Protein interactions critical to DNA repair and cell cycle control systems are often coordinated by modules that belong to a superfamily of structurally conserved BRCT domains. Because the mechanisms of BRCT interactions and their significance are not well understood, we sought to define the affinity and specificity of those BRCT modules that orchestrate base excision repair and single-strand break repair. Common to these pathways is the essential XRCC1 DNA repair protein, which interacts with at least nine other proteins and DNA. Here, we characterized the interactions of four purified BRCT domains, two from XRCC1 and their two partners from DNA ligase IIIα and poly(ADP-ribose) polymerase 1. A monoclonal antibody was selected that recognizes the ligase IIIα BRCT domain, but not the other BRCT domains, and was used to capture the relevant ligase IIIα BRCT complex. To examine the assembly states of isolated BRCT domains and pairwise domain complexes, we used size-exclusion chromatography coupled with on-line light scattering. This analysis indicated that isolated BRCT domains form homo-oligomers and that the BRCT complex between the C-terminal XRCC1 domain and the ligase IIIα domain is a heterotetramer with 2:2 stoichiometry. Using affinity capture and surface plasmon resonance methods, we determined that specific heteromeric interactions with high nanomolar dissociation constants occur between pairs of cognate BRCT domains. A structural model for a XRCC1-DNA ligase IIIα heterotetramer is proposed as a core base excision repair complex, which constitutes a scaffold for higher order complexes to which other repair proteins and DNA are brought into proximity.

Protein-protein interactions are central to the function and regulation of many proteins, but the specificity of such associations is often not well understood. Proteins have evolved to interact with one another often through specific protein interaction modules or domains. The primary focus of this study is a protein interaction module typified by the breast cancer susceptibility protein BRCA1 C-terminal (BRCT)1 domains that coordinate various protein interactions in cell cycle control and DNA repair. The BRCT family comprises over 100 members that share predicted (1–3) and observed structural similarity (4–6). Proteins can contain from one to eight divergent BRCT domains, which are present in DNA repair proteins such as DNA ligase III, poly(ADP-ribose) transferase 1 (ADPRT1 or PARP1), and the x-ray cross complementation group 1 protein (XRCC1).

Human XRCC1 protein is involved in two interconnected DNA repair pathways, base excision repair (BER) and single-strand break repair (SSBR). The former consists of two subpathways: a short-patch pathway that replaces 1 nucleotide and a long-patch pathway that replaces 2–15 nucleotides (7, 8). In the BER and SSBR pathways, XRCC1 interacts directly with at least seven other proteins. In short-patch BER, XRCC1 interacts with 8-oxoguanine glycosylase (OGG1) (9), apurinic/apyrimidinic endonuclease (Ape1) (10), polymerase β (Polβ) (11, 12), and DNA ligase IIIα (LigIIIα) (13). In long-patch BER, XRCC1 interacts with some of the same proteins, as well as proliferating cell nuclear antigen (14). In SSBR, XRCC1 interacts with PARP1 (11, 15) and polynucleotide kinase/phosphatase (16). The regions of XRCC1 that are involved in these various interactions are depicted in Fig. 1A.

Some of the interaction partners of XRCC1 also interact with each other, mediating triangular interactions, for example among Ape1, Polβ, and XRCC1. In addition, many indirect physical interactions exist, for example those of XRCC1 with binding partners of Ape1 (17–20). Recently, XRCC1 has been shown to interact with and be stabilized by apratxin, a protein associated with ataxia-oculomotor apraxia, which also may be involved in SSBR (21, 22). XRCC1 was found to interact with tyrosyl DNA phosphodiesterase, a protein associated with the neurodegenerative disease spinocerebellar ataxia with axonal neuropathy 1 (SCAN1) (23). Although the significance of this interaction is not understood, a functional connection to DNA metabolism is preserved. To add to the complexity of the multiple interactions of XRCC1, it is known to bind single strand, nick, and gap DNA (24) and double strand DNA ends (25).

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† The abbreviations used are: BRCT, BRCA1 C-terminal repeat homolog (for individual BRCT domain nomenclature see Table I); BER, base excision repair; GSH, glutathione (reduced form); GST, glutathione S-transferase; Hiso, hexahistidine; LigIIIα, DNA Ligase IIIα; MALS, multilangle light scattering; mAb, monoclonal antibody; OGG1, 8-oxoguanine glycosylase; PARP1, poly(ADP-ribose) polymerase 1; Polβ, DNA polymerase β; SEC, size exclusion chromatography; SPR, surface plasmon resonance; SSBR, single-strand break repair; XRCC1, x-ray cross complementation group 1; Ape1, apurinic/apyrimidinic endonuclease; MES, 4-morpholineethanesulfonic acid; Ab, antibody; SDS, sodium dodecyl sulfate.

This paper is available on line at http://www.jbc.org
Architecture of XRCC1 and its interactions. For each partner, a line is shown under XRCC1 corresponding to the region of its interaction. The minimal regions of XRCC1 that interact with aprataxin, polynucleotide kinase/phosphatase, and tyrosyl DNA phosphodiesterase have not been mapped. B, illustration of XRCC1 BRCT domain interactions. Structural domains are represented as shapes with BRCT domains, which were examined in this study, depicted as ovals. BRCT interactions are depicted by vertical alignment.

Through the physical interactions of XRCC1, the activity of some of its enzymatic partners is stimulated. For example, in short-patch BER, XRCC1 activates OGG1 (9) and Apel (26). In the SSBR pathway, XRCC1 stimulates polynucleotide kinase/phosphatase (16). In both pathways, XRCC1 enhances LigIIIα activity through stabilization of its protein levels (13). Based on the different types of protein interactions orchestrated by XRCC1, there is the potential for one or more large multicomponent complexes to operate in these overlapping DNA repair pathways. To gain a better understanding of the specific complexes involved in these pathways, more extensive, quantitative studies of the relevant complexes are needed.

The interactions of XRCC1 with PARP1 and LigIIIα are mediated through BRCT domains from each protein (Fig. 1B). The first BRCT domain of XRCC1 (designated X1BRCTa) associates with the BRCT domain from PARP1 (15). The second, C-terminal BRCT domain of XRCC1 (X1BRCTb) associates with the BRCT domain from LigIIIα (L3BRCT) (27–29). It is hypothesized that XRCC1 functions as a scaffold by using its N-terminal domain (X1NTD), and its two BRCT domains to tether catalytically active DNA repair proteins into multicomplexes (11). Alternatively, XRCC1 could serve as an auxiliary protein at one or more steps in a series of handoff reactions, as has been proposed for the earlier steps of BER (30, 31). The interactions of X1NTD have been characterized quantitatively using biophysical methods (24). Other XRCC1 interactions, including its BRCT domain-mediated interactions with LigIIIα and PARP1, have been assessed using biochemical approaches (32, 33).

Recent attention has focused on the role of BRCT domains as phosphopeptide-binding modules. The BRCT domain-containing proteins BRCA1, Fep1, and TopBP1 proteins bound only the phosphorylated forms of their protein targets (34). Thirty-three additional BRCT domains exhibited higher affinity for phosphopeptides than non-phosphorylated peptide controls (34). Moreover, BRCA1 and Pax transactivation domain-interacting protein bound phosphorylated peptides from a library comprising ATM/ATR phosphorylation consensus sequences (35). Although phosphopeptide binding may be common among BRCT domains, there is considerable sequence divergence among these proteins. Therefore, phosphorylation-dependent interactions may not be common to all BRCT domains (reviewed in Ref. 6). Indeed, the structures of the 53BP1-p53 complex and the DNA ligase IV-XRCC4 complex exhibit no phosphoprotein-dependent interactions. There is no evidence to indicate that phosphorylation is required for BRCT domain interactions in either BER or SSBR.

Because BRCT domains are widespread in nature yet appear to have specific cellular binding partners, we are interested in defining the molecular details of the affinity and specificity of BRCT interactions involving XRCC1. Specifically, if structurally similar BRCT domains interact through common structural motifs and both self- and heteromeric interactions are observed, what dictates the specificity of their interactions? We sought to address this question using differential affinity tagging of the four BRCT domains from XRCC1, LigIIIα, and PARP1, which enabled biochemical interaction studies among these protein domains.

Materials and Methods

Cloning—Human BRCT domain-encoding open reading frames were subcloned into expression vectors using a standard PCR procedure. The purified fusion protein was used as the immunogen for mAb production, followed by restriction digestion and ligation. All of the expression plasmids constructed used are summarized in Table I. Subcloning of the XRCC1 BRCTb domain (X1BRCTb) comprising residues 533–633 (29) and the LigIIIα BRCT domain (L3BRCT) comprising residues 838–932 (33) was reported previously. The PARP1 BRCT domain (PBRCT) was subcloned into pET28a to yield an N-terminally His6-T7 epitope-tagged protein. The XRCC1 BRCTa domain (X1BRCTa) was subcloned into pET41a to generate an N-terminal GST-His6 fusion protein. BRCT constructs are named based on previous conventions (33) according to their N-terminal fusion, if present, and an abbreviation for the domain (Table I). Details of the amino acid sequence encoded and the restriction sites used are also given.

Protein Expression and Purification—Each of the BRCT domains was expressed in Escherichia coli Rosetta (DE3) pLacI (Novagen) transformed with the corresponding expression plasmid. Terrific Broth (Sigma) containing the appropriate antibiotic was inoculated with 0.02 ml of an overnight starter culture. Large-scale (1.0–1.5 liter) cultures were grown to an optical density (A600) of 0.5 and were induced for 16 h at 22 °C with 0.001 M β-naphtylacetylglucoside. Cells were collected by centrifugation at 5,000 × g and were resuspended in 30 ml of a nonionic (Triton X-100) lysis buffer (see below). Cells were lysed at >15,000 p.s.i. in an EmulsiFlex C5 cell disruptor (Avestin). Lysates were clarified by centrifugation twice at 10,000 × g for 20 min at 4 °C followed by filtration (0.45-μm pore size) to remove particulate matter.

The non-tagged X1BRCTb domain was purified by anion exchange chromatography using Q-Sepharose (Q Trap, Amersham Biosciences) and size exclusion chromatography using Superdex 75 HiLoad 16/60 (Amersham Biosciences) according to published procedures (29). GST-L3BRCT and GST-Hisα-X1BRCTa were purified using 5 ml glutathione-Sepharose (GSTrap FF) columns (Amersham Biosciences). The column was equilibrated with 25 ml of phosphate-buffered saline (PBS) (0.043 M Na2HPO4, 0.021 M NaH2PO4, 0.1 M NaCl, pH 7.4) and 25 ml of clarified crude lysate were bound. The column was washed with 100 ml of PBS, and the bound protein was eluted with GST elution buffer (0.05 M Tris-HCl, 0.01 M glutathione, reduced form (GSH), pH 8.0). The Hisα-T7-PARP1 BRCT domain was purified using a 5-ml HiTrap chelating column (Amersham Biosciences) charged with Ni2+. The column was equilibrated with 25 ml of Ni2+ lysis buffer (0.05 M NaH2PO4, 5% glycerol, 0.3 M NaCl, 0.001 M Na2HPO4, pH 8.0 containing 0.01 M imidazole, 25 ml of clarified crude lysate were bound, the column was washed with 100 ml of Ni2+ lysis buffer containing 0.02 M imidazole, and the bound protein was eluted with a linear gradient of 0.02–0.5 M imidazole over a volume of 100 ml.

Monoclonal Antibody Production—Monoclonal antibody (mAb) 94 used in this study was raised against the recombinant human DNA Ligase IIIα BRCT domain (L3BRCT). This domain was expressed as a GST fusion protein in E. coli and purified by GSTrap chromatography. The purified fusion protein was used as the immunogen for mAb production using procedures described previously (36). Enzyme-linked immunosorbent assays were used to screen the growing hybridoma for GST-L3BRCT- and GST-specific antibodies, and clones positive for GST-L3BRCT (but negative for GST) were picked for secondary screening by immunoblotting.

L3BRCT Immunoprecipitation and Co-Immunoprecipitation—For immunoprecipitation experiments, 2–10 μg of purified protein were
incubated with 20 μg of anti-L3BRCT mAb 94 at 4 °C for 1 h. 50 μl of a 50% slurry of protein A-Sepharose 6MB (Sigma) was equilibrated in TNEN buffer (0.05 mM Tris-HCl, pH 8.0, 0.15 mM NaCl, 0.005 mM EDTA, 0.5% Nonidet P-40) and added to the protein mixture, which was incubated for an additional 2 h. The suspension was centrifuged (500 × g for 5 min at 22 °C), washed three times with 0.5 ml of TNEN, and resuspended in 25 μl of SDS sample loading buffer. The samples were boiled for 5 min, were briefly centrifuged, and 20 μl of the supernatant was loaded onto a 4–20% acrylamide gradient gel and subjected to SDS-PAGE. Proteins were visualized by staining with Coomassie Brilliant Blue R-250.

To probe the L3BRCT complex formation, purified GST-L3BRCT (10 μg) was mixed with X1BRCTb (2 μg) in a total volume of 300 μl using TNEN as a diluent. Incubations with antibody and protein A-Sepharose and electrophoresis were performed as described above. Proteins were transferred to a nitrocellulose membrane, and the blot was cut in half horizontally just above the 21-kDa marker position. The half top was probed with anti-GST Ab (Amersham Biosciences, I:5,000) and the bottom half was probed with anti-XRCC1 polyclonal Ab (37). The blot was washed and further incubated with secondary anti-goat (Santa Cruz Biotechnology, 1:7,500) and anti-mouse horseradish peroxidase conjugates (Sigma, 1:2,000). The immunoblot was detected using enhanced chemiluminescence (ECL, Amersham Biosciences) and exposed to autoradiographic film. In experiments to test the effect of order of addition (protein, protein, then mAb), the first two components were mixed first, incubated for 30 min on ice, and the third component was added last.

**Size Exclusion Chromatography—SEC** was carried out using an Akta fast protein liquid chromatography system (Amersham Biosciences) equipped with Superdex 200 or 75 HR 10/30 columns (Amersham Biosciences). The columns were equilibrated and run at a flow rate of 0.5 ml/min using GF buffer (0.01 mM NaH2P04, 0.05 M NaCl, 5% glycerol). X1BRCTb protein was also run in GF buffer containing 1 M NaCl. MALS measurements were performed using a KW-804 (Shodex) or a Superdex 75 HR 10/30 (Amersham Biosciences) column. MALS measurements were performed using a MiniDAWN in-line detector (Wyatt Technology). Protein concentration was monitored using a UV monitor at 280 nm and a refractive index detector at 690 nm (Optilab DSP, Wyatt Technology). The detectors were plumbed in the order: UV, RI, then MALS. SEC-MALS experiments employed MALS buffer (0.02 mM NaPO4, 0.1 mM NaCl, 0.001 mM EDTA, pH 7.0) and a flow rate of 0.5 ml/min. Data were analyzed using Astra software (Wyatt Technology) using the refractive index detector and a refractive index increment (dndc) value of 0.185 ml/g.

**Fusion-protein Capture Experiments**—Interacting proteins (or controls) were combined using 0.5 mg of the GST fusion proteins and equimolar partner proteins at a total concentration of 0.13 mg/ml in binding buffer (0.05 mM Tris-HCl, pH 8.0, 5% glycerol, 0.05 mM NaCl). Complexes were formed by mixing two proteins and incubating on ice for 30 min. Putative complexes were bound to 1 ml GSTTrap, HiTrap-Ni2+, or T7 tag antibody (Novagen) columns. For all resin types, the columns were washed with 5 ml of interaction buffer (0.05 mM Tris-HCl, pH 8.0, 5% glycerol, 0.02 mM imidazole) plus 0.05 mM NaCl. The bound complexes were eluted with 4.5 ml of interaction buffer plus 0.05 mM NaCl containing 0.01 M GSH, 0.5 mM imidazole, or the supplied elution buffer for the T7 tag antibody column. Relevant fractions were analyzed by SDS-PAGE on NuPAGE 4–12% acrylamide gradient gels (Invitrogen) that were run in NuPAGE MES buffer (Invitrogen).

**Surface Plasmon Resonance**—The affinity of X1BRCTb for its cognate partner L3BRCT was measured using surface plasmon resonance (SPR) with a BIACore X instrument. The GST-L3BRCT ligand was immobilized onto an anti-GST antibody (Ab) chip. An anti-GST Ab (BIACore) was first amine-coupled to a CM-5 chip (BIACore) to a density of 10,000 response units. The GST-L3BRCT ligand was diluted to 1 ng/μl in HBS-P buffer (0.01 M HEPES, pH 7.5, 0.15 M NaCl, 0.05% Tween 20) and injected over one flow cell of the anti-GST Ab-coated sensor chip at 5 μl/min until 300 response units of GST-L3BRCT was captured to the chip. A second flow cell served as a reference to account for bulk-shift responses and minor, nonspecific interactions with the anti-GST Ab, which was subtracted from the binding response. X1BRCTb was buffer exchanged into HBS-P using a Micro Bio-spin P6 (Bio-Rad Laboratories). Binding was measured for six concentrations of X1BRCTb analyte (0.05, 0.125, 0.25, 0.5, 1.0, and 5.0 μM), which were injected for 3 min at a flow rate of 20 μl/min. After measuring the off-rates for 2 min for each analyte injection, complete regeneration of the surface was achieved with one 60-s injection of 2 M NaCl and two 30-s injections of 0.05% SDS. The interaction affinity, as described by the equilibrium dissociation constant (Kd), was determined globally by fitting to the kinetic simultaneous koff, kon model, first testing a model of equimolar stoichiometry. The steady-state affinity was determined from curve fitting to a plot of the equilibrium response unit (R eq) values, derived from sensorgrams fitted locally, against the concentrations of analyte.

**Construction of the Heterotetrameric BRCT Model**—The heterotetrameric BRCT model was generated based on the known XIChTB (38) and L3BRCT structures (4) as follows. The relevant XIChTB dimerization interface was inferred from structural (38) and mutagenesis data (32). The non-crystallographic symmetry operator was used to generate the second copy of XIChTB, which interacts with the first through a helix 1. The interface of XIChTB that interacts with LigIIIa was inferred from additional mutagenesis studies (28). Crystallographic symmetry operators were used to generate the third and fourth copies of XIChTB, which associate through connecting loop 2 of the first two copies. The L3BRCT structure was then superimposed (root mean square deviation, 2.2 Å) on the third and fourth copies of XIChTB using SuperPose (39). The model is corroborated by the demonstrated involvement of a helix 1 (specifically residues Val-873 and Ala-874), the α1-β2 loop, and three residues following α2 in the dimerization interface of L3BRCT from two-dimensional and amide exchange NMR experiments (4).

**RESULTS**

To examine specificity of interactions within the DNA repair pathways requiring XRCC1, we designed bacterial expression constructs to produce and isolate differentially tagged BRCT domains separate from the parent proteins XRCC1, LigIIIa, and PARP1. From ten constructs tested, five were selected for their efficacy in protein overexpression and solubility (Table I). These constructs encoding N-terminal fusion peptides were used to purify, detect, and differentiate XRCC1 and two of its binding partners in the following experiments.

**Recognition and Co-immunoprecipitation of an LigIIIa BRCT Complex**—To probe the specificity of the L3BRCT interaction with XIChTB, a mAb specific for L3BRCT domain was developed and selected. Purified BRCT domains were tested with the antibody and analyzed by SDS-PAGE (Fig. 2A). The anti-L3BRCT mAb specifically binds to the GST-L3BRCT protein (lane 2) but does not recognize or immunoprecipitate X1BRCTa, XIChTB, or PBCT (lanes 4–6). This mAb does...
not bind to GST as determined from enzyme-linked immunosorbent assay and immunoblotting (data not shown), nor does it cross-react with the GST fusion protein GST-X1BRCTa (lane 4).

The anti-L3BRCT mAb was used to co-immunoprecipitate the X1BRCTb-GST-L3BRCT complex (Fig. 2B), which was formed from independently purified proteins. The co-precipitated proteins were detected using an anti-GST antibody (top half) and anti-XRCC1 antibody (bottom half), respectively. In this experiment, the anti-L3BRCT mAb precipitated the GST-L3BRCT fusion protein (Fig. 2B, lane 2) and the preformed X1BRCTb-GST-L3BRCT complex (lane 3). Incubation of the mAb with L3BRCT prior to the addition of X1BRCTb resulted in partial (~50%) interference with the BRCT complex formation (lane 4).

Self-association of BRCT Domains—Based on previous observations that certain BRCT domains self-associate (4, 29, 33), we tested for oligomeric associations giving rise to higher molecular mass species in each individual, purified BRCT domain. Native molecular weight (\(M_r\)) was estimated by size exclusion chromatography (SEC), in comparison to seven known proteins that fit a standard curve with an \(R^2\) value of 0.98 (\(M_r\) versus \(K_{av}\); see “Materials and Methods”). Additional SEC experiments were performed using a MALS detector for direct determination of the weight-average molar mass (\(M_w\)).

In agreement with our earlier studies performed with higher protein concentrations, the C-terminal BRCT domain of XRCC1 (X1BRCTb; see Fig. 1) was found to self-associate, with the oligomeric state dependent on ionic strength. At a low salt concentration (0.05 M NaCl), X1BRCTb is apparently tetrameric (\(M_r = 3.4\)), whereas at a high salt concentration (1 M NaCl) it behaves as a dimer (\(M_r = 2.2\)) (Table II). This salt-dependent dissociation behavior was observed previously (29); however, the association states observed under various conditions were not distinctly identified. The tetramer formation mediated by X1BRCTb is also temperature-dependent based on SEC of an XRCC1 fragment comprising both X1BRCTa and X1BRCTb domains. This protein eluted as a tetramer in SEC at 4 °C and as a dimer at 22 °C (data not shown).

To examine the association state of the first BRCT domain of XRCC1, the X1BRCTa domain was purified as a GST-His\textsubscript{6}-X1BRCTa fusion protein. This protein was subjected to SEC, and the \(M_r\) was estimated at 120,000 (Table II). The ratio of observed mass to calculated subunit mass (\(M_r/m\)) was 3.2. In comparison, the purified GST protein exhibited a \(M_r/m\) of 2.8, which is consistent with a dimeric quaternary structure (Table II). A \(M_r/m\) ratio slightly greater than 2 is expected based on the dimerization of GST (40), because deviations from a spherical shape yield slightly larger apparent masses. Considering the effects of shape and the flexible linker between fusion partners, it follows that GST-His\textsubscript{6}-X1BRCTa would also elute at slightly smaller volume (i.e., larger mass) than that expected for a spherical protein. The dimerization of GST prevented assessment of the oligomeric state of X1BRCTa, which could be monomeric or dimeric. We infer that the N-terminal GST fusion would not necessarily interfere with potential oligomer formation of X1BRCTa, because the full-length XRCC1 protein contains a domain that is N-terminal to X1BRCTa.

Next, the oligomer formation of the LigIII\textsubscript{a} BRCT domain was assessed. The \(M_r\) for GST-L3BRCT was estimated at 178,000. This is most consistent with a tetrameric structure (\(M_r/m = 4.9\)), although GST is known to dimerize (40). Accordingly, following cleavage of the fusion protein with thrombin, L3BRCT eluted in SEC as a dimer (\(M_r/m = 2.1\)), which is in agreement with earlier studies (33). This result was corroborated by the behavior of His\textsubscript{6}-L3BRCT, which also eluted as a dimer (\(M_r/m = 1.7\)). These results indicate that the GST-L3BRCT tetramer associates both through its GST and BRCT domains and that the fusion protein does not interfere with the interactions of this BRCT domain. Similarly, the

### Table II

| Protein name\(^a\) | SEC column | Subunit mass\(^b\) | SEC mass \(\times 10^{-3}\) | MALS mass \(\times 10^{-3}\) (native:subunit ratio) | Deduced oligomer |
|-------------------|------------|------------------|----------------|---------------------------------|-----------------|
| GST               | Shodex KW-804 | 36.1             | 178 (4.9)    | 141 (3.9)                  | Tetramer |
| GST-L3BRCT        | Superdex 200 | 10.0             | 22.9 (2.3)  | ND                               | Dimer |
| L3BRCT            | Superdex 200 | 12.5             | 21.6 (1.7)  | ND                               | Dimer |
| H\textsubscript{10}-L3BRCT | Superdex 200 | 13.7             | 30.5 (2.2)  | ND                               | Dimer |
| H\textsubscript{10}-X1BRCTa | Superdex 200 | 13.7             | 30.5 (2.2)  | ND                               | Dimer |
| X1BRCTb           | Superdex 200 | 10.9             | 24 (2.2)    | 22 (2.0)                      | Dimer |

\(a\) Constructs named according to their fusion partners, if applicable, and their abbreviations.

\(b\) Subunit mass calculated based on the amino acid sequence of the expressed protein, including fusion sequences.

\(c\) ND, not determined.

\(d\) All experiments performed at 0.05 M NaCl; X1BRCTb also performed at 1 M NaCl.
the influence of the GST fusion partner, GST and X1BRCTb

flow-through and wash fraction (protein and the X1BRCTb domain. The presence of X1BRCTb in the markers are as in there is no interaction under these conditions. Lane assignments and detectable X1BRCTb in the GSH elution fraction (in kilodaltons) are indicated at

complex was then eluted with GSH (Fig. 3

0.05 M NaCl.

Sepharose affinity capture was used to bind complexes based on affinity

electrophoresis. Proteins were analyzed by Coomassie Blue staining following SDS-gel

Lane 1

Lane 2

Lane 3

Lane 4

Lane 5

Lane 6

Lane 7

Lane 8

Lane 9

Lane 10

A

B

C

D

FIG. 3 .

Specificity of X1BRCTb binding by L3BRCT. GSH-Sepharose affinity capture was used to bind complexes based on affinity for GST. A, GST-L3BRCT interaction with X1BRCTb target protein. Proteins were analyzed by Coomassie Blue staining following SDS-gel electrophoresis. Lane 1, marker proteins; lane 2, purified capture protein; lane 3, purified target protein; lane 4, pre-column mixture; lane 5, column flow-through; lane 6, 0.05 M NaCl wash; lane 7, 0.15 M NaCl; lane 8, 0.5 M NaCl; lane 9, 1.5 M NaCl; lane 10, specific elution. The lack of target protein in the flow-through fraction (lane 5) or salt fractions, and the co-elution of the two proteins with 0.01 M GSH (lane 10) indicates a strong interaction. The molecular masses of marker proteins (in kilodaltons) are indicated at left. B, control experiment with GST protein and the X1BRCTb domain. The presence of X1BRCTb in the flow-through and wash fraction (lanes 5 and 6) and the absence of detectable X1BRCTb in the GSH elution fraction (lane 10) indicate that there is no interaction under these conditions. Lane assignments and markers are as in A.

PARP1 BRCT domain eluted at a volume consistent with a dimer (M, ratio = 2.2).

Specificity of Cognate BRCT Domain Interactions—BRCT domain interactions were examined in pairwise combinations of purified domains, testing for complex formation using affinity chromatography capture (Table III). For X1BRCTb association with GST-L3BRCT, GSH-Sepharose was used as the affinity matrix (Fig. 3A). The heteromeric complex formed was stable, as X1BRCTb remained bound during washes of increasing ionic strength up to 1.5 M NaCl (Fig. 3A, lanes 6–9). The complex was then eluted with GSH (Fig. 3A, lane 10). To test the influence of the GST fusion partner, GST and X1BRCTb were mixed and subjected to the same regime of binding, washes, and elution (Fig. 3B). Because X1BRCTb washed through without binding, it did not interact with either GSH-Sepharose or GST under these conditions. These results indicate that the isolated L3BRCT and X1BRCTb domains form a specific and tight interaction, consistent with prior studies (4, 33).

In another series of affinity capture experiments, the putative interactions of PARP1 BRCT were tested by affinity capture using GSH-Sepharose. This approach was used to capture PBRCT via its cognate partner X1BRCTa (Fig. 4A). This interaction was also stable in high salt, and the two proteins were co-eluted. In addition, PBRCT showed no interaction with GST or with GSH-Sepharose (Fig. 4B). Therefore, the interaction between PBRCT and X1BRCTa is also specific to the BRCT domains. Using the same approach, non-cognate BRCT domain interactions were examined for weak or nonspecific interactions. No interaction was observed either between the PARP1 BRCT and LigIIIα BRCT domains (Fig. 4C). Similarly, X1BRCTb was not captured by PBRCT (Fig. 4D).

To determine the strength of BRCT domain interactions, surface plasmon resonance (SPR) experiments were performed using immobilized L3BRCT. X1BRCTb was bound with apparent equimolar stoichiometry, a steady-state dissociation con-

FIG. 4. Specificity of the interaction between X1BRCTa and PBRCT. GSH-Sepharose affinity capture was used to bind complexes based on affinity for the GST-His6-X1BRCTa protein. Proteins were analyzed as in Fig. 3A. A, interaction of GST-His6-X1BRCTa with His6-T7-PBRCT target protein. Bound proteins were eluted with GSH. B, similar test for PBRCT binding by GST-L3BRCT in which no interaction was detected. C, X1BRCTa versus X1BRCTb. GST-His6-X1BRCTa immobilized on GSH-Sepharose did not bind X1BRCTb. D, PBRCT versus X1BRCTb. His6-T7-PBRCT immobilized on Ni affinity resin did not interact with X1BRCTb.

TABLE III

| Immobilized protein | Captured protein | Interactiona |
|---------------------|------------------|--------------|
| GST-L3BRCT          | X1BRCTb          | +            |
| GST-His6-X1BRCTa    | X1BRCTb          | –            |
| GST                 | X1BRCTb          | –            |
| His6-T7-PBRCT       | X1BRCTb          | –            |
| His6-T7-PBRCT       | GST-L3BRCTb      | +            |
| His6-T7-PBRCT       | GST-His6-X1BRCTa | –            |
| His6-T7-PBRCT       | GST              | –            |
| a +, interaction stable in 1.5 M NaCl; –, interaction not detected in 0.05 M NaCl. |
| b Performed both with T7 tag antibody and Ni affinity resins. |
shown that BRCT domains from XRCC1 and LigIII are relevant to these observations is a recent report on the importance of BRCT self-association in the Schizosaccharomyces pombe Crb2 protein, in which the intramolecular association of tandem BRCT domains was found to be essential for the DNA checkpoint mechanism (41).

Based on known structures of X1BRCTb and L3BRCT, we were further interested in which regions of these domains mediated the interactions. Our observations of a dimer of X1BRCTb are consistent with the crystal structure, which was determined with two molecules in the asymmetric unit (38). Based on this structure, the asymmetric dimer interface (i.e. the non-crystallographic symmetry axis) was proposed to coincide with the biologically relevant dimerization interface. However, the dimer in the asymmetric unit is only one of three possible dimers. Moreover, in this space group (P3_2_1), the choice of origin (and hence asymmetric dimer) is arbitrary. The three intermolecular contact regions of X1BRCTb exhibit buried surface areas of 630, 507, and 245 Å², the first of which is the proposed biological dimerization interface. Thus although the proposed dimerization interface is the most extensive one, it is not clear which of the one or more contact regions are biologically relevant based on the structure alone.

The self-interactions of isolated XRCC1 domains have implications for the intact protein. The only known self-interactions of XRCC1 are mediated by X1BRCTa (29). XINTD is monomeric in solution (42), and we did not observe an interaction between X1BRCTa and X1BRCTb. Therefore, the larger oligomers observed for full-length XRCC1, including tetramers and octamers (38) likely occur through the associations of the X1BRCTb domain. Secondary interactions may be possible between X1BRCTa domains, either directly or indirectly through PARP1 (see below).

**Strength and Specificity of Heteromeric BRCT Interactions—** Although there is limited sequence identity within the BRCT domain family (3), there is considerable structural similarity (4, 5). The BRCT domain has been characterized as a protein-protein interaction module (37, 38) and a phosphopeptide-binding motif (34, 35). Through affinity column capture and SPR experiments, we have shown that the cognate BRCT domain complexes X1BRCTa-PBRCT and X1BRCTb-L3BRCT associate specifically, are stable in high ionic strength solutions, and exhibit dissociation constants in the high nanomolar range. Furthermore, under the same conditions we did not detect interactions among non-cognate BRCT domains. These experiments demonstrate that, despite the sequence- and structural-level similarities among BRCT domains, they have evolved to mediate stable and specific interactions with their cognate partners.

XRCC1 has been shown to be multiply phosphorylated in vivo and to exhibit altered phosphorylation patterns following treatment with the DNA-damaging agent methylmethane sulfonate (43). The phosphorylation sites of XRCC1 reside in two spans of residues (459–494 and 503–546) that coincide with the linker between X1BRCTa and X1BRCTb (44). The phospho-

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**Table IV**

| Protein complex       | SEC column | Protomer mass | Native mass | Deduced oligomer |
|-----------------------|------------|---------------|-------------|-----------------|
| X1BRCTb-GST-L3BRCT    | Superdex 200 | 47.0 | 105 (2.2) | Heterotetramer |
| X1BRCTb-L3BRCT       | Shodex KW-804 | 23.4 | 39.2 (1.7) | Heterotetramer |
| X1BRCTb-H^10-L3BRCT  | Superdex 200 | 20.9 | 51.6 (2.5) | Heterotetramer |

| Protein complex       | SEC column | Protomer mass | Native mass | Deduced oligomer |
|-----------------------|------------|---------------|-------------|-----------------|
| X1BRCTb^2              |            |              |             |                 |

*a* Protomer mass calculated from the amino acid sequences of the two proteins, including fusion sequences, assuming equimolar stoichiometry.

*b* Derived from thrombin cleavage of the X1BRCTb-GST-L3BRCT complex.

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**DISCUSSION**

**Self-association of BRCT Domains—** Previously, we have shown that BRCT domains from XRCC1 and LigIIIα are oligomeric in solution. As independently purified proteins, the X1BRCTb domain is multimeric (29) and the L3BRCT domain is dimeric (4, 33). In this study, we used SEC coupled with an on-line MALS detector for absolute determination of native molar mass, and confirmed that X1BRCTb forms mixtures of dimer and tetramer that are dependent on ionic strength and temperature. These experiments clearly define the self-association properties of this domain, which was previously known only to multimerize.

Moreover, we demonstrated that the BRCT domains from LigIIIα and PARP1 proteins also form homodimers, which indicates a general trend of self-interaction for BRCT domains. Given that these are protein interaction modules, higher order heteromeric complexes are likely formed. The fusion of GST with the BRCTa domain of XRCC1 (GST-His9-X1BRCTa) causes dimerization, but we determined that this is due at least in part to the propensity of GST to self-associate. Perhaps relevant to these observations is a recent report on the importance of BRCT self-association in the Schizosaccharomyces pombe Crb2 protein, in which the intramolecular association of tandem BRCT domains was found to be essential for the DNA checkpoint mechanism (41).
phosphorylation sites were subsequently mapped to specific residues in span 1 (Ser-518, Thr-519, and Thr-523) and span 2 (Ser-461, Ser-475, Ser-485, and Thr-488), which are all contained within the linker between the two BRCT domains of XRCC1 (22). The phosphorylation of XRCC1 in span 1 affected the interaction with aprataxin and to a lesser extent with polynucleotide kinase/phosphatase and was therefore proposed to regulate the partitioning of XRCC1 into specific complexes (22). Based on the known interaction regions of XRCC1 (Fig. 1A), phosphorylation at these seven sites is unlikely to affect other interactions with the possible exception of Ape1.

The XRCC1-LigIIIα complex has been proposed to be a heterodimer (27) and to use the same contact region of X1BRCTb for self-association and for heterologous interaction (32). Accordingly, this interface was probed by site-specific mutagenesis for effects on heteromeric interactions. Two double mutants of X1BRCTb in α helix 1, T30R/A31R and R23E/R27E, interfered with the interaction of the L3BRCT domain (32). This helix is observed to mediate the interaction between the two copies of X1BRCTb observed by x-ray crystallography (38). These observations suggest that this interface is important for the interaction with LigIIIα, either directly or because a dimeric structure of X1BRCTb is needed for a high affinity interaction with L3BRCT. In another study, a 20-residue peptide from X1BRCTb that included β strand 2 bound tightly to LigIIIα (28). This peptide encompasses residues from two β strands and two connecting loops, but notably does not include α helix 1. The implication is that the interaction between X1BRCTb and L3BRCT is mediated by an interface distinct from that containing α helix 1. Combining our results with these mutagenic and structural data, we propose that the homodimer formation of XRCC1 occurs through α helix 1 of X1BRCTb and the interaction with LigIIIα occurs through a separate interface connecting loop 2.

**Stoichiometry of XRCC1 BRCT Complexes**—We demonstrated that in isolation, X1BRCTb forms dimers and tetramers and the L3BRCT domain is dimeric. Combined with the results of our earlier studies (33), we hypothesized that the complex formed by the isolated X1BRCTb and L3BRCT domains is a heterotetramer (2:2). Using SEC-MALS, we confirmed this, using relatively low concentrations of proteins, either coexpressed or independently purified and mixed. Our conclusion differs from the molecular model of a heterodimeric (1:1) XRCC1-LigIIIα complex proposed previously (32) and argues that the interfaces for homodimer formation and heteromer association are distinct. Furthermore, from our kinetics observations using SPR, the formation of these heterotetramer complexes is relatively rapid, with an on-rate ($k_+$) of 1.31 × 10^4 M⁻¹ s⁻¹. The association kinetics suggest that partitioning of homodimers into heterodimers is unlikely to occur on a relatively rapid time scale.

From a synthesis of the hydrodynamic, structural, and mutagenesis data, we propose a model for a heterotetrameric BRCT domain complex (Fig. 5, A and B). As discussed above, the crystal structure of X1BRCTb (38) shows three intermolecular contact regions, which are potential subunit interfaces. We propose that for isolated X1BRCTb, α helix 1 mediates the primary dimerization interface, in agreement with previous conclusions (38). Furthermore we propose that a second interface, including connecting loop 2, mediates homotetramer formation or alternatively the heteromer association with L3BRCT. Based on the heterotetrameric BRCT complex observed in solution, we have constructed a model by superposition of two copies of L3BRCT on the structure of a X1BRCTb tetramer (see Fig. 5, A and B). Similarly, for intact XRCC1, we infer that the biologically relevant core complex is a heterotetramer consisting of two molecules of XRCC1 and two molecules of LigIIIα (Fig. 5C). This conclusion is supported by fluorescence resonance energy transfer experiments that indicate that XRCC1 is a multimer in vivo (14).

**Implications for DNA Repair Complexes**—Based on these conclusions, a larger BER/SSBR complex likely exists than has been proposed in previous models. A PARP1 homodimer could mediate bridging interactions between the X1BRCTa domain in each molecule of XRCC1, further stabilizing the assembly (Fig. 5C). The presence of two XRCC1 molecules in this putative DNA repair complex could provide several advantages over a complex built on an XRCC1-LigIIIα heterodimer. First, subunit interactions provide thermal stability (45, 46), which in turn provides proteolytic stability (47). Indeed, the X1BRCTb-L3BRCT complex is thermally more stable, by 8–15 °C, than either isolated domain (39). Second, because XRCC1 interacts with many partners, it could provide a mechanism by which two partners that interacted with overlapping regions of XRCC1 (Fig. 1A), possibly competitively, could co-exist in the complex (e.g. Ape1, OGG1, and proliferating cell nuclear antigen). Finally, the interactions of an XRCC1 dimer with a LigIIIα dimer and two molecules of Polβ (as well as other BER components) could give rise to two sets of active centers that could serve complementary functions in BER and SSBR. Alternatively, this complex could act at sites of clustered DNA damage, such as those generated by ionizing radiation or radiomimetic agents (48, 49). Such a function could provide new testable hypotheses related to the elevated sister chromatid exchange events associated with XRCC1-deficient cells.
