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BRCT Domains: phosphopeptide binding and signaling modules

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1. ABSTRACT

The *BR*CA1 *C-t*erminus (BRCT) domains are essential for the tumor suppressor function of BRCA1, and have been found in a variety of proteins from bacteria to men. Recent studies demonstrate that the BRCT domain constitutes a novel phosphopeptide binding region. In this review we seek to discuss the recent biochemical and structural data that have helped elucidate the molecular basis of BRCT domain function and BRCT-mediated interactions, with special emphasis on the role of phospho-specific interactions in key networks that regulate DNA repair. Finally we offer predictions on additional phospho-interacting BRCT domains and potential *in vivo* binding sites for several BRCT domains.

2. INTRODUCTION

The cloning of BRCA1 and the subsequent genetic, biochemical, and cellular studies of this familial breast and ovarian cancer susceptibility gene have implicated BRCA1 in regulating important nuclear functions such as transcription, recombination, DNA repair, and checkpoint response (1-11). Mutations in the BRCA1 gene account for approximately 80-90% of all hereditary breast cancers (12). BRCA1 consists of an N-terminal RING domain and a C-terminal region comprised of two tandem BRCT domains (amino acids 1649-1859) (6,9). Most cancer-causing BRCA1 mutations result in truncated BRCA1 gene products that lack one or both BRCT domains. In addition, cancer-associated missense

mutations in the BRCA1 BRCT domains have been shown to sensitize cells to a variety of DNA damaging agents, and to specifically disrupt the G2/M cell cycle checkpoint (13,14). Furthermore, experimental data support an essential role for the BRCT domains in regulating DNA repair and binding, cell cycle, and gene expression (15-21). These findings, together with the observation that deletion of the BRCA1 BRCT repeats led to tumor development in mice (22), demonstrate that BRCT repeats play a central role in BRCA1-mediated tumor suppressor function.

Careful bioinformatic and biochemical studies have revealed the presence of BRCT repeats in a diverse array of proteins, particularly in those involved in DNA damage responses. Examples include DNA damage response or repair and cell cycle checkpoint proteins (e.g., BRCA1, MDC1, 53BP1, NBS1, and XRCC1), oncoprotein ECT2, BARD1, and the PAX transcription-activation domain-interacting protein PTIP (1,10,11). Individual BRCT repeats (~90-100 amino acids in length) are capable of folding independently, but often exist in tandem pairs separated by regions of various sizes.

It is particularly interesting to note the presence of BRCT domains in prokaryotes as well as viruses (1,23). For example, single BRCT domains have been found in bacterial DNA ligases, raising tantalizing possibilities about the evolutionary origin of BRCT domains. Perhaps the sequences and complexity of the BRCT domains have evolved over time to accommodate their increasingly complex and indispensable roles in higher organisms.

3. BRCT DOMAINS AS PHOSPHOPEPTIDE INTERACTION MOTIFS

While BRCT domains can interact with each other, as in the case of XRCC1 and DNA ligase III that interact through their respective BRCT domains (24), the most common type of BRCT-mediated interactions occurs between the BRCT domain and a non-BRCT partner (25,26). Besides BRCA1, the BRCT domains of several other proteins such as 53BP1, DNA ligase IV, and XRCC1 have also been shown to mediate phospho-independent protein-protein interactions (28-31). In addition, BRCT domains can also mediate protein-DNA interactions. For example, several of the ToPBP1 BRCT domains have been demonstrated to bind both single and double stranded DNA fragments in a sequence independent manner (32).

The activities of protein kinases and phosphatases are often modulated in response to cues such as cell cycle or DNA damage, which in turn controls the dynamics of protein complex formation. Phosphorylation and dephosphorylation are mediated through the opposing activities of these enzymes thereby regulating the signals that are relayed through phosphorylation-dependent protein-protein interactions. Recent work from several laboratories has indicated that BRCT repeats can also function as phosphopeptide-binding modules, shedding new light on how signals may be transduced from protein kinases through BRCT containing proteins (13,14,33). These findings provide a unique opportunity to understand how BRCT-containing proteins may be coupled to different downstream signaling pathways in a regulated manner. We will discuss in more detail phospho-specific interactions mediated through BRCT domains.

3.1. BRCA1

The first indication of BRCT domain involvement in phospho-dependent protein-protein interactions emerged from studies in *Saccharomyces cerevisiae* with the cell cycle protein RAD9. RAD9 was shown to homo-oligomerize preferentially with hyperphosphorylated RAD9 via its C-terminal BRCT domains (34). Recently, studies from three groups have demonstrated that BRCT domains can indeed function as phospho-interacting modules (13,14,33). Yu *et al* showed that BRCA1 BRCT could interact with phosphorylated BACH1 (BRCA1-Associated Carboxyl-terminal helicase) (33). The binding was mapped specifically to Ser990 on BACH1, and this interaction was required for DNA damage-induced checkpoint function during G2/M phase transition. Using an oriented peptide library analysis, Rodriguez *et al* arrived at similar conclusions and found that BRCA1 BRCT domains preferred a phosphoserine-aromatic-hydrophobic-Phe/Tyr motif (14). The selection for Phe was remarkably strong at the P+3 position (13,14). Notably, the region surrounding Ser990 in BACH1 matches very well with this predicted motif. Furthermore, substitution with amino acids other than Phe at the P+3 position abolished BRCA1 BRCT interaction and resulted in G2/M checkpoint defect (14,33). ATM/ATR activation in response to gamma-irradiation results in the phosphorylation of a variety of proteins including other kinases, transcription factors, scaffold and DNA repair proteins (35). Manke *et al* therefore constructed an oriented phosphopeptide library that resembled the Ser/Thr-Gln motif generated by ATM/ATR, to search for novel modular phosphoserine or phosphothreonine (pSer or pThr) binding domains involved in DNA damage response (13). The group found that the BRCT domains in BRCA1 and PTIP recognized ATM/ATR substrates such as p53 only after irradiation, indicating the requirement of phosphorylation for interaction and the role of BRCT domains as phospho-protein binding modules in DNA damage response pathways (13).

Additional BRCT domains from proteins such as MDC1 (mediator of DNA damage checkpoint 1), BARD1, DNA ligase IV, and *Saccharomyces cerevisiae* RAD9 have been examined by oriented peptide library analysis, revealing a phospho-dependent binding specificity that extends from the residues in position P+1 (with pSer/pThr as P0) to P+5, with particularly strong selections for aromatic/aliphatic residues at the P+3 position (14,33). In the study by *Rodriguez et al*, BRCT repeats from MDC1 were predicted to have a preference for pSer-X-X-Y (14). Based on this prediction, H2AX, a marker for sites of DNA breaks, may be a potential binding partner of MDC1. Indeed, these predictions have been confirmed by later studies (36,37). The recently solved crystal and NMR structures of several BRCT domains have further provided the molecular basis for recognition of specific phosphopeptides by BRCT domains (36,38-41). For

example, the residues that form the phospho-serine binding pocket in BRCA1 are conserved in several BRCT domains (Figure 2). Taken together, these observations indicate that phosphopeptide binding (with definable specificities) may be a common function of a set of BRCT repeats. For example, multiple proteins may interact with the BRCT region of BRCA1 in a phosphorylation-dependent manner and collaborate functionally with BRCA1 to participate in multiple cellular processes. The incorporation of enzymatic activities and post-translational modifications therefore afford the BRCA1 interaction networks more specificity and range in regulation.

3.1.1. Phospho-dependent BRCA1 BRCT interacting proteins

3.1.1.1. BACH1

BACH1 was originally identified in a screen for proteins that directly interact with the BRCT domain of BRCA1 (26). In fact, phosphorylation of BACH1 at Ser990 is a prerequisite for its association with BRCA1 (14,33). This interaction is cell cycle regulated and required for the G2/M checkpoint control in response to DNA damage. Mutant BRCA1 that either lacks one BRCT domain or contains missense mutations (P1749R and M1775R) fails to interact with BACH1. These data suggest that an intact BRCT domain structure is required for its interaction and function. As discussed above, Phe at position P+3 is also critical for phosphorylation-dependent interactions between BACH1 and BRCA1. These studies and the analyses of other BRCT-containing proteins indicate that phospho-dependent binding constitutes a crucial aspect of BRCT function, and demonstrate the versatility of this interaction module. The various modes of interaction (such as homodimerization and non-phospho dependent binding) should complement each other functionally and allow the BRCT-containing proteins to act in diverse pathways.

3.1.1.2. CtIP

The transcriptional suppressor CtBP binding partner CtIP was identified by two-hybrid screening as interacting with BRCA1 BRCT domains (25). Within the BRCA1-binding region of CtIP, the sequence surrounding Ser327 resembles the phosphorylation motif on BACH1 and undergoes transient phosphorylation during G2 (42). Furthermore, Ala mutation of Ser327 abolished the *in vitro* and *in vivo* interaction of CtIP with BRCT BRCA1 (42). The BRCA1/CtIP complex, which only exists during G2, is required for the G2/M transition checkpoint and DNA damage-induced Chk1 activation, suggesting that CtIP cooperates with BRCA1 in cell cycle checkpoint control (42). However, the BRCA1/CtIP complex is not required for prolonged G2 accumulation after DNA damage, a process controlled by a separate BRCA1/BACH1 complex (42). Notably, the *in vivo* interaction between BRCA1 and CtIP is completely ablated by the tumor-associated mutations of BRCA1 (A1708E and P1749R) as well as the nonsense mutation that eliminates the C-terminal 11 amino acids of BRCA1 (Y1853delta) (25). In fact, tumor derived-mutations on any BRCA1 BRCT repeats or its spacer region disrupt the BRCA1/CtIP interaction (43). Clearly, the BRCT domain assists BRCA1 function by its ability to interact with diverse partners at different cell cycle stages.

3.1.1.3. Acetyl-CoA carboxylase

Magnard *et al* identified ACCA (Acetyl Coenzyme A (CoA) Carboxylase alpha) as a BRCA1 BRCT interacting protein through a glutathione-S-transferase (GST) pull down assay with murine cells (44). ACCA is the rate-limiting step enzyme that catalyzes the carboxylation of Acetyl CoA to malonyl-CoA for the synthesis of long-chain fatty acids (45,46). A short-term regulatory mechanism for ACCA involves the phosphorylation and dephosphorylation of its Ser79 that result in its inactivation and activation. BRCA1 interacts only with the phosphorylated (i.e. inactive) form of ACCA. Sequences surrounding residue Ser1263 on ACCA strongly resemble those surrounding residue Ser990 of BACH1, and likely mediate the recognition by BRCT repeats of BRCA1. BRCA1 modulates lipid synthesis through its phospho-dependent binding to ACCA, thereby preventing ACCA from dephosphorylation and its subsequent activation (47). Importantly, this interaction is disrupted by germ-line BRCA1 BRCT mutations (e.g., A1708E, M1775R, P1749R, R1835X and Y1853X) (44). A number of studies have linked lipogenesis with cancer. For example, ACCA and another major enzyme in lipogenesis FAS (Fatty Acid Synthase), are highly expressed in several human malignancies including breast cancer (48). These observations have provided new mechanisms by which BRCA1 may exert its tumor suppressor function, and offered clues to how BRCT domains may signal in metabolic pathways.

3.1.1.4. Abraxas/CCDC98 and RAP80

Phosphopeptide affinity proteomic analysis of the BRCA1 complex revealed Abraxas/CCDC98 as directly binding to the BRCA1 BRCT domains through a pSer-X-X-Phe motif (49-51). In addition, Abraxas/CCDC98 mediates the formation of BRCA1 foci in response to DNA damage and BRCA1-dependent G2/M checkpoint activation. The binding of Abraxas/CCDC98 to BRCA1 requires phosphorylation, and is mutually exclusive with BACH1 or CtIP interaction with BRCA1. This is consistent with the observation that each of these proteins interacts with BRCA1 through the BRCT domains. Another ubiquitin-binding protein, RAP80, was found to associate with the Abraxas/CCDC98-BRCA1 complex (49,52,53). RAP80 and BRCA1 interact in a BRCA1 BRCT dependent manner, as two clinical missense mutations of BRCA1 BRCT domains (V1696L and P1749R) led to disrupted RAP80-BRCA1 binding (49,52). However, RAP80 does not contain a pSXXF motif, suggesting an indirect interaction between RAP80 and BRCA1. Indeed, several groups have shown that Abraxas/CCDC98 bridges the interaction between RAP80 and BRCA1 (49-51). After DNA damage, the Abraxas/CCDC98-RAP80 complex translocates to sites of damage, followed by the recruitment of BRCA1 via its binding to the phosphorylated C-terminus of Abraxas/CCDC98. Both Abraxas/CCDC98 and RAP80 are required for DNA damage resistance, DNA repair and

G2/M checkpoint control. The biochemical understanding of the interactions of these proteins with BRCA1 therefore not only helps to illustrate signaling pathways that recruit BRCA1 to sites of DNA damage, but also sheds light on the function and activity of other BRCT containing proteins.

3.2. MDC1

The recently identified mediator of DNA damage responses MDC1 contains tandem BRCT domains (54-56). Cells lacking MDC1 are sensitive to ionizing radiation, and fail to efficiently activate the intra S-phase and G2/M phase checkpoints. Upon treatment of cells with double-strand break inducing agents, MDC1 rapidly translocates to sites of DNA damage, where it mediates the accumulation of checkpoint and repair factors into nuclear foci together with proteins such as phosphorylated H2AX (54-56). Recent studies have shown that the BRCT domain of MDC1 directly interacts with the C-terminus of the phosphorylated H2AX that harbors the sequence SQEY (36,37). In the predicted MDC1 BRCT recognition motif (pSer-X-X-Y), tyrosine at position P+3 plays a critical role in mediating MDC1 binding (14). In the case of phospho-H2AX peptide, Ala substitution of Tyr completely abrogates its interaction with MDC1 (36). Furthermore, the addition of two extra Ala residues to the C-terminus of the peptide dramatically weakened the binding. Although the BRCT domains of MDC1 have a similar pattern of recognition compared to those of BRCA1 BRCT, biochemical and crystallographic data highlight the importance of a free carboxyl terminus for the overall sequence specificity (36,37). Since H2AX is the only MDC1 BRCT-interacting protein identified so far, the possibility that MDC1 BRCT may be able to recognize internal sequences cannot be ruled out.

The SQEY sequence on H2AX is also conserved in budding yeast and known to be phosphorylated by ATM-family kinases (57). Hammet *et al.* reported that the BRCT protein RAD9 binds to phosphorylated H2A in yeast and regulates the G1 check point (58), suggesting MDC1 may have evolved to function similarly in yeast and mammalian cells.

3.3. PTIP

The transcriptional regulatory protein PTIP appears to play an important role in regulating genome stability and mitosis (59-61). PTIP is required for the survival of cells exposed to ionizing radiation. Manke *et al* have shown that a pair of tandem BRCT repeats of PTIP has specific and high-affinity binding for peptides with the pS/T-Q-V-F sequence (13). It appears that BRCT-phosphopeptide interaction is responsible for PTIP localization to nuclear foci that contain 53BP1 and phospho-H2AX. Phosphorylated Ser25 on 53BP1 is bound by the BRCT repeats on PTIP after DNA damage, which requires ATM-dependent phosphorylation (61). Interestingly, this pair of tandem BRCT domains on PTIP can bind to ATM-phosphorylated epitopes other than the pS/T-Q-V-F sequence as well, highlighting the versatility of the BRCT domains in binding different phosphorylated targets.

3.4. MCPH1

MCPH1, also known as BRIT1 (78), contains 3 predicted BRCT domains, two of which are located at the C-terminus of MCPH1. MCPH1 was cloned as a gene that shows correlation with microcephely brains in animals (62). While its implication in controlling brain size is still controversial, recent evidence favors its role in DNA damage responses and mitosis (63-67, 79). MCPH1 appears to mediate early DNA damage responses, because RNAi knockdown of MCPH1 prevented the recruitment of BRCA1 and 53BP1 to damage foci. MCPH1 itself is known to localize to DNA damage foci through the interaction between its C-terminal BRCT repeats and the phosphorylated SQEY motif on H2AX (66). Mutations of MCPH1 BRCT repeats have been found in breast cancer (65), again underlining the importance of BRCT domains in protecting cells from genomic instability and cancer.

4. CANCER-ASSOCIATED BRCA1 MUTATIONS

Structural and sequence analysis of BRCA1 BRCT domains reveals that the residues that form or stabilize the binding pockets for phosphoserine and phenylalanine residues are among the most highly conserved among BRCA1 orthologs (68). And these regions strongly correlate with the location of cancer-associated mutations. Interestingly, clinically relevant missense mutations have been found within the two BRCT motifs of BRCA1 (27), implying a link between the function of BRCT domains and BRCA1-mediated tumor suppression (Table 1). Based on data from the Breast Cancer Information Database http://research.nhgri.nih.gov/bic/, the amino acids most frequently targeted for missense mutations in the BRCT repeats are Met1652, Arg1699, Ala17008, and Met1775. Interestingly, Arg1699 and Met1775 residues directly interact with residues in the BACH1 phosphopeptide, therefore these mutations may result in disrupted interactions between BRCT domains and their phosphorylated substrates.

Some cancer-associated mutations may destabilize the global BRCT fold whereas others are more likely to specifically interfere with ligand binding (3,27,69-71). Several mutations have been tested for binding to Ser-X-X-Phe and BACH1 peptides (summarized in Table 1) (40). Highly destabilizing truncation or missense mutations led to disrupted binding to phosphorylated peptides, demonstrating that correct folding is essential for the recognition of the phosphorylated target (39). In the instances where the missense mutations have little or no folding defects, the specificity or affinities of the interaction may be affected. For instance, the M1775R mutation that prevents BRCA1/BACH1 interaction, has exhibited not only decreased affinity for the BACH1 peptide, but also altered specificity, where a tyrosine residue at P+3 is preferred over Phe (40).

Clearly, examination of the effects of other missense mutations on the ability of BRCA1 BRCT to bind phosphorylated physiological targets will provide another

Table 1. Effect of BRCA1 BRCT missense mutations on BRCT folding and/or phosphopeptide binding

Missense mutations[1]	Position	pSer-X-X-Phe peptide binding[2]	BACH1 peptide binding[2]	Folding Defect[3]
M1652I	BRCT 1	+	?	-
F1695L	BRCT 1	+	?	-
R1699Q	BRCT 1	+/-	+/-	-
T1720A	BRCT 1	+	?	-
Y1853C	BRCT 2	+/-	-	++
C1697R	BRCT 1	+/-	?	++
A1708E	BRCT 1	+/-	?	++
S1715R	BRCT 1	+/-	?	++
W1718C	BRCT 1	+/-	?	++
P1749R	BRCT 2	+/-	-	++
G1738E	Linker	+/-	-	++
G1738R	Linker	+/-	?	++
V1809F	BRCT 2	+/-	?	+
D1692Y	BRCT 1	+/-	?	+
R1699W	BRCT 1	+/-	~ weak	+
R1751Q	BRCT 2	+	?	+
M1775R	BRCT 2	-	~ weak	+
M1783T	BRCT 2	+	?	+
S1655A	BRCT 1	?	-	?
S1655F	BRCT 1	-	-	?
K1702M	BRCT 1	?	-	?

[1]The mutants are grouped based on their effects solely on protein folding or peptide binding. [2]Ability to bind to phospho-peptide compared to non-phosphorylated counterpart: (+) bind specifically to phosphopeptide; (-) no binding; (+/-) no specific binding; (?) not determined (36,49,52,55). [3]Proteolytic sensitivity of the protein fold: (-) stability of the mutant indistinguishable from WT; (+) moderate destabilized; (++) severely destabilized; (?) not determined (70).

platform for studying the biochemical effects and clinical consequences of BRCT mutations in DNA damage proteins.

5. STRUCTURAL BASIS FOR PHOSPHOPEPTIDE INTERACTIONS

The first structural information for BRCT domains came from the crystal structure of the C-terminal BRCT domain of XRCC1 (72). In general, a single BRCT repeat forms a compact domain composed of a parallel four-stranded beta-sheet surrounded by a pair of alpha-helices (alpha1 and alpha3) on one side and a single alpha helix (alpha2) on the other (27,72) (Figure 1 and 2). Studies of the tandem BRCT domains of BRCA1 have shown that both repeats stack closely against each other through a large hydrophobic interface, giving rise to a deep surface cleft (27) (Figure 1). Sequence comparison and structural analysis revealed that this surface cleft is highly conserved among BRCA1 orthologs (68). In addition, the repeats are connected by a relatively flexible linker and packed in a head-to-tail manner that is conserved between human and rat BRCA1, as well as in the BRCT domain of p53BP1. To date, structural data have become available for the BRCT domains of BRCA1 (27,38-41), 53BP1 (29,30), DNA ligase III and IV (73,74), a NAD+-dependent DNA ligase (75), MDC1 (36,37), and BARD1 (76). To understand the molecular basis for phosphorylation-dependent interactions, we will focus on the BRCT structures that have been co-crystallized with phosphopeptides.

5.1. Structures of the BRCA1/BACH1 and MDC1/H2AX phosphopeptide complexes

The crystal and NMR structures of the BRCA1 tandem BRCT repeats in complex with the pSer BACH1 containing peptide have been solved, helping to illustrate the mechanism by which phosphopeptide recognition occurs (38-41). Overall, the structures of the unbound and BACH1-bound BRCT repeats are similar, with only a slight relative rotation of each BRCT domain and a translation of the N-terminal BRCT helix α1 toward the cleft between the domains in the presence of the peptide (40). The phosphorylated BACH1 peptide binds to this cleft, interacting with residues from both repeats, consistent with the requirement of both domains for efficient phosphopeptide binding (Figure 1 and 2). In addition, the structure illustrates the BRCT domain preference for pSer over pTyr, since the phospho recognition pocket appears too shallow to accept a bulky phenyl ring.

The structure also provides evidence for the observation that the binding of tandem BRCT domains to phosphorylated BACH1 requires both pSer and a Phe at the P+3 position (38-41). The N-terminal half of the BRCA1 interface has a basic pocket where pSer990 from BACH1 binds, through three hydrogen bonds involving the side chains of Ser1655 and Lys1702 and the backbone amide of Gly1656 located in the first BRCT repeat. Additionally, the C-terminal half of the interface has the aromatic side chain of Phe (at P+3) from BACH1 interacting with the hydrophobic pocket formed by the side chains of Phe1704, Met1775 and Leu1839. Additional hydrogen bonding with the main chain of Phe is supplied by main and side chain atoms from Arg1699.

Several residues (Ser1655, Lys1702, Phe1704, Met1775 and Leu1839) responsible for the recognition are conserved in BRCA1 orthologs, suggesting that phosphopeptide recognition may be an evolutionarily conserved function among BRCA1 proteins (Figure 2). In fact, Ala mutations of either Ser1655 or Lys1702 abolished the interaction with BACH1 and resulted in loss of function of BRCA1 in DNA damage-induced checkpoint control

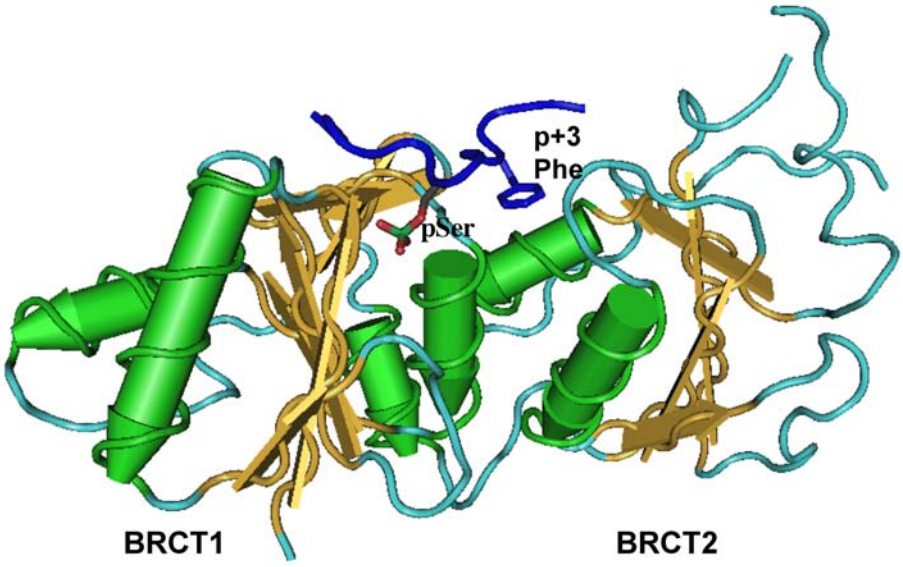

Figure 1. Structure of the BRCA1 tandem BRCT repeats bound to BACH1 phosphopeptide (38-41). The BRCT alpha helices are green, the beta strands are gold, and the phosphopeptide is blue (stick representation). The two BRCT repeats are connected by a linker region and arranged in tandem. The pSer-binding pocket is formed by residues on the BRCT1, whereas Phe at p+3 position sits into a hydrophobic groove formed by residues from both BRCT domains. Model was constructed using Cn3D4.1.1.

(38). However, the various members of this large family must be able to differentiate among the multitude of phosphorylated substrates in order to perform their distinct functions. A number of studies have examined the sequence conservation of several BRCT-motif containing proteins, including those from BARD1, BRCA1, and MDC1 (36-38,40,41,68,76). As expected, the residues in the BRCA1 BRCT domain that coordinate binding to pSer990 of BACH1 are highly conserved among these four proteins, whereas the residues that interact with Phe993 of BACH1 seem less well conserved (38-41) (Table 2). Such sequence variability may help to explain why different BRCT containing proteins were found to prefer different phosphopeptides, with the strongest selection at the P+3 position (13,14).

Notably, pSer990 of BACH1 exclusively interacts with the first BRCT domain, whereas Phe993 primarily interacts with the second BRCT through *van der Waals* contacts (38-41). It is therefore not surprising that tandem, rather than single, BRCA1 BRCT repeats are required for BACH1 phosphopeptide binding. For the few single BRCT domain containing proteins such as telomeric protein RAP1, the mechanism of how they achieve specificity remains unclear. On the other hand, single BRCT domains may form homo- or hetero-dimers (72,77). One possibility is that single BRCT containing proteins would homo- or hetero-dimerize to bind phosphorylated sequences in a manner similar to tandem BRCT repeats (38).

The recently solved crystal structure of MDC1 BRCT repeats bound to the H2AX phosphopeptide offers additional clues about phosphopeptide recognition by BRCT domains (36,37,41). The overall structure of MDC1-H2AX complex is similar to that of BRCA1-BACH1, where the BRCT-phosphate interaction interfaces are highly conserved. However, unlike BRCA1, MDC1 prefers Tyr residue at the P+3 position that is at the C-terminus of the H2AX peptide. The free carboxyl group of H2AX forms two salt bridges with Arg1933 on MDC1. Extension of the H2AX peptide by adding amino acids to the C-terminus of P+3 Tyr greatly reduced binding affinity, consistent with an essential role of the interaction between the free carboxyl group and MDC1 BRCT domains.

6. PREDICTIONS FOR PHOSPHOPEPTIDE RECOGNITION BY BRCT DOMAINS

6.1. Candidate phosphopeptide binding BRCT domains in human cells

While it remains unclear whether the BRCT domains as yet studied can mediate phosphorylation-dependent interactions as well, structural analyses of phosphopeptides-bound BRCT domains have provided us the opportunity to predict BRCT-phosphopeptide interactions. As discussed in detail in Sections 5, phosphopeptide recognition is achieved primarily through two key binding pockets formed by the tandem BRCT domains. The phosphoserine recognition pocket is formed by three residues on Lbeta1alpha1 and alpha2 from the first BRCT domain (Table 2). All the BRCT repeats known to bind phosphopeptides contain a (T/S)G motif and a K/N residue within Lbeta1alpha1 and alpha2 respectively. Based on these observations, we have predicted 5 additional putative phosphopeptide-binding BRCT repeats from human BRCTD1, TOPBP1, ECT2, and XRCC1. These BRCT repeats harbor either the (T/S)G or a closely related (T/S)S motif at the corresponding Lbeta1alpha1 positions (Table 2).

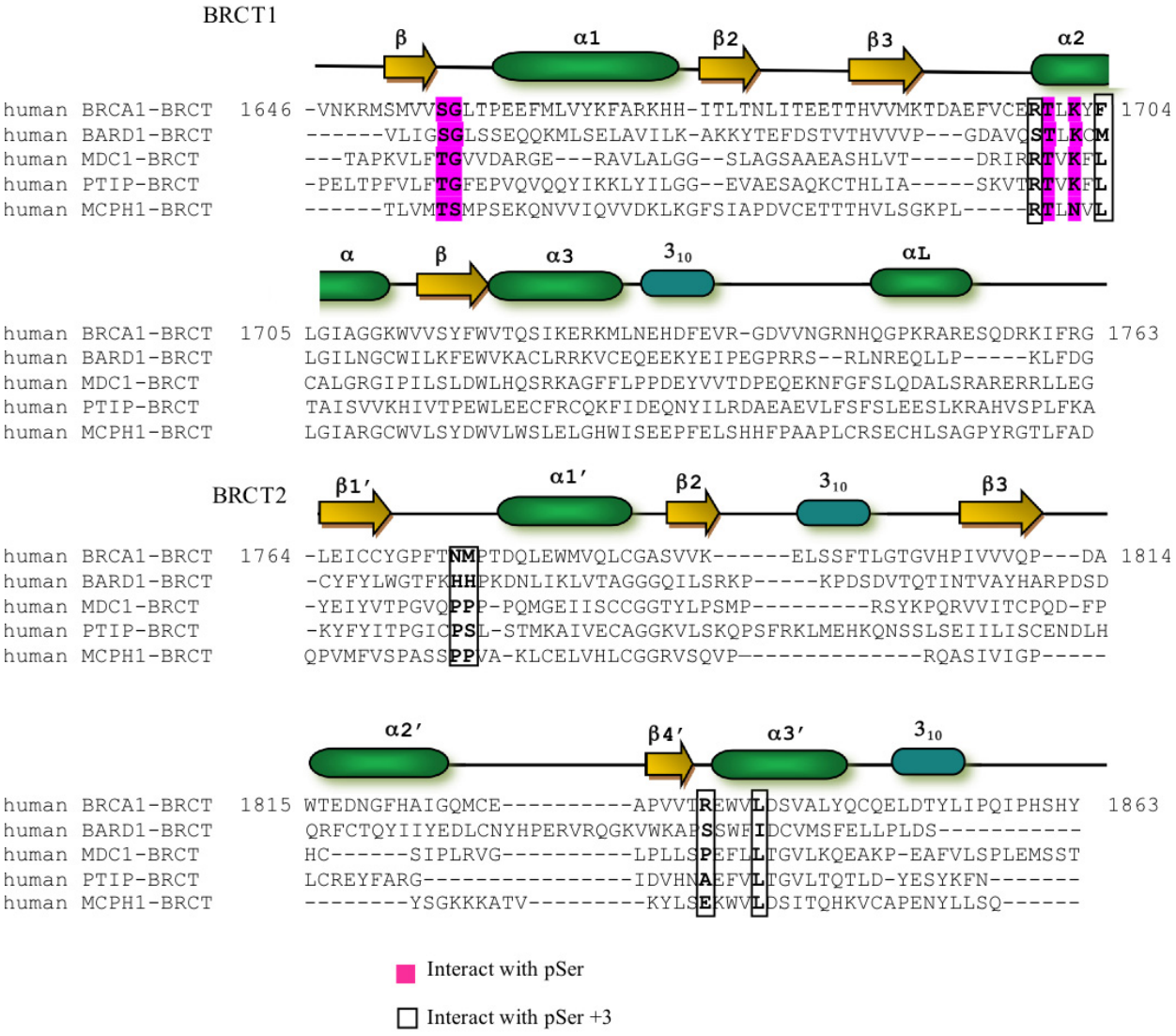

Figure 2. Amino acid sequence alignment of the BRCT repeats of BRCA1, BARD1, MDC1 PTIP, and MCPH1. The secondary structure and numbering are indicated for human BRCA1. Residues involved in binding to pSer are highly conserved among these proteins (pink shadow), whereas residues that interact with Phe at P+3 position seem less well conserved (black boxes) The positions of the BRCT BRCA1 missense mutations from Table1 are indicated here by asterisks. The alignment was generated using ClustalW from EMBL.

The other key binding pocket is involved in specificity determination of BRCT-phosphopeptide interaction. As revealed by the structures of phosphopeptides binding to BRCA1 or MDC1 BRCT domains, the P+3 residue (relative to pSer) plays an important role in governing the specificity of BRCT repeats (38-41). BRCA1 and MDC1 prefer Phe and Tyr respectively at this position (14). Unlike the phosphoserine-binding pocket that is mainly formed by residues from the first BRCT domain, the P+3 pocket is formed by residues from both the first and second BRCT domains (Table 2). In the BRCA1 BRCT structure, the Phe residue from alpha2, Met residue from Lbeta1'alpha1', and Leu residue from alpha3' contribute to Phe recognition at the pSer+3 position. In comparison, Leu of alpha2, Pro of Lbeta1'alpha1', and Leu of alpha3' help to coordinate the recognition of Tyr at the P+3 position in the MDC1-H2AX peptide structure. Interestingly, MCPH1 contains the same residues as MDC1 in the P+3 pocket and was shown recently to bind the phospho-H2AX peptide (66). These data lend support to utilizing the residues that make up the pSer+3 binding pocket for specificity prediction of BRCT domains. For example, the P+3 pocket residues from PTIP BRCT repeats (residues 560-757) are similar to those of MDC1. Accordingly, PTIP was shown to bind with high affinity peptides with Phe at the P+3 position (13). It is also possible that PTIP BRCT domains

Table 2. Predicted phosphopeptide-binding pockets for BRCT domains

BRCT domains	pSer pocket		P+3 pocket			Known Motif or peptides
	Lbeta1	alpha1 alpha2	Lbeta1'	alpha1' alpha2	alpha3'	
BRCA1	SG	K	M	F	L	pSPTF
MDC1	TG	K	P	L	L	pSQEY
MCPH1	SG	N	P	L	L	pSQEY
BARD1	SG	K	H	M	I	pSEDE?
ECT2	TG	K	E	R	W	?
PTIP (560-757)	TG	K	P	L	L	pSQVF pSQEY?
TOPBP1 (22-207)	TS	K	L	L	F	?
TOPBP1 (1177-1401)	SS	K	E	L	A	?
XRCC1	SG	K	E	S	Y	?
BRCTD1	TG	K	K	L	G	?
DNL4	SG	K	T	I	T	pSYYI?

Table 3. Potential *in vivo* binding sites for human BRCA1, MDC1, and PTIP BRCT domains

Phosphorylation sites	Protein	Potential BRCT domains
SPVY	ABL1M1	PTIP, MDC1
SQSY	ATM	PTIP, MDC1
SQSY	CD19	PTIP, MDC1
SQDY	CHD5	PTIP, MDC1
SQRY	FLJ10726	PTIP, MDC1
SQSY	FLJ12949	PTIP, MDC1
SQGY	MSH2	PTIP, MDC1
SISY	RNF19	PTIP, MDC1
SQDY	USP28	PTIP, MDC1
SQDF	ATE1	PTIP, BRCA1
SQKF	CENTB2	PTIP, BRCA1
SQQF	DDX17	PTIP, BRCA1
SQAF	KIAA2018	PTIP, BRCA1
SQEF	BCORL1	PTIP, BRCA1
SQKF	KIAA1840	PTIP, BRCA1
SQAF	LRG6	PTIP, BRCA1
SQDF	PFS2	PTIP, BRCA1
SQRF	PNKD	PTIP, BRCA1
SQNF	RAD23	PTIP, BRCA1
SQDF	RAD50	PTIP, BRCA1
SQSF	RIC8	PTIP, BRCA1
SQDF	SCML2	PTIP, BRCA1
SQSF	53BP1	PTIP, BRCA1
SQRF	VPS26B	PTIP, BRCA1

may interact with the phosphorylated tail of H2AX (Table 2). This may explain the finding that PTIP is targeted to phospho-H2AX DNA damage foci (13).

6.2. Potential *in vivo* sites for human BRCT domains

Many of the BRCT-containing proteins have been implicated in DNA damage and repair pathways. Because ATM and ATR are major regulators of DNA damage and repair, it is likely that BRCT domains bind to phosphorylated Ser or Thr sites that are substrates of these two kinases. Recent advances in mapping endogenous phosphorylation sites by ATM and ATR kinases in human cells have provided a reservoir of potential binding sites for BRCT repeats (35). It would be of great interest in the near future to match these phosphorylation sites with different BRCT domains and assemble a phosphorylation-mediated interaction network, in order to better understand signal transduction initiated by DNA damages. In addition to bench work, one can start to predict such interactions based on known properties of different BRCT domains. In Table 3, we have listed potential interacting sites of BRCA1, MDC1, and PTIP BRCT domains based on the data set from Matsuoka *et al.* These known phosphorylation sites contain either Phe or Tyr at the P+3 position.

7. SUMMARY AND PERSPECTIVE

Among the majority of the proteins studied so far, tandem BRCT domains appear to be required for binding to phosphopeptides. However, single BRCT repeats as found in the DNA polymerase REV1, the phosphatase FCP1, NBS1 and DNA ligase III have also been proposed to interact with phosphopeptides based on *in vitro* binding experiments (33). Therefore the function of single BRCT domain and whether they (perhaps in conjunction with other domains) can bind phosphorylated sequences remain unknown. Here we have also listed a number of candidate BRCT domains including the tandem BRCT domains of XRCC1 that are predicted to interact with phosphopeptides. It will be of great interest to determine whether these predictions are correct and if so, the specificities of these interactions.

Due to the lack of space, we did not discuss at all the DNA-binding activities of BRCT domains. One important question to ask is how BRCT modules might function as a molecular sensor by direct or indirect recognition of particular DNA structures. As noted above, some cancer relevant mutations appear to affect the affinity as well as specificity of BRCT-mediated interactions. Are

these changes pertinent to cancer? Numerous studies have helped to map out the vast interaction networks mediated by BRCT domain containing proteins such as BRCA1 and MDC1. How do phospho-dependent interactions regulate the assembly/dis-assembly of the complexes as well as the ordered recruitment/translocation of signaling molecules to sites of responses? Many of the BRCT proteins were found at the DNA damage foci and known to bind phosphorylated H2AX tail. What is the order of recruitment of different BRCT domain proteins? Do they function as nucleating sites to initiate different signals or are they recruited at different times during the repair process? These questions and many more await for further biochemical and cellular studies.

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