylalanine binding pocket, suggesting that this mutant is impaired in binding pSer-X-X-Phe targets [17] (Figure 1).

The M1775R variant structure reveals a pair of anions to be stably bound to the surface of the protein near the site of the mutation [14]. The anions probably stabilize the substituted arginine side chain within this positively charged portion of the surface. Intriguingly, these anions bind near the positions of the +2 and +3 residues in the wild type BRCT-peptide structure. This suggested to us that while the M1775R might not bind the natural pSer-X-X-Phe target, it might bind alternative peptides with negatively charged residues at the +2 or +3 positions. Indeed, comparison of the binding affinities of pSer peptides with all possible sequences at the +3 position reveals a clear preference for acidic residues at this position [18]. Thus, it is possible that M1775R may not only be deficient in the recognition of its appropriate partner, but it may also interact with inappropriate phospho-proteins, perhaps acting as a dominant negative.

We also found that certain mutations distant from the peptide binding site resulted in reduced peptide binding (Table 1). One of these, V1809F, is stably folded and therefore the loss of peptide binding associated with this mutation cannot be attributed to a loss in the overall structural stability of the domain. We were able to crystallize and determine the structure of this variant; comparison of its structure with that of the wild type protein revealed a subtle structural rearrangement responsible for the loss of peptide binding (Figure 1). Val 1809 occupies a closely packed hydrophobic pocket in the core of the C-terminal BRCT. Replacement of this residue with the larger Phe is accommodated by a rearrangement of the neighboring side chains. Most importantly, Leu 1780 is shifted into a different rotamer by the mutation. The movement of Leu 1780 brings it into contact with Met 1775, pushing this side chain into the phenylalanine binding pocket. Like the M1775R mutation, V1809F occludes the Phe recognition pocket, and this probably explains the loss of peptide binding for this variant. Unlike M1775R, there is insufficient pedigree data to assess the cancer risks associated with V1809F. Our data suggest that this variant is deficient in a major function associated with the BRCT domain and is therefore likely to be a cancer-predisposing mutation.

Significant advances have been made in the understanding of the molecular function of the BRCA1 BRCT repeats, and these studies have allowed us to understand the molecular defects associated with a number of previously uncharacterized sequence variants in this region of the protein. However, it remains to be shown what the function of phospho-serine binding is in the context of the intact protein. Do these interactions facilitate rearrangements in the overall structure and organization of the BRCA1 complex? Do these interactions affect that ubiquitin ligase activity of BRCA1? Answers to these questions will not only help to detail molecular mechanisms that underlie hereditary breast cancer, but will help to define mechanisms that regulate the cellular response to DNA damage.

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