Homo-oligomerization Is the Essential Function of the Tandem BRCT Domains in the Checkpoint Protein Crb2*

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BRCT (BRCA1 C terminus) domains are frequently found as a tandem repeat in proteins involved in DNA damage responses, such as *Saccharomyces cerevisiae* Rad9, human 53BP1 and BRCA1. Tandem BRCT domains mediate protein-protein and protein-DNA interactions. However, the functional significance of these interactions is largely unknown. Here we report the oligomerization of *Schizosaccharomyces pombe* checkpoint protein Crb2 through its tandem BRCT domains. Truncated Crb2 without BRCT domains is defective in DNA damage checkpoint signaling. However, addition of either of two heterologous dimerization motifs largely restores the functions of truncated Crb2 without BRCT domains. Replacement of Crb2 BRCT domains with a dimerization motif also renders cells resistant to the dominant negative effect of overexpressing Crb2 BRCT domains. These results demonstrate that the crucial function of the tandem BRCT domains is to oligomerize Crb2.

The BRCT* domain was first identified as a two-copy repeat in the C-terminal regions of mammalian BRCA1, 53BP1, and *Saccharomyces cerevisiae* Rad9 (1). Subsequently this conserved domain was found in many more proteins, most of which are involved in DNA damage responses (2, 3). BRCT domains participate in binding to both BRCT and non-BRCT proteins. For example, *S. cerevisiae* Rad9 homo-oligomerizes through its tandem BRCT domains (4). Human DNA repair proteins XRCC1 and DNA ligase III engage in heterodimeric interactions through their C-terminal regions, each containing a single BRCT domain (5). The tandem BRCT domains in 53BP1 are necessary and sufficient for its interaction with the non-BRCT protein p53 (6, 7). Probably the ultimate example of the binding versatility of BRCT domains is the tandem BRCT domains in BRCA1, which interact with more than ten different proteins (8, 9). Recent studies showed that BRCT domains preferentially bind to phosphorylated protein partners as well as peptide libraries with phosphoserine or phosphothreonine residues (10, 11), indicating that BRCT domains may act as phosphopeptide binding modules. In addition to the roles in protein interactions, BRCT domains have also been shown to bind broken DNA ends (12, 13). The accumulating knowledge on the binding capabilities of BRCT domains calls for a better understanding of the functional significance of these interactions. However, out of a myriad of interactions that BRCT domains are postulated to participate in, few have been indisputably linked to the physiological functions of the proteins bearing these domains.

*Schizosaccharomyces pombe* Crb2/Rhp9 was identified in a methylethylmethane sulfonate-sensitive mutant screen and in a screen for Cut5-interacting proteins (14, 15). It shares sequence and functional similarity with *S. cerevisiae* Rad9, human 53BP1, and BRCA1. The strongest homology among these proteins is in their C-terminal tandem BRCT domains (14, 15). Structural studies have shown that the tandem BRCT domains in BRCA1 and 53BP1 adopt very similar structures and the two BRCT repeats pack onto each other to form a single globular unit (6, 7, 16). The conservation of the key residues in the inter-BRCT repeat interface has led to the notion that Crb2, Rad9, 53BP1, and BRCA1 share a common ancestor with tandem BRCT domains (7). Functionally, these BRCT proteins have been proposed to act as adapters or mediators that recruit a subset of substrates to the ATM and ATR family checkpoint kinases (17–19). In addition, BRCA1, 53BP1, and Crb2 have been shown to relocalize to DNA damage-induced nuclear foci and are likely to participate in checkpoint signaling and/or DNA repair at sites of damage (20–22). We have recently shown that large scale recruitment of Crb2 to sites of DNA damage requires its association with histone H2A that has been phosphorylated at its C terminus by Rad3 and Tel1, the fission yeast homologs of ATR and ATM, respectively (23).

While the tandem BRCT domains are the most prominent structural feature of Crb2 and related proteins, the exact roles of the BRCT domains in these proteins remain unclear. Here we report the ability of the tandem BRCT domains of Crb2 to homo-oligomerize. We show that a heterologous dimerization motif can effectively substitute for the tandem BRCT domains in Crb2, demonstrating that the crucial function of the BRCT domains is to mediate a Crb2-Crb2 interaction.

**EXPERIMENTAL PROCEDURES**

*Plasmids—*Crb2 yeast two-hybrid constructs containing amino acids 1–275, 1–520, 1–778, 276–520, 276–778, and 521–778 were produced by PCR amplification of the corresponding DNA sequences followed by insertion into pGBK7 (bait vector, Clontech) and pGADT7 (prey vector, Clontech). GST and Crb2 overexpression constructs were made by cutting pKM283 (24) or Crb2 two-hybrid plasmids with NdeI/BamH1 and ligating the insert into pREP1 (25). The N-terminal TAP tag was generated by PCR using pBS1479 as template (26). For cotransformation with pREP1-based plasmids or for transforming Leu† marker (28). The *CRB2* promoter and terminator constructs were based on the vector pJK148, which contains the LEU2 marker in some pREP1-based constructs was changed to *his3* by swapping in a *PstI-EcoV* fragment from pJR1-3XH (27). The crb2* integrating constructs were based on the vector pBS1479 as template (26). For cotransformation with pREP1-based plasmids or for transforming Leu† marker (28). The *CRB2* promoter and terminator constructs were based on the vector pJK148, which contains the LEU2 marker in some pREP1-based constructs was changed to *his3* by swapping in a *PstI-EcoV* fragment from pJR1-3XH (27).

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sequences between crb2$^+$ and its neighboring genes. DNA encoding the
Gen4 leucine zipper motif was obtained by PCR using S. cerevisiae
genomic DNA as template. The C-terminal leucine zipper fusion tag
contains the last 22 amino acids of Gen4 (amino acids 250–281) pre-
ceded by a Gly-Gly-Gly-Ala-Ala linker. Chkl-leucine zipper fusion con-
struct was based on pAL130 provided by A. Lopez-Girona. Plasmid
pAL130 contains the LEU2 marker, a genomic sequence including 450
bp 5'-untranslated region and chkl coding regions, a Myc2-HA-His$^+$
tag fused in-frame at the C terminus, and the nmt1 terminator.

Yeast Strains—Strains used in this study are listed in Table I. Crb2
integrating plasmids were cut with Nru1 and integrated at the leu1–32
locus. The chkl–myc-LEU2 and chkl–myc-LZ-LEU2 plasmids were cut with NdeI and integrated at the chkl::ura4$^+$ locus. Culturing
conditions were as described (29).

Yeast Two-hybrid Screen—The screen was carried out according to
the manual of the MATCHMAKER System 3 (Clontech). AH109 cells
containing the bait plasmid were transformed with a S. pombe cDNA
library (Clontech) and His$^+$, Ade$^+$ colonies were selected.

GST Pull-down Assay—Overexpression of proteins with the nmt1
promoter was achieved by growing cells in EMM medium without
thiamine for 15 h at 30 °C. Cells were broken with FastPrep system
(Qbiogene) in lysis buffer (50 mM HEPES-NaOH, pH 7.4, 100 mM NaCl,
1% Nonidet P-40, 10% glycerol, 1 μM pepstatin). Lysates were
beads (Amersham Biosciences). After washing five times with lysis
buffer, bound proteins were eluted by boiling in SDS-PAGE loading
buffer, separated on a 10% gel, and transferred onto nitrocellulose
membrane. GST and GST-tagged proteins were detected by a polyclonal
anti-GST antibody (Amersham Biosciences). TAP-tagged proteins were
detected by a peroxidase-antiperoxidase complex (Sigma).

Microscopy—Fluorescence microscopy was performed as described
using a DeltaVision optical sectioning microscope (22). Cells were
grown in liquid EMM medium at room temperature and photographed
at eight Z-sections 0.5-μm apart. The Z sections were projected into one
image using the maximum intensity method.

RESULTS

In an attempt to identify Crb2 interacting proteins in S. pombe, we carried out a yeast two-hybrid screen. We found that
full-length Crb2 (778 amino acids) and a N-terminal fragment of
Crb2 (amino acids 1–275) had a tendency to self-activate reporter
gene expression. Thus, we used a N-terminally truncated Crb2
containing amino acids 276–778 as bait for our screen. One positive
plasmid was isolated 9 times in the screen. It contained a C-terminal
portion of the coding region and the 3'-untranslated region of crb2$^+$. The Crb2 coding region in this plasmid started at
glycine 465. Therefore, the two interacting fragments of Crb2

\begin{table}
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\begin{tabular}{ll}
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Strain   & Genotype                                  \\
\hline
KS1598   & h$^-$ leu1–32                            \\
LLD3259  & h$^-$ leu1–32 ura4-D18 crb2-D2::ura4$^+$  \\
LLD3423  & h$^-$ leu1–32 ura4-D18 his3-D1 crb2-D2::ura4$^+$  \\
LLD3424  & h$^-$ leu1–32::crb2(1–320)-leu1 ura4-D18 crb2-D2::ura4$^+$  \\
BM3425   & h$^-$ leu1–32::crb2(1–520)-LZ-leu1 ura4-D18 crb2-D2::ura4$^+$  \\
LLD3426  & h$^-$ leu1–32::GST-crb2(1–520)-leu1 ura4-D18 crb2-D2::ura4$^+$  \\
LLD3427  & h$^-$ leu1–32 ura4-D18 chkl1–myc::ura4$^+$  \\
LLD3428  & h$^-$ leu1–32 ura4-D18 chkl1–myc::ura4$^+$  \\
LLD3429  & h$^-$ leu1–32::crb2(1–520)-leu1 ura4-D18 chkl1–myc::ura4$^+$  \\
LLD3430  & h$^-$ leu1–32::crb2(1–520)-LZ-leu1 ura4-D18 chkl1–myc::ura4$^+$  \\
LLD3431  & h$^-$ his3-D1                                \\
LLD3432  & h$^-$ leu1–32::GST-crb2(1–520)-leu1 ura4-D18 his3-D1 crb2-D2::ura4$^+$  \\
LLD3433  & h$^-$ leu1–32::crb2(1–520)-LZ-leu1 ura4-D18 his3-D1 crb2-D2::ura4$^+$  \\
LLD3434  & h$^-$                                          \\
LLD3435  & h$^-$ leu1–32 ura4-D18 chkl1::ura4$^+$ chkl1–myc::LEU2  \\
LLD3436  & h$^-$ leu1–32 ura4-D18 chkl1::ura4$^+$ chkl1–myc-LZ-LEU2  \\
LLD3437  & h$^-$ leu1–32 ura4-D18 chkl1::ura4$^+$ chkl1–myc::LEU2 crb2-D2::ura4$^+$  \\
LLD3438  & h$^-$ leu1–32 ura4-D18 chkl1::ura4$^+$ chkl1–myc-LZ-LEU2 crb2-D2::ura4$^+$  \\
LLD3439  & h$^-$ leu1–32::crb2(1–520)-leu1 ura4-D18 chkl1::ura4$^+$ chkl1–myc-LZ-LEU2 crb2-D2::ura4$^+$  \\
LLD3440  & h$^-$ leu1–32::crb2(1–520)-leu1 ura4-D18 chkl1::ura4$^+$ chkl1–myc-LZ-LEU2 crb2-D2::ura4$^+$  \\
LLD3496  & h$^-$                                          \\
LLD3498  & h$^-$ leu1–32::2×YFP-crb2–leu1 ura4-D18  \\
               & crb2-D2::ura4$^+$  \\
LLD3499  & h$^-$                                          \\
LLD3496  & h$^-$ leu1–32::2×YFP-crb2(1–520)-LZ-leu1 ura4-D18 crb2-D2::ura4$^+$  \\
\hline
\end{tabular}
\caption{S. pombe strains used in this study}
\end{table}

\begin{figure}
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\includegraphics[width=\textwidth]{fig1}
\caption{Crb2 interacts with itself through C-terminal tandem BRCT domains. A. Crb2 BRCT domains self-interact in a yeast two-hybrid assay. AH109 cells (Clontech) transformed with bait constructs were mated with Y187 cells (Clontech) transformed with prey constructs, and the mated cells were replica-plated onto selective plates (SC-Trp-Leu-His) supplemented with 2.5 mM 3-amino triazole, which allow growth only when bait and prey interact. B, TAP-tagged Crb2 BRCT domains copurified with GST-tagged Crb2 BRCT domains but not with GST. C, Id lysates made from crb2-D3 strains over-expressing these proteins with nmt1 promoter were incubated with glutathione beads and the bound proteins were eluted with SDS-PAGE loading buffer.}
\end{figure}
both contained the C-terminal tandem BRCT domains. This result suggested that Crb2 may interact with itself through its BRCT domains and prompted us to examine the functional consequence of Crb2 homo-oligomerization.

To directly test whether the BRCT domains of Crb2 self-interact, we constructed two-hybrid plasmids expressing a C-terminal fragment containing only the tandem BRCT repeats (Crb2-(521–778)). This fragment was able to self-interact in a two-hybrid assay (Fig. 1A), indicating that the tandem BRCT domains are sufficient to mediate oligomerization of Crb2. As controls, this fragment did not interact with other known Crb2-interacting proteins, such as Cut5 and Chk1 in our two-hybrid assay, whereas the N-terminal fragment of Crb2 interacted with Cut5 and the longer fragments encompassing the middle region interacted with Chk1 (Fig. 1A). Rad3 has been shown to interact with the BRCT domains of Crb2 (30). However, Rad3 did not interact with any Crb2 fragments in our two-hybrid assay, although it did interact with Rad26, a known Rad3-interacting protein (Fig. 1A).

To confirm the interaction mediated by the BRCT domains with an independent method, we expressed Crb2-(521–778) fused at N terminus with a Schistosoma japonicum GST tag and Crb2-(521–778) fused at N terminus with a tandem affinity purification (TAP) tag together in a crb2/H9004 strain. When we precipitated GST-tagged BRCT domains from a cell lysate with glutathione beads, TAP-tagged BRCT domains were precipitated as well (Fig. 1B). As a control, when GST was expressed with TAP-Crb2-(521–778), we did not detect the TAP-tagged

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**Fig. 2.** BRCT domains are important for the function of Crb2 and substitution of BRCT domains with a heterologous dimerization motif largely maintains the function of Crb2. A, GST-tagged Crb2 fragment missing the BRCT domains overexpressed from a pREP1-based plasmid rescued the UV sensitivity of crb2Δ strain effectively, whereas overexpressed TAP-tagged fragment only partially rescued. B, Crb2 fragments missing the BRCT domains were stably expressed from an integrated plasmid with the crb2 promoter. Top, Western blot of cell lysates probed with anti-Crb2-(1–275) antibody. Bottom, Ponceau S stain of the same membrane used for the Western blot. Strains used were: wild-type, K81598; crb2Δ, LLD3259; crb2(1–520), LLD3424; crb2(1–520)-LZ, BM3425; GST-crb2(1–520), LLD3428. C, UV sensitivity of strains stably expressing Crb2 fragments scored by percentage of cells that survived the UV treatment. D, UV sensitivity of strains stably expressing Crb2 fragments scored by a spot assay. 5-fold serial dilutions of cells were spotted on YES plates and irradiated. E, BRCT domains are required for efficiently arresting cell cycle progression in response to DNA damage caused by bleomycin, and leucine zipper or GST can substitute for the BRCT domains. Strains stably expressing Crb2 fragments were treated with 5 milliunits/ml bleomycin in YES medium at 30 °C for 2.5 h. Cells were then fixed with formaldehyde and stained with DAPI (4,6-diamidino-2-phenylindole). Arrowheads indicate dividing cells with septa. Bar, 5 μm. F, BRCT domains are required for efficient Chk1 modification in response to IR treatment, and leucine zipper can substitute for the BRCT domains. Strains stably expressing Crb2 fragments and Myc-tagged Chk1 were treated with or without 90 Gy of γ-irradiation. Cell lysates were separated on SDS-PAGE and probed with anti-Myc (9E10) antibody. Strains used were: wild-type, LLD3427; crb2Δ, LLD3428; crb2(1–520), LLD3429; crb2(1–520)-LZ, LLD3430.
fragment in the pull-down fraction (Fig. 1B). We also tried to detect interactions between two differently tagged full-length Crb2 proteins in cell lysates. No significant interaction was observed either with or without ionizing radiation (IR) treatment. We note here that the majority of full-length Crb2 in cell lysates is insoluble and it is possible that the oligomerized Crb2 is predominantly distributed in the insoluble fraction.

To assess the functional significance of BRCT-domain-mediated oligomerization, we first overexpressed truncated Crb2 without BRCT domains from the nmt1 promoter of an episomal pREP1-based plasmid in a crb2Δ background. N-terminally TAP tagged Crb2(1–520) fragment only partially rescued the UV sensitivity of crb2Δ cells (Fig. 2A), consistent with a recent report showing that a BRCT truncation impairs the checkpoint function of Crb2 (30). However, N-terminally GST tagged Crb2(1–520) rescued the UV sensitivity of crb2Δ cells to wild-type level with the UV doses that we tested (Fig. 2A). One possible explanation for this dramatic difference between the two fusion proteins is that GST is known to form homodimer in solution and has been used as a heterologous dimerization module (31, 32). Therefore we hypothesized that the essential function of tandem BRCT domains might be to oligomerize Crb2, and this function can be fulfilled by a heterologous dimerization domain.

We wanted to make certain that the ability of GST-Crb2(1–520) to complement crb2Δ was not caused by overexpression or due to a property of GST other than its ability to dimerize. To this end, we created strains that stably expressed different Crb2 fragments under the control of the crb2Δ promoter by integrating plasmids into chromosomes of crb2Δ cells (Fig. 2B). In the control strain, Crb2(1–520), a fragment of Crb2 without its BRCT domains, was expressed at a level a few fold higher than that of endogenous Crb2 in a wild-type strain. In another strain, GST-Crb2(1–520) was expressed at a level similar to that of endogenous Crb2. We also constructed a strain that expressed Crb2(1–520) fused at its C terminus to the leucine zipper motif (LZ) of S. cerevisiae Gcn4, which is known to mediate homodimerization (33). Crb2(1–520)-LZ was expressed at a level similar to that of Crb2(1–520).

We examined the sensitivity of these strains to UV radiation (Fig. 2, C and D). The crb2(1–520) strain was only slightly more resistant than the crb2Δ strain, whereas both GST-crb2(1–520) and crb2(1–520)-LZ strains were as resistant to UV radiation as wild type up to the dose of 100 J/m². At 150 J/m², GST-crb2(1–520) was more sensitive than wild type, suggesting that GST can not completely replace the BRCT domains. We further tested the ability of these strains to arrest cell cycle progression in the presence of DNA damage. When the radiomimetic drug bleomycin was applied to cells, GST-crb2(1–520) and crb2(1–520)-LZ strains behaved like wild type, uniformly arresting as mononuclear elongated cells, whereas crb2(1–520) and crb2Δ strains failed to arrest and generated dividing cells with unevenly distributed DNA (Fig. 2E), a morphology typical of checkpoint deficient S. pombe mutants treated with bleomycin (34). Crb2 is required for the activation of checkpoint kinase Chk1 (15), therefore we examined whether substituting BRCT domains with a heterologous dimerization motif affects the ability of Crb2 to activate Chk1. In response to IR, we observed a slow migrating form of Chk1 when lysates from wild-type and crb2(1–520)-LZ strains were run on SDS-PAGE, indicating that Chk1 was activated in response to DNA damage in these strains. No shift of Chk1 upon IR treatment was observed with crb2(1–520) and crb2Δ strains. Taken together, these results strongly indicate that the essential function of Crb2 BRCT domains can be carried out by a heterologous dimerization motif.

Fluorescence microscopy study showed that Crb2 forms distinct nuclear foci in response to DNA damage and these foci represent sites of DNA damage (22). Crb2 focus formation requires histone H2A C-terminal tail phosphorylation by Rad3 or Tel1 kinases (23). However, the lack of Crb2 focus formation does not prevent H2A phosphorylation site mutant from robust checkpoint activation in response to DNA damage. To examine whether BRCT domains are needed for Crb2 focus formation, we tagged full-length and truncated Crb2 with YFP and visualized live cells with fluorescence microscopy (Fig. 3A). Full-length Crb2 formed damage-induced nuclear foci after 36 Gy of IR treatment. In contrast, neither Crb2(1–520) nor Crb2(1–520)-LZ formed discernible foci before or after IR treatment, suggesting that BRCT domains are required for efficient Crb2 focus formation and leucine zipper motif cannot substitute for this nonessential function.

In response to DNA damage, Crb2 undergoes hyperphosphorylation manifested by reduced mobility on SDS-PAGE (15). To investigate whether BRCT domains are needed for Crb2 hyperphosphorylation, we treated cells expressing full-length and truncated Crb2 with IR and examined the mobility of Crb2 on SDS-PAGE (Fig. 3B). Only marginal mobility change of Crb2(1–520), Crb2(1–520)-LZ, and GST-Crb2(1–520) was observed after 180-Gy IR treatment, as opposed to the far more pronounced shift displayed by full-length Crb2. Our results are consistent with a recent report showing that hyperphosphorylation of Crb2 requires the BRCT domains (30). Histone H2A C-terminal tail phosphorylation site mutations not only largely abolish Crb2 focus formation, but also significantly diminish
the DNA damage-induced mobility change of Crb2 on SDS-PAGE, suggesting that phosphorylation of Crb2 may be facilitated by its accumulation at sites of DNA damage (23). It is possible that the loss of focus formation makes the truncated Crb2 less accessible to upstream kinases. Alternatively, truncation of BRCT domains may have removed certain phosphorylation sites. The dimerization motifs can largely rescue the checkpoint defects of BRCT truncated Crb2 but fail to rescue the focus formation and hyperphosphorylation defects, indicating that neither focus formation nor the majority of hyperphosphorylation are critical for the checkpoint functions of Crb2.

If the BRCT domains of Crb2 are sufficient for its oligomerization, and if oligomerization of Crb2 is needed for its function, one would expect that overexpression of BRCT domains in wild-type background would likely disrupt the oligomeric structure of wild-type full-length Crb2 and cause a DNA damage-sensitive phenotype. As expected, when Crb2-(521–778) fragment was overexpressed under the control of nmt1 promoter, cells with wild-type Crb2 became more sensitive to UV radiation (Fig. 4). In contrast, when the same fragment was overexpressed in cells with Crb2-(1–520)-LZ or GST-Crb2-(1–520) as the only copy of Crb2, the UV sensitivity of these cells did not change significantly, suggesting that overexpressed BRCT domains bring about the dominant negative effect through the interaction with full-length Crb2, but not with other cellular targets. Moreover, we found that overexpression of GST specifically made the GST-crb2-(1–520) strain more sensitive to UV radiation, likely due to the disruption of GST-Crb2-(1–520) dimer. This result demonstrated again that GST can substitute for BRCT domains because of its ability to dimerize.

Crb2 is required for the activation of Chk1 and it directly interacts with Chk1 (15), therefore we hypothesized that forcing Chk1 to dimerize may rescue the loss of Crb2 BRCT domain. To test this idea, we C-terminally tagged Chk1 at its own chromosomal locus with either Myc tag alone or Myc tag plus the leucine zipper motif of Gcn4. Neither tag changed the UV sensitivity of wild-type or crb2Δ strains (Fig. 5). However, tagging Chk1 with Gcn4 leucine zipper motif significantly boosted the ability of crb2-(1–520) strain to survive UV radiation, indicating that constitutively dimerizing Chk1 partially bypassed the requirement for Crb2 BRCT domains.

**DISCUSSION**

In this report we showed that the BRCT domains of Crb2 oligomerize. We demonstrated that the main function of the BRCT domains is to oligomerize Crb2 since they can be replaced by a heterologous dimerization motif.

The Crb2 homolog in S. cerevisiae, Rad9, oligomerizes through its tandem BRCT domains (4), suggesting that the oligomerization function of BRCT domains is conserved between these checkpoint proteins in two distantly related fungi species. The Rad9 study used mutations at the aromatic residues most invariant in BRCT domains to demonstrate a correlation between the loss of Rad9 oligomerization and the loss of its checkpoint function. In the crystal structures of the BRCT domains of XRCC1 and BRCA1, these conserved aromatic residues are part of the hydrophobic core of the BRCT fold and provide crucial structure-stabilizing roles (5, 16, 35). Mutating these residues probably impairs the folding of the BRCT repeat as a whole and may be less likely to disrupt a specific binding site on the surface of the protein. Hence these mutants may fail to engage not only the Rad9-Rad9 interaction, but also potential interactions with other unknown Rad9 partners. The possibility that the defects of these mutants are due to the loss of certain unknown interactions therefore cannot be ruled out.

In contrast, our results here provide explicit evidence that the essential function of BRCT domains in Crb2 is to homo-oligomerize the protein.

Crb2 undergoes damage-induced hyperphosphorylation (15). The sites of damage-induced phosphorylation on Crb2 have not been mapped. Our results suggested that BRCT domains are required for damage-induced hyperphosphorylation of Crb2 but BRCT-dependent hyperphosphorylation of Crb2 is largely dispensable for the checkpoint functions of Crb2, particularly the activation of downstream kinase Chk1. This finding is consistent with several previous observations. First, the mutation of a Cdc2 phosphorylation site on Crb2, T215A, significantly diminishes the damage-induced hyperphosphorylation of Crb2 but does not affect Chk1 activation (36). Second, histone H2A C-
terminal phosphorylation site mutations also markedly reduce the hyperphosphorylation of Crb2 but leave the checkpoint arrest response largely intact (23). In addition, mutations of the S/T/Q cluster in S. cerevisiae Rad9 abolish most of the damage-induced hyperphosphorylation of Rad9 but have no effect on the activation of S. cerevisiae Chk1 (37). It was shown recently that the BRCT domains in BRCA1 and other BRCT proteins specifically associate with phosphopeptides (10, 11). It is unclear to what extent this type of phosphorylation dependent binding accounts for the known interactions involving BRCT domains. S. cerevisiae Rad9 BRCT domains appeared to preferentially interact with hyperphosphorylated Rad9 generated after DNA damage (4). The BRCT domain-dependent hyperphosphorylation of Crb2 may also regulate the homo-oligomerization of wild type Crb2. Such regulation, if it exists, seems to be dispensable because BRCT domains can be effectively substituted by the heterologous dimerization motifs. However, it is formally possible that the dimerization motif-mediated Crb2-Crb2 interactions are still regulated in a damage-dependent manner through residual phosphorylation of Crb2 (1–520), or the interactions among Cut5, Chk1, and Crb2 (1–520).

Crb2 and its related BRCT proteins have been proposed to act as adaptors to transduce the checkpoint signal from upstream ATM and ATR family kinases to downstream effector kinases (17, 18). In S. cerevisiae, the protein complex containing hyperphosphorylated Rad9 appears to facilitate the activation of the effector kinase Rad53 by recruiting multiple Rad53 molecules and thereby enhancing their intermolecular autophosphorylation (38). According to this model, oligomerization of Rad9 through BRCT domains may be needed to generate a high local concentration of Rad53. In S. pombe, Chk1 is the effector kinase acting downstream of Crb2, and the two proteins physically interact with each other (15, 30). The oligomerization of Crb2 through BRCT domains therefore would therefore be able to bring multiple Chk1 molecules together. It is known that DNA damage-induced activation of Chk1 requires the phosphorylation of Chk1 by Rad9 (39, 40). We speculate that the maximal level of activation of Chk1 may also require intermolecular autophosphorylation, which can be aided by the oligomerization of Crb2. Alternatively, the higher order complex containing multiple Crb2 and Chk1 molecules may adopt a specific conformation better suited for the phosphorylation by Rad3.

Even though a heterologous dimerization motif can effectively substitute for the tandem BRCT domains in Crb2, we have observed differences between the dimerization motif fusion proteins and the wild-type protein. The dimerization motifs cannot rescue the defects of Crb2(1–520) in DNA damage induced focus formation and hyperphosphorylation. These defects do not seem to severely compromise the main functions of Crb2 in checkpoint activation and resistance to genotoxic insult. However, subtle phenotypic difference caused by these defects may be sufficient to reduce the biological fitness of the organism in its native environment. Thus, these nonessential functions of BRCT domains still warrant further study. Because Crb2 focus formation also requires histone H2A tail phosphorylation, it will be of interest to investigate whether BRCT domains are involved in the interactions between Crb2 and phosphorylated H2A (23).

The tandem BRCT domains in both S. pombe Crb2 and S. cerevisiae Rad9, proteins from two distantly related organisms, mediate homo-oligomerization important for their function. These findings suggest that oligomerization may be required for the function of other related checkpoint proteins. Even though the BRCT domains in human 53BP1 and BRCA1 do not seem to engage in homotypic association, other domains of these proteins or their binding partners may have taken over the role of the oligomerization motif during evolution. For example, the N-terminal region of BRCA1 is able to mediate homodimerization (41). It will be of interest to evaluate the functional importance of oligomerization for these human proteins.

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