Role of a BRCT domain in the interaction of DNA ligase III-α with the DNA repair protein XRCC1

Richard M. Taylor, Bill Wickstead, Sam Cronin and Keith W. Caldecott

The BRCT domain (for BRCA1 carboxyl terminus) is a protein motif of unknown function, comprising approximately 100 amino acids in five conserved blocks denoted A–E. BRCT domains are present in the tumour suppressor protein BRCA1 [1–3], and the domain is found in over 40 other proteins, defining a superfamily that includes DNA ligase III-α and the essential human DNA repair protein XRCC1. DNA ligase III-α and XRCC1 interact via their carboxyl termini, close to or within regions that contain a BRCT domain [4]. To examine whether the primary role of the carboxy-terminal BRCT domain of XRCC1 (denoted BRCT II) is to mediate the interaction with DNA ligase III-α, we identified the regions of the domain that are required and sufficient for the interaction. An XRCC1 protein in which the conserved D-block tryptophan was disrupted by point mutation retained the ability to interact with DNA ligase III-α, so this tryptophan must mediate a different, although conserved, role. XRCC1 in which the weakly conserved C-block was mutated lost the ability to interact with DNA ligase III-α. Moreover, 20 amino acids spanning the C-block of BRCT II conferred full DNA ligase III-α binding activity upon an unrelated polypeptide. An XRCC1 protein in which this 20mer was deleted could not maintain normal levels of DNA ligase III-α in transfected rodent cells, a phenotype associated with defective repair [5]. In summary, these data demonstrate that a BRCT domain can mediate a biologically important protein–protein interaction, and support the existence of additional roles.

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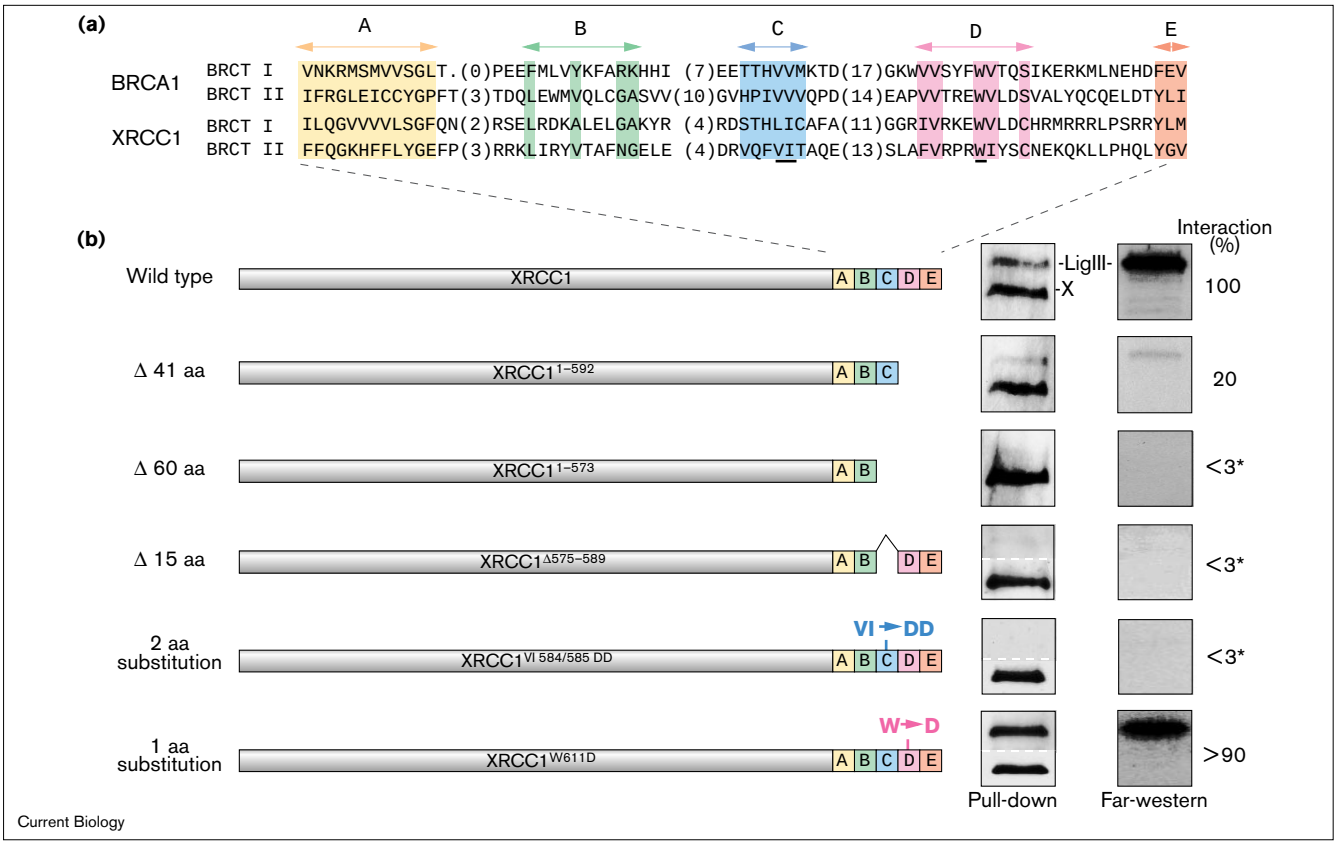
Results and discussion

To test the proposal that BRCT domains serve to mediate protein–protein interactions [1,2,6], we have disrupted a BRCT domain. We generated targeted deletions and point mutations within the BRCT II domain of XRCC1 to identify the motifs that are required and sufficient for the interaction of XRCC1 with DNA ligase III-α [4,7,8]. Wild-type and mutant histidine (His)-tagged human XRCC1 polypeptides were purified from *Escherichia coli* and compared for their ability to interact with human DNA ligase III-α by 'pull-down' assays and 'far-western' blotting (Figure 1). The interaction of DNA ligase III-α with a truncated XRCC1 polypeptide, $XRCC1^{1–592}$, was reduced fivefold compared with full-length XRCC1, and the interaction of DNA ligase III-α with two other truncated XRCC1 molecules, $XRCC1^{1–573}$ and $XRCC1^{\Delta 575–589}$, was reduced more than 30-fold (Figure 1b). These results indicate that the region of BRCT II downstream of amino acid 573 is important for interaction with DNA ligase III-α, and that the region between residues 573 and 592 is critical for this interaction. Residues 573–592 span the C-block motif of BRCT II, which is one of the five conserved blocks (Figure 1) [1,2]. The importance of the C-block for interaction with DNA ligase III-α was confirmed by the activity of the $XRCC1^{VI584/585DD}$ polypeptide, in which two of the non-charged residues characteristic of C-block motifs were substituted with aspartate. This mutant had less than 3% of the DNA ligase III-α binding activity exhibited by wild-type XRCC1 (Figure 1b). In contrast, substitution of the D-block residue Trp^{611} with aspartate ($XRCC1^{W611D}$) did not significantly disrupt the interaction with DNA ligase III-α (Figure 1b). It is intriguing that Trp^{611} is dispensable, as it is one of the two most conserved amino acids in BRCT domains and is a defining feature of the D-block motif, which is itself the most conserved motif in BRCT domains [1,2].

These results indicate that the C-block motif of BRCT II is critical for the interaction of XRCC1 with DNA ligase III-α. To examine whether the C-block is sufficient for interaction with DNA ligase III-α, a peptide comprising the 20 amino acids that span this region was tested for its ability to confer the property of binding the DNA ligase upon an unrelated polypeptide. An oligonucleotide duplex encoding residues 573–592 of XRCC1 was cloned into the *E. coli* expression vector pMAL-cR1 downstream of an open reading frame (ORF) encoding the bacterial maltose binding protein (MBP). The resulting construct encoded a tripartite polypeptide comprising MBP, $XRCC1^{573–592}$ and the α-fragment of β-galactosidase (Figure 2a). This polypeptide, denoted MBP–$X^{573–592}$, was compared with one expressed from the pMAL-cR1 vector (MBP–β-galactosidase α, denoted MBP, Figure 2a) for the ability to interact with His-tagged DNA ligase III-α immobilised on agarose beads in a pull-down assay. His–DNA ligase III-α bound to agarose was incubated with either MBP or MBP–$X^{573–592}$ and the

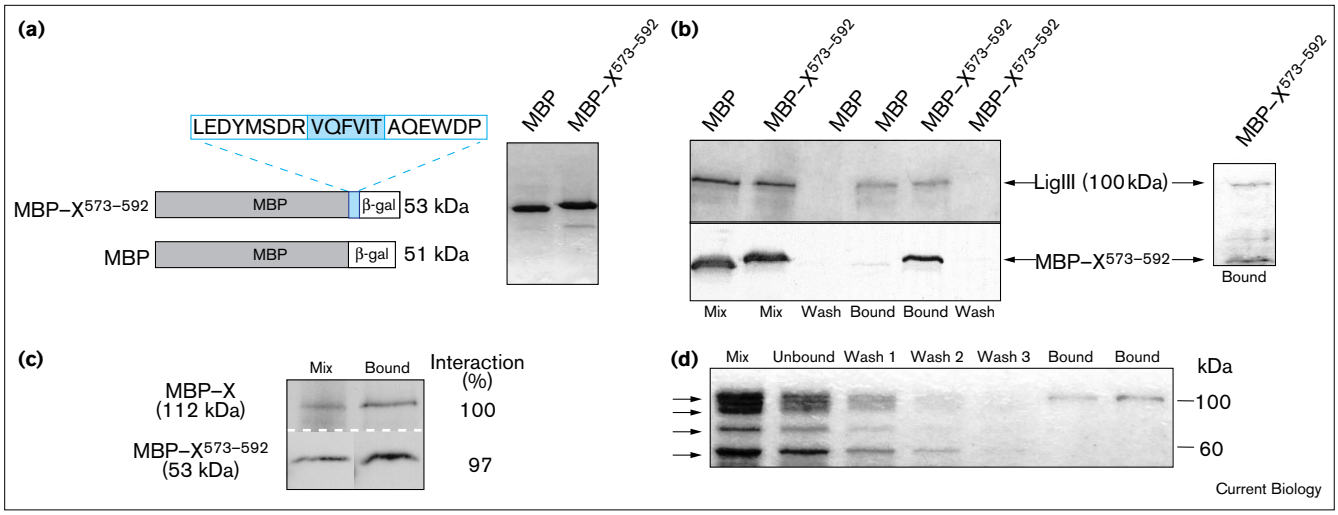
Figure 1



Amino acids spanning the C-block of BRCT II are required for interaction with DNA ligase III-α. **(a)** The BRCT domains present in BRCA1 and XRCC1 are aligned as previously depicted [2]; conserved blocks A–E are boxed and the numbers of residues between blocks are indicated. Underlined residues were targeted for substitution mutation, as depicted in (b). **(b)** The interaction of mutant XRCC1 molecules with DNA ligase III-α measured by pull-down assays and far-western blotting. The BRCT II amino-acid residues (aa) and conserved blocks mutated by substitution or deletion are indicated. For far-western blots, radiolabeled XRCC1 polypeptides were incubated with nitrocellulose blots containing DNA ligase III-α previously fractionated by SDS–PAGE and protein complexes were detected by autoradiography. For pull-down assays, His–XRCC1 bound to agarose beads and DNA ligase III-α polypeptides were mixed to facilitate protein–protein interactions and protein complexes recovered by centrifugation. XRCC1 and DNA ligase III polypeptides were detected on immunoblots with appropriate antibodies used individually or in combination. The positions of XRCC1 (X) and DNA ligase III-α (ligIII) polypeptides are indicated. The efficiency of interaction with DNA ligase III-α is indicated for each XRCC1 polypeptide, as a percentage relative to full-length XRCC1, and is the average of values obtained from multiple pull-down and far-western experiments. Values indicated by an asterisk are maximum estimates, as the measurement of interaction efficiencies below 3% was beyond the sensitivity of the assays.

beads recovered by centrifugation. After extensive washing, His–DNA ligase III-α and any associated MBP fusion proteins were fractionated by SDS–PAGE and detected by immunoblotting (with anti-MBP antisera) or staining with Coomassie blue. These experiments revealed that 25-fold more MBP–$X^{573–592}$ than MBP was bound by His–DNA ligase III-α (Figure 2b, left panel). In control experiments, MBP–$X^{573–592}$ was not bound by the agarose beads in the absence of His–DNA ligase III-α (data not shown). Staining for total protein suggested that His–DNA ligase III-α and MBP–$X^{573–592}$ were recovered in approximately stoichiometric amounts, suggesting that His–DNA ligase III-α was fully bound by MBP–$X^{573–592}$ (Figure 2b, right panel). To quantitate the relative binding efficiency of MBP–$X^{573–592}$ with DNA ligase III-α, we compared the behaviour of MBP–$X^{573–592}$ with MBP fused to full-length XRCC1 (denoted MBP–X [8]) in a pull-down assay. MBP–$X^{573–592}$ and MBP–X bound to His–DNA ligase III-α on agarose beads with equal efficiency (Figure 2c), indicating that the 20mer peptide spanning the C-block is sufficient for full DNA ligase III-α binding activity when inserted into MBP. The behaviour of the XRCC1 protein that had BRCT II sequences carboxy-terminal to the C-block deleted ($XRCC1^{1–592}$) had suggested that this carboxy-terminal region significantly influences interaction with DNA ligase III-α (Figure 1b). It is possible that the region carboxy-terminal to the C-block normally places the C-block in a context suitable for interaction, a role which when the

Figure 2



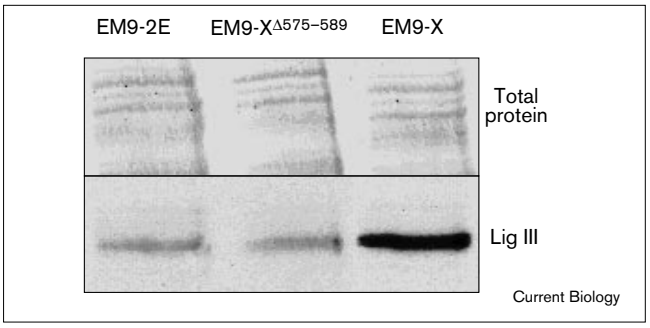
A 20mer peptide module that spans the C-block of BRCT II is sufficient for full and specific interaction with DNA ligase III-α when inserted into an unrelated polypeptide. **(a)** A diagram of MBP–$X^{573–592}$ and MBP is on the left with the 20mer peptide boxed in blue. The highlighted VQFVIT sequence defines the C-block within the BRCT domain, the rest is flanking sequence. The recombinant proteins following purification from *E. coli*, SDS–PAGE and staining with Coomassie blue are shown on the right. **(b)** MBP–$X^{573–592}$ binds DNA ligase III-α stoichiometrically in pull-down assays. DNA ligase III-α immobilised on agarose beads was mixed with MBP or MBP–$X^{573–592}$ and the agarose–protein complexes were recovered by centrifugation. Aliquots sampled during the experiment were subjected to SDS–PAGE. DNA ligase III-α (ligIII) was detected with Coomassie blue and MBP fusion proteins with either anti-MBP antisera (left panel) or Coomassie blue (right panel). Aliquots on the left panel are DNA ligase III-α agarose beads and MBP or MBP–$X^{573–592}$ (mix), samples recovered from the last agarose-bead wash (wash), and DNA ligase III-α agarose beads with any associated MBP fusion proteins (bound). On the right panel, the total protein (Coomassie blue staining) in an aliquot from the MBP–$X^{573–592}$ pull-down (bound) is shown to examine stoichiometry. **(c)** MBP–$X^{573–592}$ and MBP–X bind His–DNA ligase III-α with equal efficiency. MBP–$X^{573–592}$ was compared with MBP–X for the ability to bind His–DNA ligase III-α in a pull-down assay. Aliquots of the 'mix' and 'bound' samples are shown following SDS–PAGE and immunoblotting with anti-MBP antisera. The efficiency (%) at which MBP–$X^{573–592}$ interacts with His–DNA ligase III-α relative to MBP–X is shown. **(d)** MBP–$X^{573–592}$ binds the carboxyl terminus of His–DNA ligase III-α. In addition to full-length His–DNA ligase III-α, a mixture of carboxy-terminal-truncated polypeptides are generated when this protein is expressed in *E. coli* at high levels due to incomplete translation. Such a preparation was mixed with MBP–$X^{573–592}$ agarose beads and agarose–protein complexes were recovered by centrifugation. Included on the gel were samples of MBP–$X^{573–592}$ agarose beads and His–DNA ligase III-α polypeptides (mix), samples containing polypeptides that failed to bind MBP–$X^{573–592}$ beads (unbound), samples recovered from the last three agarose-bead washes (wash), and samples containing polypeptides eluted from MBP–$X^{573–592}$ beads with 1 M NaCl (bound). The positions of full-length and truncated His–DNA ligase III-α polypeptides are indicated by arrows on the left-hand side.

C-block region is inserted into MBP is either not required or is fulfilled by the β-galactosidase fragment.

To examine the specificity of the interaction between His–DNA ligase III-α and MBP–$X^{573–592}$, we determined whether MBP–$X^{573–592}$ bound His–DNA ligase III-α within the same region bound by full-length XRCC1. We used a preparation of recombinant His–DNA ligase III-α that contained truncated His–DNA ligase III-α polypeptides lacking the normal carboxyl terminus, in addition to containing the full-length protein (see Figure 2d legend). We reasoned that because full-length XRCC1 interacts with the carboxyl terminus of DNA ligase III-α, MBP–$X^{573–592}$ should bind only full-length DNA ligase III-α if it also targets the carboxyl terminus. Indeed, full-length DNA ligase III-α was efficiently separated from the truncated polypeptides by MBP–$X^{573–592}$ bound to agarose beads in a pull-down assay (Figure 2d).

The XRCC1 mutant CHO cell lines EM9 and EM-C11 possess reduced levels (4–6-fold) of DNA ligase III-α protein and cannot efficiently rejoin DNA single-strand breaks arising from ionising radiation and certain alkylating agents [5,7–10]. Moreover, EM-C11 cell extract cannot efficiently ligate DNA single-strand breaks during DNA base-excision repair *in vitro*, a deficiency that is complemented by the addition of recombinant DNA ligase [5]. To examine whether the C-block of BRCT II is required to maintain normal cellular levels of DNA ligase III-α and consequently required for normal DNA repair, we compared XRCC1 and $XRCC1^{\Delta575–589}$ proteins for their ability to increase the level of DNA ligase III-α in EM9 transfectants. Immunoblots probed with anti-DNA ligase III polyclonal antibody revealed that whereas full-length XRCC1 did increase DNA ligase III-α to normal levels, $XRCC1^{\Delta575–589}$ failed to elevate the level of the ligase above levels present in EM9 cells transfected with 'empty' vector (Figure 3 and data not

Figure 3



XRCC1$^{\Delta 575-589}$ fails to correct the reduced levels of DNA ligase III-α in EM9 cells. Total cell extract from stable EM9 transfectants harbouring either pcD2E vector (EM9-2E), pcD2EX encoding full-length XRCC1 (EM9-X), or pcD2EX$^{\Delta 575-589}$ encoding XRCC1$^{\Delta 575-589}$ (EM9-X$^{\Delta 575-589}$) were fractionated by SDS–PAGE. Fractionated polypeptides were either stained with Coomassie blue (top panel) or transferred to nitrocellulose and immunoblotted with an anti-DNA ligase III (ligIII) antibody (bottom panel). The level of DNA ligase III-α present in the EM9-X cell extract is similar to that present in an extract from wild-type parental AA8 cells ([8] and data not shown).

shown). Thus, the C-block motif of BRCT II is required to maintain normal cellular levels of DNA ligase III-α.

The results described here demonstrate that the BRCT II domain of XRCC1 facilitates the physical interaction of this essential DNA repair protein with DNA ligase III-α and indicate that this interaction is required to maintain normal cellular levels of the ligase. These data thus demonstrate that the BRCT II domain facilitates a biologically important protein–protein interaction, and support the hypothesis that the role of BRCT domains is to mediate such interactions [1,2,6]. It seems likely, however, that BRCT II also fulfils another role, as it is unlikely that such a large and complex structure is required solely for interaction with DNA ligase III-α. Consistent with this, the highly conserved tryptophan that in part defines the D-block of BRCT domains was dispensable for this interaction. Thus, it is likely that at least some amino acids located carboxy-terminal to the C-block in BRCT II fulfil another role, which is conserved in other BRCT domains. One possibility is that they have a role in signal transduction. For example, each BRCT domain could mediate an interaction that is unique to that domain (for example, via the weakly conserved C-block motif) in combination with an interaction that is common to all domains (for example, via the highly conserved D-block motif). In this scenario, BRCT domains could behave as adaptor molecules capable of linking different cellular processes (such as DNA repair and cell cycle control) to a common or related signal transduction pathway.

Materials and methods

Far-western blotting was conducted as described [8,11]. Briefly, recombinant human XRCC1 was phosphorylated with casein kinase II (Boehringer Mannheim) in the presence of γ-[^{32}P]ATP. Labelled XRCC1 probes (1 μg at 50 ng/ml) were incubated with nitrocellulose blots containing renatured human DNA ligase III-α that had previously been fractionated by SDS–PAGE. XRCC1–DNA ligase III-α complexes were detected by autoradiography. Pull-down assays were conducted as described [4,8,11]. Briefly, His–DNA ligase III-α (1 nM) or His–XRCC1 (1 nM) bound to nickel–NTA agarose beads, or MBP–X$^{573-592}$ (1 nM) bound to agarose beads, were incubated with purified MBP fusion proteins (1 nM), DNA ligase III-α (~1 nM) in *E. coli* extract, or a heterogeneous mixture of purified His–DNA ligase III-α polypeptides (1 nM), respectively. After 20–30 min, agarose beads were recovered by low-speed centrifugation, washed extensively, and bead-bound proteins eluted with 200 mM imidazole for nickel–NTA agarose complexes, or 1 M NaCl for agarose–MBP–X$^{573-592}$ complexes. Following SDS–PAGE, gels were stained with Coomassie blue to detect total protein or with appropriate antibodies (singly or in combination) to detect XRCC1 and MBP fusion proteins. In control experiments, the XRCC1 mutations described did not affect the interaction of XRCC1 with DNA polymerase-β, a polypeptide that interacts with XRCC1 outside the BRCT II domain ([12,13] and data not shown).

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