RAD51C Interacts with RAD51B and Is Central to a Larger Protein Complex in Vivo Exclusive of RAD51*

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RAD51B and RAD51C are two of five known paralogs of the human RAD51 protein that are thought to function in both homologous recombination and DNA double-strand break repair. This work describes the in vitro and in vivo identification of the RAD51B/RAD51C heterocomplex. The RAD51B/RAD51C heterocomplex was isolated and purified by immunoaffinity chromatography from insect cells co-expressing the recombination proteins. Moreover, co-immunoprecipitation of the RAD51B and RAD51C proteins from HeLa, MCF10A, and MCF7 cells strongly suggests the existence of an endogenous RAD51B/RAD51C heterocomplex. We extended these observations to examine the interaction between the RAD51B/RAD51C complex and the other RAD51 paralogs. Immunoprecipitation using protein-specific antibodies showed that RAD51C is central to a single large protein complex and/or several smaller complexes with RAD51B, RAD51D, XRCC2, and XRCC3. However, our experiments showed no evidence for the inclusion of RAD51 within these complexes. Further analysis is required to elucidate the function of the RAD51B/RAD51C heterocomplex and its association with the other RAD51 paralogs in the processes of homologous recombination and DNA double-strand break repair.

The human RAD51 protein functions in homologous recombination and DNA double-strand break repair (1–4). Five paralogs of human RAD51 have been identified: RAD51B (hREC2, RAD51L1), RAD51C (RAD51L2), RAD51D (RAD51L3), XRCC2, and XRCC3; these proteins share ~25% amino acid sequence identity with one another and RAD51 (5–9). DT40 chicken cell knockouts have been generated for each paralog, all of which exhibit a lack of RAD51 foci formation as well as enhanced radiation and cisplatin sensitivity, consistent with a deficiency in homologous recombination and DNA double-strand break repair (10, 11). These results are consistent with previous work, which showed that the XRCC3 knockout CHO cell line (irs1SF) is deficient in RAD51 foci formation, suggesting that XRCC3 is required for RAD51 function (12). Similarly, XRCC2-defective cell lines also fail to form damage-dependent RAD51 foci (13). Furthermore, both XRCC2 and XRCC3 have been shown to be required for repair of double-strand breaks by homologous recombination in vivo (14–16). Initially by sequence and now by association and function, it has become increasingly evident that these RAD51 paralogs participate together in recombination and repair processes.

Extensive yeast two- and three-hybrid analysis suggests that there are a variety of putative protein-protein interactions between the RAD51 paralogs. These include XRCC2/RAD51D (17, 18) and XRCC3/RAD51 (19). Moreover, interactions have been suggested between RAD51C/RAD51B, RAD51C/RAD51D, RAD51C/XRCC3, and RAD51C/RAD51 (8, 18). More recent biochemical evidence has corroborated the interaction between XRCC2 and RAD51D by co-purification of the recombinant proteins and co-elution of the native proteins by gel filtration from mammalian cell extracts; RAD51D was further shown to be a DNA-stimulated ATPase (17). In addition, a stable heterocomplex was demonstrated for recombinant RAD51C and XRCC3 proteins, and this complex was shown to stimulate homologous pairing and strand exchange in the absence of RAD51 (20). Similarly, Masson et al. showed the integrity of the XRCC3/RAD51C interaction by co-expression of the proteins and identification of the endogenous complex in HeLa cells (21).

Using a comparable approach, here we extend the observation that the RAD51B and RAD51C proteins interact and examine the putative protein complex in vitro and in vivo. By co-infection of insect cells with recombinant RAD51B and RAD51C baculoviruses followed by immunoaffinity purification, we have shown that the RAD51B and RAD51C proteins interact. The recombinant RAD51B/RAD51C heterocomplex has been purified to near homogeneity, and this interaction has been shown to be stable under partial denaturing conditions. More importantly, immunoprecipitation using RAD51B and RAD51C antibodies showed that the proteins co-purify from several mammalian cell lines indicating the presence of the native complex. In addition, we have examined the RAD51B/RAD51C complex within the context of the other RAD51 paralogs and show evidence for the existence of a single large complex and/or several smaller complexes containing RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3; but interestingly, exclusive of RAD51.

Experimental Procedures

Recombinant Expression of RAD51B and RAD51C—The full-length RAD51B cDNA (5) was cloned into unique BamHI and EcoRI sites in a modified pFastBacTM HT vector (Invitrogen). This vector allows for the
incorporation of an N-terminal glutamate tag (EE (22)). The RAD51C cDNA (8) was cloned by enzymatic digestion into SalI-NcoI sites in the pFastBac1™ vector (Invitrogen). This vector does not provide the recombinant protein with an epitope tag. Recombinant baculoviruses containing either the RAD51B or RAD51C cDNA were produced using the Bac-to-Bac™ expression system (Invitrogen). For recombinant insect production, Sf9 cells (1.5 × 10^6 cells/ml) were infected at a multiplicity of infection of 5 with either recombinant RAD51B or RAD51C virus. Forty-eight hours postinfection, the cells were harvested by low speed centrifugation (1,056 × g) for 20 min at 4 °C. The resulting cell pellet was lysed in cold hypotonic TE buffer (20 mM Tris pH 8.1, 1 mM EDTA, 1 mM MgCl₂, 1 mM dithiothreitol, 10 μg/ml leupeptin, 1 mM colchicine) for 1 h. The concentration of NaCl in the lysate was brought up to 100 mM, and the lysate was centrifuged for 30 min at 100,000 × g at 4 °C. The supernatant was collected and 0.2% N-octylglucoside added. The lysate was incubated at room temperature for a further 10 min, and soluble recombinant protein was obtained after centrifugation at 100,000 × g for 30 min at 4 °C. For additional experiments, recombinant RAD51B and RAD51C baculoviruses were co-infected into Sf9 insect cells at approximately equivalent multiplicities of infection, and recombinant proteins were produced as described above.

**Immunocytochemistry**—Immunocytochemistry was used for the purification of the recombinant RAD51B, RAD51C, and co-infected RAD51B/RAD51C proteins. The supernatant obtained after lysis was applied to an immunocytochemistry column containing the monoclonal antibody to the glutamate epitope tag (EE, monoclonal antibody) bound to Sepharose (22). The column was washed several times in Tris buffer containing various detergents and salts to remove unbound proteins (Wash 1, Buffer A (Tris pH 8.0, 150 mM NaCl, 2 mM β-mercaptoethanol, 0.5% Nonidet P-40); Wash 2, Buffer A with 4 mM NaCl; Wash 3, Buffer A without NaCl, with 1 mM LiCl; Wash 4, Buffer A; Wash 5, 30 mM Na₂CO₃, pH 10, 150 mM NaCl, 2 mM β-mercaptoethanol, 0.25% CHAPS). Purified recombinant RAD51B, RAD51C, or RAD51B/RAD51C proteins were eluted by peptide competition (50 μM/ml with 500 μM β-mercaptoethanol, 0.25% CHAPS). Purified recombinant RAD51B, RAD51C, or RAD51B/RAD51C proteins were eluted by peptide competition (50 μM/ml with 500 μM β-mercaptoethanol, 0.25% CHAPS). The eluant from the column was dialyzed against Tris-buffered saline with 0.1% Tween 20 (Bio-Rad) and visualized by Western blot analysis (Biomolecular Sequencing Facility, UCSF, San Francisco, CA). The RAD51C cDNA was also cloned into the pAcG1 transfer vector (23) containing the N-terminal glutamate epitope tag (EE). Recombinant RAD51C virus was produced by the co-transfection of the RAD51C-pAcG1 transfer vector and Autographa californica baculoviral DNA into Spodoptera frugiperda (Sf9) insect cells. For recombinant insect cell production, Sf9 cells (1.5 × 10^6 cells/ml) were infected at a multiplicity of infection of 5 with either recombinant RAD51B or RAD51C virus. Forty-eight hours postinfection, the cells were harvested by low speed centrifugation (1,056 × g) for 20 min at 4 °C. The resulting cell pellet was lysed in cold hypotonic TE buffer (20 mM Tris pH 8.1, 1 mM EDTA, 1 mM MgCl₂, 1 mM dithiothreitol, 10 μg/ml leupeptin, 1 mM colchicine) for 1 h. The concentration of NaCl in the lysate was brought up to 100 mM, and the lysate was centrifuged for 30 min at 100,000 × g at 4 °C. The supernatant was collected and 0.2% N-octylglucoside added. The lysate was incubated at room temperature for a further 10 min, and soluble recombinant protein was obtained after centrifugation at 100,000 × g for 30 min at 4 °C. For additional experiments, recombinant RAD51B and RAD51C baculoviruses were co-infected into Sf9 insect cells at approximately equivalent multiplicities of infection, and recombinant proteins were produced as described above.

**Cell Culture**—HeLa S3, MCF10A, and MCF7 cells were obtained from the American Type Culture Collection (Manassas, VA). HeLa cells were cultivated in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 300 μg/ml l-glutamine, and antibiotics. MCF10A cells were cultured in Dulbecco’s modified Eagle’s medium/nectarine growth medium (1:1) supplemented with 0.5% hydrocortisone, 0.1 mg/ml bovine insulin (Sigma), 0.5 μg/ml hydrocortisone, 20 ng/ml murine epidermal growth factor (Becton-Dickinson; Franklin Lakes, NJ), 300 μg/ml l-glutamine, and antibiotics. MCF7 cells were grown in Dulbecco’s modified Eagle’s medium α supplemented with 10% heat-inactivated fetal bovine serum, 300 μg/ml l-glutamine, 0.01 mg/ml insulin, and antibiotics. Cells were maintained in a humidified atmosphere at 37 °C and 5% CO₂. All media and supplements were obtained from Invitrogen unless otherwise specified.

**Cellular Lysates and Immunoprecipitation**—Cell extracts were prepared from exponentially growing cells in lysis buffer (50 mM Tris pH 7.5, 50 mM NaCl, 0.5% Nonidet P-40 with protease inhibitors), incubated on ice for 30 min, and centrifuged at 14,000 × g for 5 min to pellet cellular debris, and the supernatants were collected. One milligram of recombinant protein G-agarose was precleared with 300 μg of protein G-agarose (Invitrogen). Samples were incubated with 25 μg of appropriate antibody or antibody preadsorbed with the appropriate peptide. To preadsorb, 25 μg of antibody was incubated with 50 μg of competing peptide. Experiments with RAD51 antibody C-20 (Santa Cruz) used 1 μg of antibody and 2 μg of competing peptide. An additional RAD51 antibody (Oncogene Research) was used to immunoprecipitate XRCC2 because of conflicting species specificity of the C-20 antibody; preimunoaffinity chromatography was chosen for recombinant protein G-agarose was added, and the samples were incubated for 1 h and washed three times with 500 μl of cold lysis buffer. Subsequently, 2 × SDS-PAGE loading buffer was added, and samples were subjected to electrophoresis. For experiments containing 500 μl or 1 ml NaCl, immunoprecipitations were performed as above with extracts prepared in lysis buffer containing 50 μl NaCl that were incubated for 1 h at 37 °C and the concentration, and washed were performed with lysis buffer containing 500 μl or 1 ml NaCl.

Nuclear extracts were prepared as previously described (25). Briefly, cells were scraped into Buffer A (10 mM Hepes pH 7.5, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, with protease inhibitors), frozen in a dry ice/ethanol bath, and quickly thawed. An aliquot of the cells was used for analysis of the nuclei. Samples routinely contain > 95% intact nuclei as determined by trypan blue staining. Samples were centrifuged for 2 min at 15,800 × g to pellet the nuclei, and the supernatant was removed. Nuclei were resuspended in Buffer C (20 mM Hepes pH 7.5, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol plus protease inhibitor mixture) and incubated for 30 min on ice. Samples were centrifuged for 2 min at 15,800 × g to pellet cell debris. Nuclear extracts (~500 μg) were used for immunoprecipitation experiments as described above.

**RESULTS**

**Purification of RAD51B and RAD51C**—Baculoviral expression was chosen for recombinant protein expression because it allows for eukaryotic post-translational modification and robust production of proteins resembling native mammalian counterparts. Recombinant baculoviruses were constructed to contain either the RAD51B or RAD51C cDNA for production of the corresponding recombinant proteins. RAD51B and RAD51C were independently cloned into a modified pFastBac™ vector such that each of the recombinant proteins would contain an N-terminal EE epitope tag for protein purification. Recombinant baculoviruses were generated using the BAC-to-Bac™ method (Invitrogen). Insect cells were infected with either the RAD51B or RAD51C baculovirus and incubated for 48 h. The cells were lysed, and the recombinant proteins were purified on an immunoaffinity column consisting of the EE antibody bound to Sepharose followed by purification on pep-
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To demonstrate the stability of the RAD51B/RAD51C interaction, the recombinant RAD51B and RAD51C baculoviruses were again co-infected into insect cells, co-purified by immunoaffinity chromatography in the presence of 1 M KCl and eluted in increasing concentrations of detergent. The RAD51B/ RAD51C heterocomplex remains stable in the presence of 0.1–1% SDS demonstrating a strong interaction between the two proteins (data not shown). This experiment was devised in a manner similar to experiments by Sung, which showed that the yeast RAD55/RAD57 complex was stable under mild denaturing conditions (4). The interaction of the RAD51B and RAD51C proteins was further demonstrated using in vitro transcription/translation and immunoprecipitation (data not shown).

RAD51B and RAD51C Form a Stable Heterocomplex in Vivo—Immunoprecipitation experiments were performed using HeLa whole cell extracts to determine whether an interaction between the endogenous RAD51B and RAD51C proteins could be identified. Using the RAD51C monoclonal antibody, we performed immunoprecipitation experiments to determine whether RAD51B could be identified from HeLa cell extracts in a complex with RAD51C. RAD51B was detected by Western blot analysis with the RAD51B-specific antibody following immunoprecipitation using the RAD51C antibody co-precipitated the endogenous RAD51B/RAD51C heterocomplex (Fig. 2B). Based on the premise that RAD51B and RAD51C proteins participate in homologous recombination, we examined the endogenous complex in the nucleus where the proteins are most likely to function. The immunoprecipitation experiments were repeated with HeLa nuclear fractions with identical results shown in Fig. 2, C and D. Immunoprecipitation using the RAD51C antibody co-precipitated the endogenous RAD51B protein (Fig. 2C), and immunoprecipitation using the RAD51B antibody co-precipitated the RAD51C protein (Fig. 2D). These results demonstrate the presence of the endogenous RAD51B/RAD51C heterocomplex in mammalian cells.
RAD51C interacts with RAD51B within a larger complex in vivo

The RAD51B/RAD51C interaction was shown to be specific because preincubation of antibody with competing peptide disrupts co-immunoprecipitation of protein partners, resulting in a loss of immunostaining by Western blot analysis (Fig. 2, compare lanes 1 and 2). Western blot analysis of Hela cell extracts demonstrates the presence of the native RAD51B and RAD51C proteins (Fig. 2, A, B, and D, lane 3). The recombinant RAD51B-E-E protein expressed from baculovirus was used as a control in panel C, lane 3. The recombinant protein migrates more slowly by SDS-PAGE than its native mammalian counterpart, this is most likely caused by the inclusion of the EE epitope tag on the recombinant protein.

RAD51B and RAD51C form complexes with multiple paralogs—We extended these observations to examine whether the other RAD51 paralogs (RAD51D, XRCC2, XRCC3, or RAD51 itself) could be detected in an endogenous complex with RAD51B and/or RAD51C. The RAD51B polyclonal antibody was used to immunoprecipitate putative protein partners from HeLa whole cell extracts under varying stringencies. In addition to RAD51C, RAD51D and XRCC2 were found to co-precipitate with RAD51B by Western blot analysis using paralog-specific antibodies; neither XRCC3 nor RAD51 were found to complex with RAD51B (Fig. 3, lane 1, A–E). Moreover, immunoprecipitation of these complexes containing RAD51B, RAD51C, RAD51D, and XRCC2 were stable in the presence of 1 M NaCl (as shown in Fig. 3, lanes 1–3, B–D, respectively), suggesting strong interactions between the proteins as the complexes appear to remain intact under conditions of high ionic strength. Similar to the experiments in Fig. 2 described above, competition with RAD51B-specific peptide showed no co-precipitation of protein partners (Fig. 3, lane 4, A–E). The endogenous RAD51 paralogs and RAD51 itself were detectable by direct Western blot analysis demonstrating that the proteins were present in HeLa cells (see Fig. 3 lane 5, A–E). The RAD51 paralogs have similar molecular masses and migrate between 36 and 50 kDa.

We performed additional immunoprecipitation experiments using the RAD51C monoclonal antibody to examine protein complex partners. In addition to RAD51B, we identified RAD51D, XRCC2, and XRCC3 in a complex with RAD51C (Fig. 4, lanes 1–3, A–E). Preincubation with RAD51C-specific peptide showed no co-precipitation of other protein partners (Fig. 4, lane 4, A–E). Western blot analysis was used to confirm the presence of the endogenous proteins (Fig. 4, lane 5, A–E). Interestingly, RAD51 was not detected in the RAD51C immunoprecipitant in the Hela whole cell extracts. Protein complexes immunoprecipitated with RAD51C remained intact under conditions of high ionic strength similar to those detailed above (Fig. 4, lane 1–3). We expanded these experiments to examine these paralog complexes in two additional cell lines, MCF10A mammary epithelial cells and MCF7 breast cancer cells, to confirm the integrity of protein interactions. Comparable with our results in HeLa cells, complexes containing RAD51B, RAD51D, XRCC2, and XRCC3 (but not RAD51) were found to immunoprecipitate with RAD51C in the MCF10A and MCF7 cells (Fig. 5, panels I and II, lane 1, A–E). Specificity of these protein interactions was further demonstrated by preincubation of the RAD51C antibody with RAD51C-specific peptide that showed no complex formation, yet all six proteins were detectable in the extracts (Fig. 5, panels I and II, lanes 2 and 3). Our observations that neither RAD51B nor RAD51C appeared to be in a complex with RAD51D in any of the three cell lines examined led us to perform reciprocal immunoprecipitation using a RAD51C-specific antibody for examination of the presence of the RAD51B and RAD51C proteins in the precipitant. We did not detect an interaction between RAD51D and RAD51B or RAD51C, or any other RAD51 paralogs (RAD51D, XRCC2, or XRCC3), but we were able to identify RAD51 in the immunoprecipitant (Fig. 6, A–F, lane 1). Control experiments showed no evidence of immunoprecipitiation of RAD51 or any of the paralogs (Fig. 6, A–F, lane 2). Direct Western blot analysis detected all six proteins from HeLa cell extract (Fig. 6, A–F, lane 3). Interestingly, these data do not support a direct interaction between RAD51D and XRCC3 that has been previously suggested using a HeLa cell line overexpressing XRCC3 (19).

**DISCUSSION**

Using multiple methods (both in vitro and in vivo) we have shown that RAD51B and RAD51C proteins form a heterocomplex. These studies are consistent with the results of Schild et al. (8, 18) and Sung (26), indicating an interaction between RAD51B and RAD51C. Unique RAD51B and RAD51C baculoviruses were generated and co-infected into insect cells to pro-
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**Fig. 5. Immunoprecipitation of the RAD51 paralogs RAD51B, RAD51D, XRCC2, and XRCC3 with RAD51C**

Panel I, MCF10A cells (normal breast epithelium); panel II, MCF7 cells (malignant breast epithelium). Lane 1, cell extracts were immunoprecipitated with RAD51C antibody; lane 2, cell extracts immunoprecipitated with RAD51C antibody preadsorbed with RAD51C-specific peptide; lane 3, immunoprecipitation 1 x NaCl; lane 4, HeLa extracts immunoprecipitated with RAD51C antibody preadsorbed with RAD51C-specific peptide; lane 5, direct Western detection of endogenous protein (100 μg). Each panel was individually incubated with antibody and visualized by ECL followed by autoradiography. A, RAD51 polyclonal antibody (1:2000); B, RAD51B polyclonal antibody (1:1000); C, RAD51D polyclonal antibody (1:2000); D, XRCC2 polyclonal antibody (1:500); E, XRCC3 polyclonal antibody (1:5000).

**Fig. 6. Immunoprecipitation of RAD51 does not co-precipitate the RAD51 paralogs.** None of the RAD51 paralogs was found to co-precipitate with RAD51. Lane 1, HeLa whole cell extracts were immunoprecipitated with a RAD51 goat polyclonal antibody (c20 Santa Cruz); lane 2, HeLa extracts immunoprecipitated with RAD51 antibody preadsorbed with peptide; lane 3, direct Western detection of endogenous protein (100 μg). Each panel was individually incubated with antibody and visualized by ECL followed by autoradiography. A, RAD51 rabbit polyclonal antibody (1:2000); B, RAD51B polyclonal antibody (1:1000); C, RAD51C monoclonal antibody (1:1000); D, RAD51D polyclonal antibody (1:2000); E, XRCC2 polyclonal antibody (1:500); F, XRCC3 rabbit polyclonal antibody (1:5000).

None of the RAD51 paralogs was found to co-precipitate with RAD51. Our data propose an endogenous relationship between the RAD51 paralogs. None of the RAD51 paralogs was found to co-precipitate with RAD51. Lane 1, HeLa whole cell extracts were immunoprecipitated with a RAD51 goat polyclonal antibody (c20 Santa Cruz); lane 2, HeLa extracts immunoprecipitated with RAD51 antibody preadsorbed with peptide; lane 3, direct Western detection of endogenous protein (100 μg). Each panel was individually incubated with antibody and visualized by ECL followed by autoradiography. A, RAD51 rabbit polyclonal antibody (1:2000); B, RAD51B polyclonal antibody (1:1000); C, RAD51C monoclonal antibody (1:1000); D, RAD51D polyclonal antibody (1:2000); E, XRCC2 polyclonal antibody (1:500); F, XRCC3 rabbit polyclonal antibody (1:5000).

**FIG. 4. Immunoprecipitation of the RAD51 paralogs RAD51B, RAD51D, XRCC2, and XRCC3 with RAD51C.** RAD51B, RAD51D, XRCC2, and XRCC3 form stable complexes with RAD51C. Lanes 1–3, HeLa whole cell extracts were immunoprecipitated with RAD51C antibody. Lane 1, immunoprecipitation in 50 mM NaCl; lane 2, immunoprecipitation in 500 mM NaCl; lane 3, immunoprecipitation 2 x NaCl; lane 4, HeLa extracts immunoprecipitated with RAD51C antibody preadsorbed with RAD51C-specific peptide; lane 5, direct Western detection of endogenous protein (100 μg). Each panel was individually incubated with antibody and visualized by ECL followed by autoradiography. A, RAD51 polyclonal antibody (1:500); B, RAD51B polyclonal antibody (1:2000); C, RAD51D polyclonal antibody (1:2000); D, XRCC2 polyclonal antibody (1:500); E, XRCC3 polyclonal antibody (1:5000).

Previous studies have noted several putative interactions between the RAD51 paralogs (18). We have extended our observations of the formation of the RAD51B/RAD51C heterocomplex in vivo to identify additional interactions with the other RAD51 paralogs. Our studies show that RAD51B, RAD51D, XRCC2, and XRCC3 co-precipitate with RAD51C, yet only RAD51C, RAD51D, and XRCC2 were observed to co-precipitate with RAD51B. The reproducibility of the immunoprecipitation results in multiple cell lines demonstrates that these protein complexes are not specific to HeLa cells and are identical in the different mammalian cell types examined. Schidl et al. suggests that RAD51B binds only to RAD51C; this report also showed an interaction between RAD51C, RAD51B, and RAD51D in concert as well as RAD51D, RAD51C, and XRCC2 (18). Taken together, these studies imply that RAD51B binds to the other paralogs via RAD51C, indicating the possibility of a single large multiprotein complex composed of RAD51B, RAD51C, RAD51D, and XRCC2. However, this evidence does not preclude the possibility of additional dimeric and trimeric paralog subcomplexes in vivo. Furthermore, these complexes appear to be stable and remain intact under conditions of high ionic strength. Preliminary experiments examining the association of RAD51B/RAD51C heterocomplex in the presence of DNase I suggests that there may be a DNA-associated heterocomplex pool as well as the existence of the RAD51B/RAD51C heterocomplex independent of DNA (data not shown). Further studies are required to examine the interaction of the paralog complexes and their association with DNA. In contrast to the evidence suggesting an interaction between RAD51 and other paralogs by yeast two-hybrid studies (8, 18), RAD51 was not detected in these complexes by immunoprecipitation in HeLa cell extracts using either the RAD51B polyclonal or RAD51C monoclonal antibodies. Furthermore, reciprocal immunoprecipitation using a RAD51-specific antibody showed no evidence for an interaction between RAD51 and the RAD51B/RAD51C proteins or between RAD51 and any of the other paralogs. We believe it is unlikely that this observation is due to the masking of the binding sites of these interactions by the RAD51 antibody because reciprocal experiments, using the RAD51B and RAD51C antibodies, gave similar negative results. Moreover, the RAD51C antibody used in these studies was polyclonal, thus providing multiple binding sites on the protein which made it unlikely that an interaction would be masked. Previous studies have reported an interaction between RAD51 and XRCC3 (19); this finding was not reproduced in our results and requires further investigation. It is plausible that the RAD51 paralogs interact with RAD51 through additional proteins only in the presence of a DNA double-strand break, or perhaps the interaction is transient or weak and therefore not detectable using these methods. Evidence from DT40 knockout experiments have shown that the RAD51 paralogs are critical for RAD51 foci formation, implicating not only a functional relationship with RAD51 but also that the paralogs are likely to participate together in recombinational repair (10, 11). Although our evidence suggests that RAD51 does not directly associate with these proteins, this does not discount a functional relationship.

Our data propose an endogenous relationship between RAD51B, RAD51D, XRCC2, and XRCC3 with RAD51C, which appears to be central to complex formation. Similar to our findings, an endogenous XRCC3/RAD51C complex has been identified (21). This complex has been shown to perform homologous pairing in the absence of RAD51 (20). In a recent study...
the RAD51B/RAD51C proteins have been shown to participate in recombinational repair processes similar to the RAD55-RAD57 complex in vitro (26). This would suggest that RAD51C has multiple roles in vivo, pairwise with XRCC3 and RAD51B, or in a larger complex with the other RAD51 paralogs possibly dependent on specific cell type (as has been suggested for the XRCC3/RAD51C complex in brain). The specific role of the RAD51B/RAD51C heterocomplex alone or within a larger multiprotein complex remains to be determined. Additional analyses will be needed to address the intricate molecular biochemistry underlying this complex DNA repair system. Ultimately this will be revealed by in vitro reconstitution of the entire recombinational repair process.

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