

RAD51AP2, a novel vertebrate- and meiotic-specific protein, shares a conserved RAD51-interacting C-terminal domain with RAD51AP1/PIR51

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ABSTRACT

Many interacting proteins regulate and/or assist the activities of RAD51, a recombinase which plays a critical role in both DNA repair and meiotic recombination. Yeast two-hybrid screening of a human testis cDNA library revealed a new protein, RAD51AP2 (RAD51 Associated Protein 2), that interacts strongly with RAD51. A full-length cDNA clone predicts a novel vertebrate-specific protein of 1159 residues, and the RAD51AP2 transcript was observed only in meiotic tissue (i.e. adult testis and fetal ovary), suggesting a meiotic-specific function for RAD51AP2. In HEK293 cells the interaction of RAD51 with an ectopically-expressed recombinant large fragment of RAD51AP2 requires the C-terminal 57 residues of RAD51AP2. This RAD51-binding region shows 81% homology to the C-terminus of RAD51AP1/PIR51, an otherwise totally unrelated RAD51-binding partner that is ubiquitously expressed. Analyses using truncations and point mutations in both RAD51AP1 and RAD51AP2 demonstrate that these proteins use the same structural motif for RAD51 binding. RAD54 shares some homology with this RAD51-binding motif, but this homologous region plays only an accessory role to the adjacent main RAD51-interacting region, which has been narrowed here to 40 amino acids. A novel protein, RAD51AP2, has been discovered that interacts with RAD51 through a C-terminal motif also present in RAD51AP1.

INTRODUCTION

The eukaryotic *RAD51* gene was first identified in baker's yeast through analysis of mutations that result in recombination deficiency and sensitivity to DNA-damaging agents (1). Genetic studies in yeast demonstrated that the RAD51 protein

plays a prominent role in both mitotic and meiotic recombination, and in DNA repair (2–5). In meiosis, RAD51 is thought to promote homologous chromosome synapsis and interhomolog recombination (6–8). Sequence and structural analysis of the yeast and mammalian RAD51 proteins revealed extensive similarity to the key bacterial recombinase RecA. The extensive biochemical analysis of yeast and human RAD51 proteins highlighted the functional similarities between these proteins and the RecA protein (9–12). The *RAD51* gene in vertebrates has been shown to be essential for cell proliferation. Mice with a targeted disruption of the *RAD51* gene die early during embryogenesis (13,14). Furthermore, repression of human *RAD51* is lethal in a chicken *rad51*^{-/-} DT40 cell lines expressing an inducible HsRAD51, probably due to the accumulation of chromosome breaks, supporting a role for HsRAD51 in the repair of DNA breaks arising during replication (15).

The normal activity of RAD51, both in homologous recombinational DNA repair in mitotic cells and in meiotic recombination, necessitates its physical interactions with a number of other proteins. Indeed, mammalian RAD51 has been shown to interact *in vitro* and/or *in vivo* with the recombination proteins RAD52 and RAD54 (16,17), the tumor suppressors TP53 and BRCA2 (18–23), the protein kinase c-ABL (24,25), the SUMO-1-conjugating enzyme UBC9 (26–28), the RAD51 paralogs XRCC3 and RAD51C (29,30), MDC1 (mediator of DNA damage checkpoint) (31), RPA (replication protein A) (32,33), CHK1 (34), nucleolin (35) and several other proteins [for review see (12,36)]. The interactions of RAD51 with TP53, RPA and the BRC repeats of BRCA2 are relatively well understood (see Discussion). Much remains to be learned about other RAD51 protein–protein interactions, and clearly further biochemical and genetic analyses of these interactions will facilitate our understanding of the function and regulation of the RAD51 protein.

In a previous study, we identified a novel RAD51-interacting protein, RAD51AP1/PIR51 through a yeast two-hybrid (Y2H) human cDNA library screen (37). (Please note: the HUGO committee on nomenclature has renamed *PIR51* as *RAD51AP1* for *RAD51 Associated Protein 1*.)

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Using the same approach, Mizuta *et al.* (19) independently identified the mouse *Rad51ap1* homolog (originally called *Rab22*). The interaction of human and mouse RAD51AP1 with RAD51 was confirmed by *in vitro* experiments, and *in vivo* co-localization of mouse RAD51AP1 to RAD51 foci was shown using ectopical overexpression of tagged constructs (19,37). In the current study, we present data on the identification of another novel human protein, RAD51AP2 (RAD51 Associated Protein 2), which interacts strongly and specifically with human RAD51. Interestingly, human *RAD51AP2* expression is detected only in adult testis and fetal ovary, which suggests that its gene product may play a role in RAD51-mediated meiotic recombination. Moreover, we have identified a common motif present in both RAD51AP2 and RAD51AP1 that mediates their interaction with RAD51. A sequence that shares some homology with this motif is also present in the RAD54 protein, but seems to play at most only a minor role in the interaction of RAD54 with RAD51.

MATERIALS AND METHODS

Two-hybrid system

The Gal4-based Y2H system was essentially as described previously (37). All the vectors and yeast strains were obtained from Clontech. To screen for RAD51-interacting proteins, a human testis cDNA library in vector pACT2 was used. Yeast strain HF7c was first transformed with a RAD51 bait construct, pEG918, followed by the library DNA. Interacting clones were selected as His⁺ transformants, and were subsequently tested for activation of *lacZ* reporter in strain SFY526. The clone containing the partial *RAD51AP2* gene sequence, whose protein product interacts with RAD51, was designated p23-1. Vectors pGBT9 and pGADGH were used to make additional two-hybrid constructs for human RAD51AP1, RAD51AP2, RAD54 and DMCI1, and for *Saccharomyces cerevisiae* RAD51. Vector pGBKT7 was used to make the RAD51AP2-C33 construct for the experiment in Figure 5C. For Y2H analyses of some of the RAD51AP1 and RAD51AP2 mutants and for the RAD54 truncation analyses, the yeast strain Y190-ura⁻ was used (29). The QuikChange II Site-directed Mutagenesis Kit (Stratagene) was used for mutagenesis, and each mutated/truncated construct was sequenced across the complete insert.

Cloning of the full-length *RAD51AP2* ORF

To amplify the full length *RAD51AP2* open reading frame (ORF), primers were designed from the 5'-untranslated region (5'-UTR) (5'-GCGGAATTCGACAGATCCTTTCCATTGCTGTC-3'; the underlined EcoRI site was added for subcloning) and from the 3'-UTR (5'-CGCGGATCCATGCCCAAACCCCAAGCTGGAAGAAC-3'; the underlined BamHI site was added for subcloning). GenBank contains three expressed sequence tag (EST) clones (loci DB088408, DB096210 and DB449196) that are reported to be full length and have sequence data from the 5' end of the *RAD51AP2* cDNA. Since these clones were not available, we used their partial GenBank sequence data from the 5'-end and our own DNA sequence data for the 3'-end to design primers to

amplify the entire ORF from Marathon-Ready total cDNA from human testis (Clontech). Since no PCR product was observed using these two primers, two additional internal primers were designed for use in combination with the original primers to separately amplify the 5'-region and the 3'-region of the *RAD51AP2* ORF (5'-CGTAACTGTCCAGACATTCTGCTTCTCCC-3' was used in combination with the original 5' primer, and 5'-GAAAATGACTACCCATCACTCAGTAGCC-3' was used in combination with the original 3' primer). PCR was performed with high-fidelity *Pfu* DNA polymerase (Stratagene) using 30 cycles. The resulting two PCR products overlap by ~100 bp, and each product includes the unique PstI site in the middle of the *RAD51AP2* ORF. Following restriction digestion, the EcoRI to PstI fragment from the 5'-end and the PstI to BamHI fragment from the 3'-end were each subcloned into the EcoRI to BamHI sites of a single pBluescript SK+ vector, reconstituting the full-length *RAD51AP2* ORF. The complete DNA sequence of the insert in this plasmid (pDS443) was determined for both stands.

Northern analysis of the *RAD51AP2* expression

A human multiple tissue Northern blot II (Clontech) was used to analyze *RAD51AP2* messenger RNA (mRNA) expression. The sequence of the *RAD51AP2* insert in clone p23-1 was amplified by PCR. The resulting fragment was labeled with α -³²P-dCTP by random priming and used as the hybridization probe following conditions suggested by Clontech.

PCR analysis of *RAD51AP1* and *RAD51AP2* transcripts in different human tissues

PCR was used to determine if the *RAD51AP1* and *RAD51AP2* transcripts are present in cDNA samples from 16 different adult human tissues (Clontech), 5 different fetal human tissues (BioChain) and 7 different human cell lines (Clontech). The PCR primers used for *RAD51AP1* were AP1-5': 5'GATGACAAGCTCTACCAGAGAGAC3' and AP1-3': 5'CTTGCTTTCAGCTGAAGGACTGCG3' and these amplify a 630 bp fragment. The primers for *RAD51AP2* were AP2-5': 5'CTGGTCTGAGTGAAGGGAATGATG3' and AP2-3': 5'GCGGTCGTACTCTTGAAATGCCATG3', and these amplify a 570 bp fragment. For each primer pair, the two different primers were from different exons, thus avoiding the possible problems with contaminating genomic DNA, since this DNA would result in a much larger PCR product. PCR was performed using *Taq* polymerase, with 35 cycles of 50°C 60 s, 72°C 120 s and 94°C 60 s.

Interaction of RAD51 and RAD51AP2 proteins in mammalian cells

To produce a RAD51AP2 protein fragment in mammalian cells, the sequence encoding the 293-amino acid RAD51AP2 polypeptide from p23-1 was subcloned into vector pCGT (obtained from Dr H. Zhang, Yale University) as a fusion with a T7 epitope, producing plasmid pOK80. Also, RAD51AP2-C293 sequence lacking its last 57 amino acids (RAD51AP2- Δ C57) was cloned into pCGT, producing plasmid pOK90. Fortunately, the vector pCGT used for cloning contained the sequence of human herpesvirus Vmw65

protein. Cloning of RAD51AP2 sequences into this vector placed the inserts upstream of the Vmw65 sequences, effectively eliminating expression of the latter. Thus, when used as a control in transfection experiments, vector pCGT produced the T7-tagged 65 kDa Vmw65 polypeptide, whereas plasmids pOK80 and pOK90 produced only the expected T7-tagged RAD51AP2 protein fragments.

For transient transfection and co-immunoprecipitation experiments, pCGT, pOK80 and pOK90 were introduced into cultured human HEK293 cells using the calcium phosphate procedure, and cells were incubated for 36 h. In some experiments, DNA damage was introduced to transfected cells 3 h before the cells were harvested by adding 100 µg/ml methylmethane sulfonate or 0.5 µg/ml mitomycin C to the medium. As a control, cycloheximide, a protein synthesis inhibitor, was added at 10 µg/ml in these experiments. After harvest, cells were lysed in buffer containing 20 mM Na-phosphate (pH 7.4), 150 mM NaCl, 0.5% NP-40, and a cocktail of protease inhibitors (Roche) for 20 min on ice. Lysates were used for immunoprecipitation with monoclonal antibody directed against the T7 tag (Novagene) followed by protein A-agarose beads. The beads were washed several times with lysis buffer and the immunoprecipitated material was analyzed by SDS-PAGE followed by immunoblotting with anti-T7 and anti-RAD51 antibodies. Secondary anti-mouse or anti-rabbit antibodies were conjugated with alkaline phosphatase, and detection was carried out with NBT (nitroblue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolylphosphate p-toluidine salt) chromogenic substrates (Life Technologies), or with the more sensitive Renaissance® chemiluminescence reagent (NEN Life Science Products). Bands produced on an X-ray film after chemiluminescence detection were scanned and quantitated using a Molecular Dynamics densitometer.

RESULTS

Isolation of the human RAD51AP2 gene

Using the human RAD51 protein as a bait, a Y2H screen of a human testis cDNA library resulted in the isolation of an ~1.2 kbp insert encoding 321 amino acids, a stop codon and a 3'-UTR (clone p23-1). The complete ORF was PCR amplified as two overlapping fragments (see Material and Methods). The two PCR products were used to reconstruct the full-length ORF, which was cloned into pBluescript SK+ and sequenced (GenBank accession # DQ860102). This sequence data shows that this ORF is predicted to encode a protein of 1159 amino acids, and its C-terminus is identical to our Clone p23-1. We have named this protein RAD51AP2 (RAD51 Associated Protein 2) (Figure 1B). Comparing the ORF sequence with the genomic sequence indicates that RAD51AP2 maps to human chromosome 2 and the ORF consists of three exons, one very large one encoding 1083 amino acids, followed by two much smaller ones encoding 27 amino acids and 49 amino acids (Figure 1A). Our DNA and predicted amino acids sequence data are completely consistent with a GenBank hypothetical cDNA (XM_496545) and protein (XP_496545).

Sequence analysis of the mouse genome predicts a homologous protein of 976 amino acids (GenBank

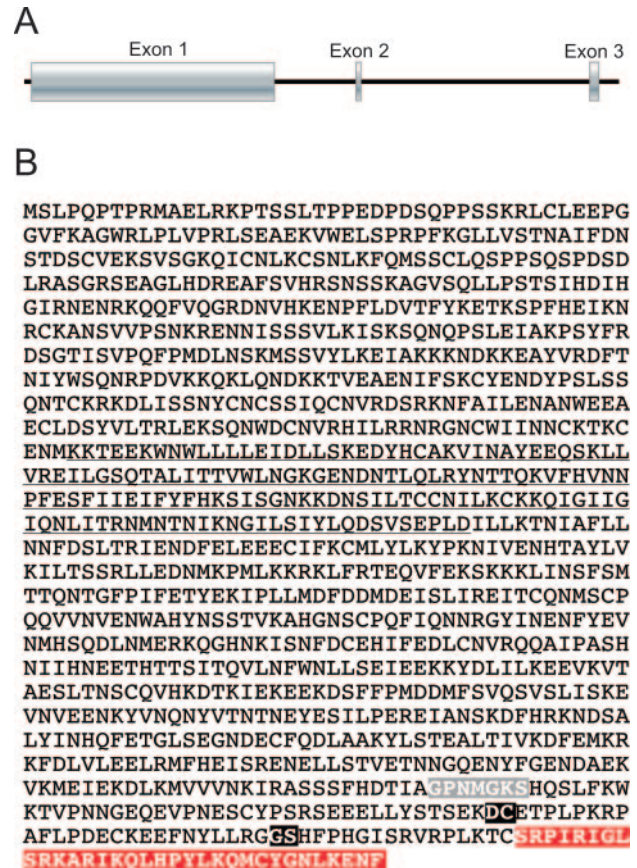


Figure 1. Structure of the human RAD51AP2 gene, and sequence of the human RAD51AP2 proteins. (A) Comparing the cDNA sequence we have determined with the genomic sequence in GenBank, it was determined that RAD51AP2 consists of one very long exon followed by two short exons, the last of which completely encodes the RAD51-interacting domain. (B) The amino acid sequence of the entire human RAD51AP2 proteins predicted from our RAD51AP2 cDNA sequence (GenBank accession # DQ860102). Residues similar to a Walker A-type nucleotide-binding motif in human RAD51AP2 are in the light gray box. The long underlined region is missing in the mouse homolog, and the short black boxed residues are the two boundaries among the amino acids encoded by the three exons. The C-terminal 33 amino acids (C33) in the red box are sufficient for RAD51-interaction in the Y2H system.

XP_147640; the predicted cDNA XM_147640 maps to mouse chromosome 12). The sequence alignment of the predicted human and mouse homologs indicates that the human protein has an extra region of 154 amino acids in the middle of exon 1 (underlined in Figure 1B). This extra region does not appear to be an extra unexcised intron, since it does not have a sequence resembling the consensus for a splice junction, and this extra 154 amino acids region is also present in the genomic sequence of *Pan troglodytes* (chimpanzee) (data not shown). Even the *Rattus novogicus* genomic sequence is only missing 45 of the 154 amino acids compared to the human protein. At the very C-terminus the human and chimpanzee proteins also encode 11 amino acids that are not present in either the mouse or rat protein. GenBank also contains a partial sequence of a *Gallus gallus* (chicken) homolog that also appears to be missing the 11 amino acids at the C-terminus (Figure 5A).

The predicted 1159 amino acid sequence of human RAD51AP2 shows no significant overall homology to any other protein in the GenBank, and has no apparent protein motifs, except for a sequence Ala-xxxx-Gly-Lys-Ser in the C-terminus that resembles the Walker A-type nucleotide binding motif (consensus Ala/Gly-xxxx-Gly-Lys-Thr/Ser), but RAD51AP2 lacks any obvious Walker B motif. The mouse, rat and partial chicken RAD51AP2 homologs all lack any sequence similar to a Walker A or B motif. Although the human RAD51AP2 protein lacks any other apparent protein motif, the C-terminal region does show some interesting properties. This region is positively charged: among the last 55 residues, there are 7 arginines, 5 lysines and 3 histidines, with only one single negatively charged residue (glutamic acid). In addition the C-terminal region shares homology with RAD51AP1 (presented below).

RAD51AP2 gene expression occurs in meiotic tissue only

Northern analysis of *RAD51AP2* gene expression was performed using a panel of human mRNAs from various organs. A strong hybridization signal was detected only in testis, with no signal present in thymus, spleen, prostate, uterus, colon, small intestine, and peripheral blood leukocytes (Figure 2). The size of the hybridizing transcript is ~ 4.2 kb, which is long enough that it could accommodate the size of the

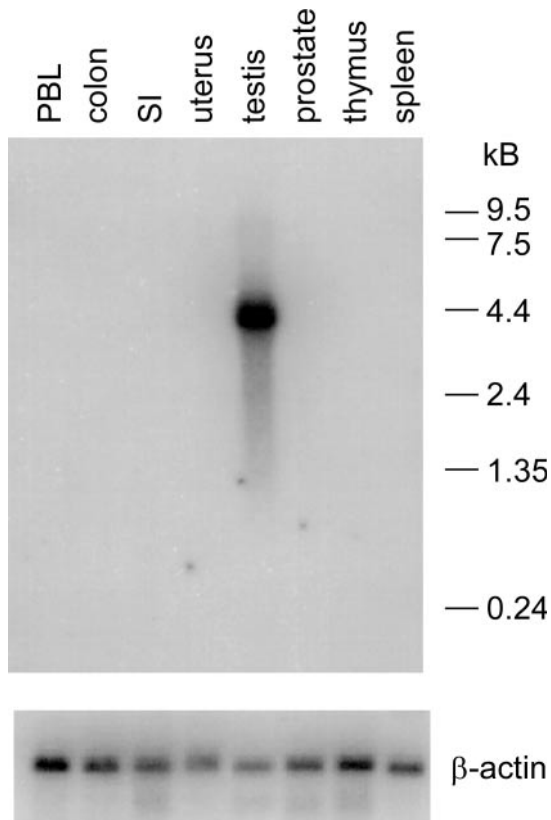


Figure 2. Northern blot analysis of the *RAD51AP2* gene in human tissues. Lanes contain ~ 2 μ g of poly A⁺ RNA from the human tissues indicated. PBL, peripheral blood leukocytes; SI, small intestine. Upper panel, hybridization with *RAD51AP2* cDNA probe. Lower panel, hybridization with human β -actin cDNA probe. The positions of RNA size markers, in kilobases, are indicated on the right.

RAD51AP2 ORF described above. The meiotic specificity of *RAD51AP2* gene expression is supported by our PCR analysis, as we were not able to amplify any *RAD51AP2* sequences from cDNA sources other than adult human testis and fetal ovary. No PCR product was observed in 15 additional human tissues, or 4 additional fetal tissues (Figure 3A and B, and additional data not shown for adult brain, heart, kidney, liver, lung, pancreas, placenta and skeletal muscle). No PCR product was observed with seven human cell lines (HEK293, SKOV-3, Saos2, A431, Du145, H1299 and MCF7) (data not shown). As a control, a PCR product from the *RAD51AP1/PIR51* transcript was amplified, and shown to be present in each of these same human 16 adult tissues, 5 fetal tissues and 7 cell lines (Figure 3C–E and additional data not shown). It should be noted that 35 PCR cycles were used to ensure that even rare transcripts would be amplified. This data can be used to determine if a transcript is present in a sample, but can not be used to quantify the transcript level, if it is present.

Information about the expression of new genes can also be obtained by examining the EST database at GenBank, which includes sequences from different cDNA libraries. There are currently only 11 human EST clones in the database with sequence identity to parts of *RAD51AP2*. Six EST clones are from testis libraries, including three that appear to encode the 5'-end (see Materials and Methods), and another EST clone was isolated from a germ cell tumor. The remaining four are from the Athersys RAGE (random activation of gene expression) library constructed by using specialized integration vectors that result in the artificial transcription of even normally silenced genes (38). Therefore, data from the EST database is consistent with expression of *RAD51AP2* in testis and germ cells, with no expression in mitotic tissues. On the other hand, the EST database has numerous *RAD51AP1* EST clones from many different human tissues, supporting the ubiquitous expression of this gene.

RAD51AP2 is not expressed in mitotic cells even after DNA damage

Although expression of *RAD51AP2* was not observed in mitotic cells, it seemed possible that this transcript might be induced by DNA damage. However, treatment of human osteosarcoma-derived U2OS cells (from ATCC) with 2 Gy X-rays still resulted in no *RAD51AP2* transcript (Figure 3F), and the same result was also observed using HeLa cells (data not shown).

Interaction of RAD51AP2 protein with RAD51 in the Y2H system

The 321-amino acids RAD51AP2 fragment was subcloned from p23-1 into two-hybrid vectors as fusions with the DNA-binding (DBD) and activation domain (AD) of Gal4, to reconfirm the interaction with human RAD51 and to test for possible additional interactions. When fused to Gal4-AD, RAD51AP2-C321 (containing the C-terminal 321 amino acids) showed strong and specific interaction with RAD51 (Table 1). No interaction was observed with human DMC1 or the yeast RAD51 protein, both highly homologous to human RAD51, or with human RAD51AP1, another

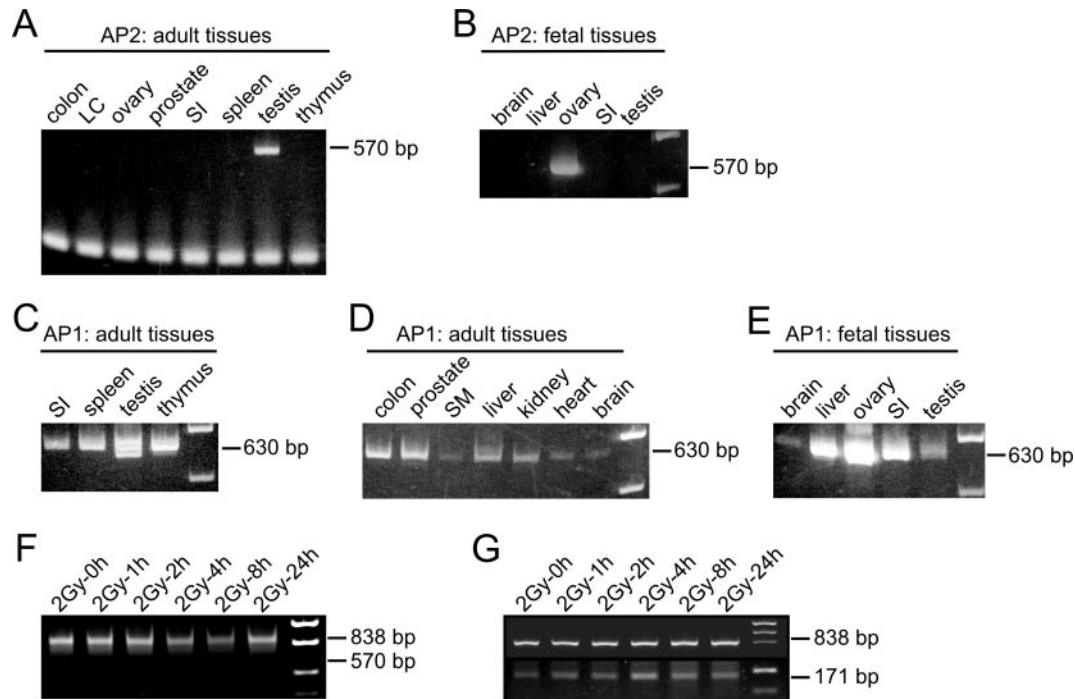


Figure 3. PCR analysis showing meiotic-specific expression of human *RAD51AP2* in adult testis and fetal ovary, but ubiquitous expression of *RAD51AP1*. (A) *RAD51AP2* (AP2 in figure) transcript in human adult tissues (note: *RAD51AP2* is top band in testis sample only and PCR primers are the bottom bands). LC—leukocytes, SI—small intestine. The size and approximate mobility (based on size standards) of the PCR products are indicated at the right side of each panel. (B) *RAD51AP2* transcripts in human fetal tissues. (C and D) *RAD51AP1* (AP1 in figure) transcript in human adult tissues. SM—smooth muscle. (E) *RAD51AP1* transcripts in human fetal tissues. Note: *RAD51AP1* has multiple variants in adult testis and fetal ovary. (B–E) DNA size markers (400 and 800 bp) in right lane. (F) *RAD51AP2* transcript is not induced in U2OS cells exposed to 2 Gy X-rays. End-point RT–PCR was carried out from 1st strand cDNA samples in a multiplex PCR reaction amplifying both a 838 bp product for β -actin and a 570 bp product for *RAD51AP2* (as in A and B). A product for *RAD51AP2* was not obtained (as indicated by the absence of a 570 bp fragment). DNA size markers (1200, 800 and 400 bp) in right lane. (G) Same samples as in F: End-point RT–PCR for β -actin and *GADD45A* (171 bp positive control for the induction of transcript after X-rays). DNA size markers (2000, 1200, and 800 bp in top panel, and 200 and 100 bp in bottom panel) in right lane.

Table 1. Specific interaction of *RAD51AP2* with *RAD51* in the two-hybrid system is mediated by the carboxy-terminus of *RAD51AP2*

Gal4 DBD ^a plasmid	Gal4 AD plasmid	Gal4 DBD protein fusion	Gal4 AD protein fusion	Units of β -galactosidase
pEG918	pOK42	<i>RAD51</i>	<i>RAD51AP2</i> -C321	300.9
pOK21	pOK42	<i>DMC1</i>	<i>RAD51AP2</i> -C321	<0.3
pEG978	pOK42	Sc <i>RAD51</i>	<i>RAD51AP2</i> -C321	<0.3
pOK31	pOK42	<i>RAD51AP1</i>	<i>RAD51AP2</i> -C321	<0.3
pEG918	pOK77	<i>RAD51</i>	<i>RAD51AP2</i> - Δ C57 ^b	<0.5
pEG918	pOK40	<i>RAD51</i>	<i>RAD51AP2</i> -C92	143.4

^aDBD and AD stand for the DNA-binding and activation domain of Gal4, respectively. The units of β -galactosidase are average values from at least four liquid cultures. All inserts are human except Sc*RAD51*, which is from yeast.

^bThis plasmid actually only encodes the C321 fragment deleted for the last 57 amino acids, and similarly in Table 2.

interacting partner of *RAD51*. Also, no *RAD51AP2*-C321 interaction with human UBC9, lamin C or mouse p53 protein was found (data not shown). The *RAD51*-interaction is mediated by the extreme C-terminus of *RAD51AP2*-C321. The *RAD51AP2*-C321 protein in which the last 57 residues are deleted (designated as *RAD51AP2*- Δ C57) fails to interact with *RAD51*. In addition, the last 92 amino acids of *RAD51AP2* (C92) are sufficient for a strong interaction with *RAD51*, although at only ~48% of the strength of the 321 amino acid fragment (Table 1). In light of our results on *RAD51AP2*- Δ C57, it seems likely that the decreased strength of the *RAD51AP2*-C92 interaction is due to reduced stability of this shorter fusion or masking of the interacting region, although we can not rule out the possibility that

additional residues outside of the last 92 amino acids of *RAD51AP2* are necessary for full interaction with *RAD51*. When present as a fusion with Gal4-DBD in a yeast reporter strain, the 321 amino acid *RAD51AP2* polypeptide weakly activates transcription of *lacZ* gene on its own (3–5 units of β -gal; data not shown). Since the *RAD51AP2*-C92 fragment retains the *RAD51* interaction capability in the two-hybrid system and has no transcription activation ability of its own, it was used in all subsequent two-hybrid studies as a Gal4-DBD fusion of *RAD51AP2*. The original cloned 321 amino acid *RAD51AP2* encoding sequence was used as a Gal4-AD fusion.

The full length *RAD51AP2* ORF and three long C-terminal fragments (C661, C830 and C661) were also cloned into the

Y2H vectors and tested for their interaction with human RAD51. All four interacted with RAD51, although more weakly than the C321 fragment, and the longer the protein/fragment, the weaker the interaction (data not shown). Our favored interpretation of this data is that the full-length 1159 amino acid human RAD51AP2 protein is probably not stably expressed at a very high level in yeast, and even the shorter fragments are not expressed well, although better than the full-length protein. An alternative interpretation is that longer fragments of RAD51AP2 contain regions that mask the interaction of RAD51AP2 with RAD51, at least in the Y2H system. Full-length RAD51AP2 and the three truncations were also tested for interaction with human DMC1, but no interaction was observed (data not shown).

Ectopically expressed RAD51AP2 interacts with RAD51 in human mitotic cells

To test if the RAD51-RAD51AP2 interaction could occur *in vivo*, the 293 amino acid RAD51AP2 polypeptide (RAD51AP2-C293) or its C-terminally truncated derivative (RAD51AP2- Δ C57), both tagged with the T7 epitope, were transiently overexpressed in HEK293 cells, and immunoprecipitation with anti-T7 antibody was carried out (Figure 4A). A fraction of endogenous RAD51 protein readily co-precipitated with RAD51AP2-C293, but not with RAD51AP2- Δ C57 or with a control protein (Figure 4A, lanes 7–9). This result further stresses the importance of the extreme C-terminus of RAD51AP2 for the interaction and provides evidence that when recombinant RAD51AP2 is ectopically expressed in mitotic human cells it can associate with RAD51.

Next, we asked whether the RAD51-RAD51AP2 interaction in human cells could be modulated in response to DNA damage. To test this, RAD51AP2-C293 was transiently overexpressed in HEK293 cells as described above, and DNA damage was introduced by adding methyl methanesulfonate (MMS) or mitomycin C (MMC) 3 h before harvesting cells for co-IP analysis (Figure 4B). Results from three independent experiments indicate that co-precipitation of RAD51 with RAD51AP2-C293 is stimulated \sim 7- and \sim 17-fold by MMS or MMC treatment, respectively (Figure 4B, lanes 5–7; and 4C). Previously, it was shown that these agents, at the concentrations used, induce formation of nuclear RAD51 foci in a number of mammalian cell lines, with up to 60% of cells being stained with anti-RAD51 antibody (39,40). These foci presumably mark sites where RAD51-dependent repair of DNA damage takes place. In contrast, cycloheximide, a protein synthesis inhibitor, does not induce RAD51 foci (40) and does not affect significantly co-precipitation of RAD51 with RAD51AP2-C293 (Figure 4B, lane 8; and 4C). Although DNA damage increases the interaction between these two proteins, we do not feel that RAD51AP2 is involved in DNA repair since it is not expressed in mitotic cells (see Discussion).

RAD51AP2 and RAD51AP1 share a common C-terminal motif necessary for interaction with RAD51

Examination of the sequence of the RAD51AP2 C-terminus revealed an intriguing homology with RAD51AP1, a protein we previously isolated because of its interaction with human

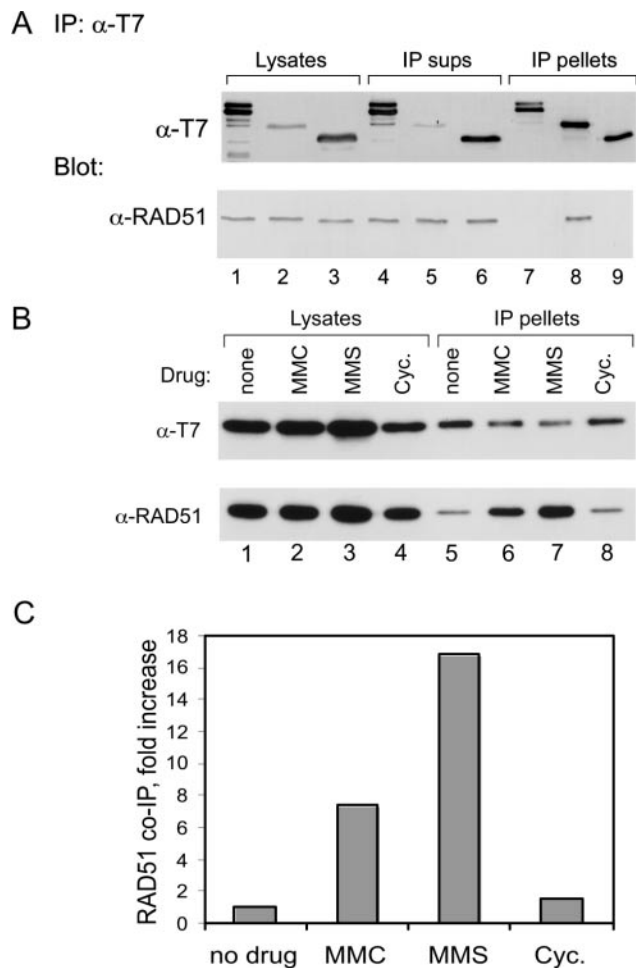


Figure 4. RAD51AP2 and RAD51 proteins interact in human cells, and the interaction is enhanced by DNA damage. (A) Human HEK293 cells were transiently transfected with plasmids encoding the following T7 epitope-tagged proteins: Vmw65 protein of herpesvirus (lane 1); 293 amino acid RAD51AP2 polypeptide (lane 2); RAD51AP2 protein deleted for its last 57 residues (lane 3). Immunoprecipitation with anti-T7 antibody followed by protein A-agarose beads was carried out, and fractions of cell lysates before IP, supernatant of IP, and IP pellets were analyzed by Western blotting using anti-T7 (top panel) or anti-RAD51 antibody (lower panel). Secondary antibodies were conjugated to alkaline phosphatase, which was detected using chromogenic substrates. (B) Transfection with plasmid encoding the 293 amino acid RAD51AP2 polypeptide was performed as in panel A, and 0.5 μ g/ml MMC, 100 μ g/ml MMS or 10 μ g/ml cycloheximide (Cyc.) was added to individual cultures 3 h before harvesting cells for IP. IP and Western blotting were done as in panel A, except that chemiluminescent substrates were used to detect peroxidase conjugated to secondary antibodies. To facilitate the relative comparison of RAD51 signals from IP pellets (shown in panel C), the amount of these fractions loaded on the gel was adjusted in an effort to get similar intensities of the T7-tagged RAD51AP2 signal in each lane. The residual differences were normalized to the control (untreated cells) for comparison of the amounts of RAD51 that coprecipitated with RAD51AP2. (C) Quantitation of RAD51-RAD51AP2 coimmunoprecipitation from panel B. The signals from precipitated RAD51 in IP pellet fractions were measured using a densitometer and then normalized for the signal from anti-T7 analysis of the same fractions. The relative increase of RAD51 signal from treated cells as compared to the signal observed from untreated cells (artificially set at 1.0 in left lane) is shown.

RAD51 (37). The homology between RAD51AP2 and RAD51AP1 spans a region of 35 amino acids located near the C-terminus of the two proteins (Figure 5A), and in this region, 14 amino acids are identical between the two

Table 2. C-terminal fragments of RAD51AP2 and RAD51AP1 proteins interact with RAD51

Gal4 DBD plasmid	Gal4 AD plasmid	Gal4 DBD protein fusion	Gal4 AD protein fusion	Units of β -galactosidase ^a
pEG918	pOK42	RAD51	RAD51AP2-C321	300.9
pEG918	pOK77	RAD51	RAD51AP2- Δ C57	<0.5
pEG918	pOK40	RAD51	RAD51AP2-C92	143.4
pOK76	pEG960	RAD51AP2-C92	RAD51	521.0
pOK92	pEG960	RAD51AP2-C33	RAD51	395.4
pEG918	pOK18	RAD51	RAD51AP1	111.0
pEG918	pOK35	RAD51	RAD51AP1-C89	24.6
pOK31	pEG960	RAD51AP1	RAD51	590.3
pOK62	pEG960	RAD51AP1-C89	RAD51	419.6
pOK81	pEG960	RAD51AP1-C25	RAD51	473.3

^aThe units of β -galactosidase are average values from at least four liquid cultures.

human proteins and 5 are highly conserved. The region of strongest homology is the 16 amino acids C-terminal part of the region encompassing 11 identical and 3 highly conserved residues between RAD51AP1 and RAD51AP2. It is important to note that RAD51AP1 and RAD51AP2 are of very different sizes (335 and 1159 amino acids, respectively) and that these proteins share not other regions of homology.

Deletion analysis was used to further map the region of RAD51AP2 responsible for the interaction with RAD51 (Table 2). As mentioned above, deletion of the last 57 amino acids of RAD51AP2 (RAD51AP2- Δ C57) completely eliminates interaction with RAD51 in yeast and human cells. In contrast, the 92 and 33 amino acids C-terminal fragments of RAD51AP2 show strong two-hybrid interaction with RAD51, in particular when fused to Gal4-DBD (Table 2). This result indicates that a small region at the very end of the cloned RAD51AP2 sequence is its primary site responsible for interaction with RAD51.

As shown above, RAD51AP2 is homologous in its C-terminus to RAD51AP1. Moreover, we demonstrated previously that RAD51AP1 also interacts with RAD51 through its C-terminus (37). To further define the region of RAD51AP1 involved in RAD51-binding, the C-terminal fragments of RAD51AP1 analogous to those of RAD51AP2 were tested for their interaction with RAD51. In particular, fragments RAD51AP1-C89 and RAD51AP1-C25 were used, which roughly correspond to RAD51AP2-C92 and RAD51AP2-C33, respectively. As shown in Table 2, RAD51AP1-C89 and RAD51AP1-C25 protein fragments are highly efficient in their interaction with RAD51. Both RAD51AP2-RAD51 and RAD51AP1-RAD51 interactions occur when the inserts are in either orientation with regards to the Y2H vectors, but are strongest when RAD51 is present as a Gal4-AD fusion. Thus, RAD51AP2 and RAD51AP1 are both using short sequences at their C-termini to bind to RAD51. This

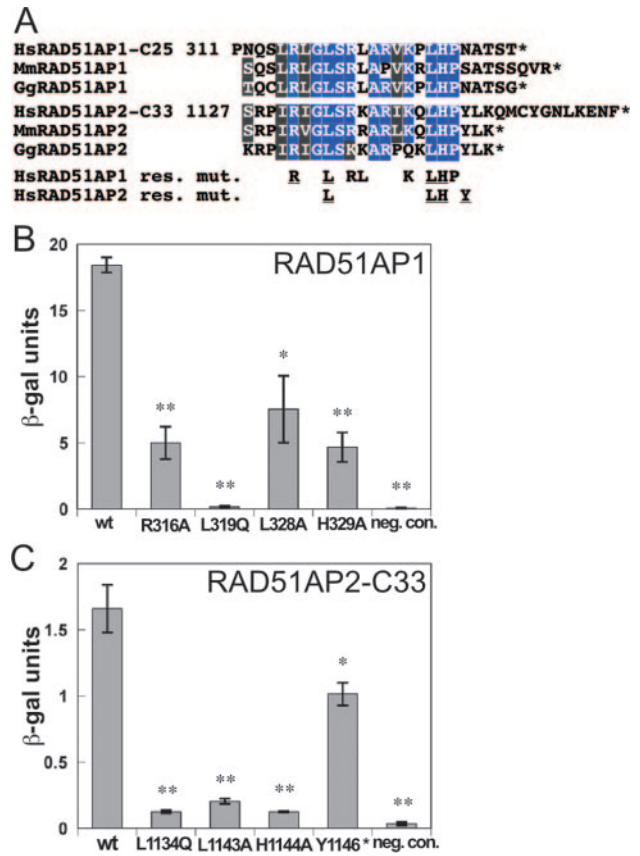


Figure 5. Sequence homology between the RAD51AP2 and RAD51AP1 and mutational analysis of their shared RAD51-interacting motifs. (A) Amino acid identities and similarities are indicated by dark blue and gray squares, respectively. Just the C-terminal domains of RAD51AP1 and of RAD51AP2 are shown. Under the sequences are the original residues that were mutated by site-directed mutagenesis. The underlined mutated residues significantly reduce the interaction with RAD51 (see panels B and C). Species abbreviations are as follows: Hs—*Homo sapiens*; Mm—*Mus musculus*; Gg—*Gallus gallus*. (B) Interactions in the Y2H system between RAD51 (i.e. pEG960) and both RAD51AP1 (i.e. pOK31, with all of RAD51AP1) and site-specifically mutated RAD51AP1 (in pOK31). Results are the average from three different colonies, with the standard error of the mean. (C) Interactions in the Y2H system between RAD51 (i.e. pEG960) and both RAD51AP2 (i.e. pDS439; pGBKT7-RAD51AP2-C33) and site-specifically mutated RAD51AP2 (in pDS439). Results are the average from two different colonies, with the standard error of the mean. * Indicate $P < 0.05$ and ** for $P \leq 0.001$ using the student *T*-test. Please note: the Y190-ura⁻ Y2H strain was used in these studies, and it gives lower β -galactosidase activity than the HF7c Y2H strain used in Table 1 and 2, and in addition, the RAD51AP2-C33 construct is in the pGBKT7 vector that also results in lower β -gal activity than similar pGBT9-derived constructs. The negative control was the original empty vector used for that construct, together with RAD51.

conclusion is further supported by our mutational analysis of the RAD51AP2/RAD51AP1 homology region (see below).

Conserved residues within the RAD51AP1 and RAD51AP2 C-terminal regions facilitate interaction with RAD51

Site-specific mutagenesis of residues in the C-terminal domains of RAD51AP1 and RAD51AP2 was performed to determine whether the residues shared in common by these

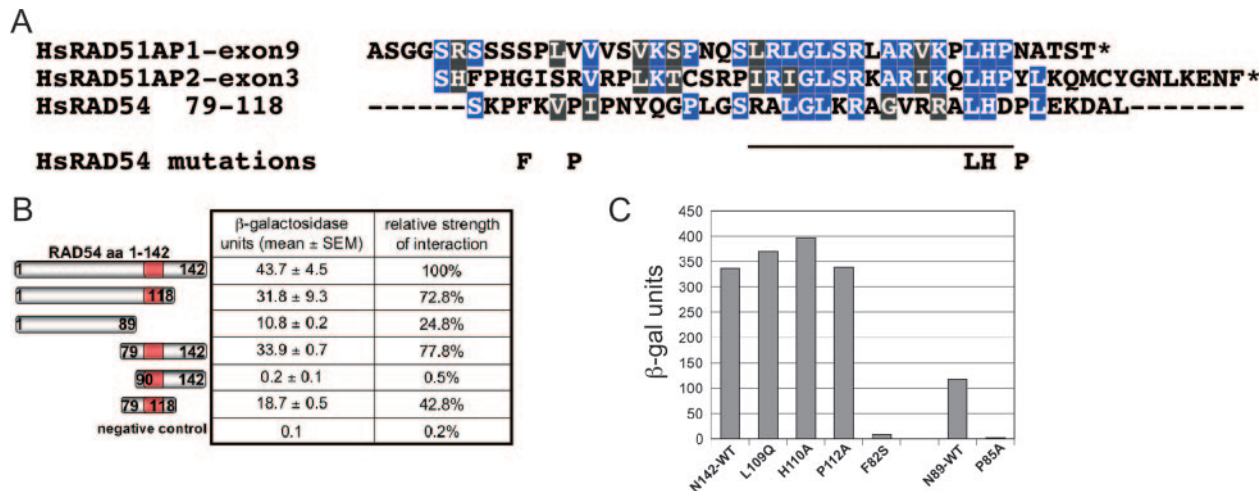


Figure 6. Truncation mapping and mutational analysis of the human RAD54-RAD51 interaction. (A) Amino acid sequence homology between human RAD54 and the RAD51-interacting domains of RAD51AP1 and RAD51AP2. Amino acid identities and similarities are indicated by dark blue and gray squares, respectively. Under the sequences are the original RAD54 residues that were mutated by site-directed mutagenesis. (B) PCR was used to make different truncation constructs of RAD54-N142 (amino acids 1–142) in pGBT9, and these truncations were tested for interaction with RAD51 (pEG960) in the Y2H strain Y190-ura⁻. Results are the average from three different colonies, with the standard error of the mean shown. Only one colony of the negative control (pGBT9 with pEG918) was quantified, but similar negative controls always give about this same result. (C) Mutations were introduced into the N-terminal fragments of RAD54 consisting of 142 or 89 residues (N142 and N89, respectively) and tested for their interaction with RAD51 in the two-hybrid system, using Y2H strain HF7c. The P85A mutation was made in RAD54-N89, while all the rest were made in RAD54-N142. WT, wild-type sequence. For identical constructs, the Y2H strain Y190-ura⁻ gives lower β -galactosidase activity than HF7c.

two proteins are important for their interactions with RAD51. Eight residues in RAD51AP1-CTD were mutated (Figure 5A), and four amino acid substitutions (R316A, L319Q, L328A and H329A) significantly decreased the interaction with RAD51 in the Y2H system using both the qualitative X-gal assay (data not shown) and the quantitative ONPG assay for β -galactosidase activity (Figure 5B). In addition, the double mutant RAD51AP1-H329A/P330A and the double L328/H329 deletion both showed decreased interaction with RAD51 to a similar extent as the individual L328A and H329A mutations in the qualitative assay (data not shown). Amino acid substitutions at other residues did not affect the interaction with RAD51; the RAD51AP1-R321A and -K326A substitutions in residues conserved with human RAD51AP2 each had no significant effect on RAD51 interaction, nor did the RAD51AP1-L322S mutation in a residue not conserved with RAD51AP2 (data not shown).

Amino acid substitutions in RAD51AP2 were made in three residues that are conserved with RAD51AP1 and that greatly reduced the RAD51AP1-RAD51 interaction. In RAD51AP2, these amino acid changes (RAD51AP2-L1134Q, -L1143A and -H1144A) all also greatly decreased the interaction with RAD51 (Figure 5C). A stop codon replacing RAD51AP2-Y1146 also significantly decreased the interaction with RAD51, but to a much lesser extent. It seems reasonable to speculate that the Tyr-Leu-Lys residues shared by human, mouse and chicken RAD51AP2 might be important for full RAD51 interaction, although these residues are not conserved in RAD51AP1.

Refined mapping of the region of human RAD54 that interacts with RAD51

Amino acid sequence comparisons of the shared RAD51-interacting motif (described above) with the sequence of

other RAD51-interacting partners revealed an interesting sequence homology with human RAD54. In the most highly conserved region shared by RAD51AP1 and RAD51AP2, out of 16 amino acids, RAD54 shares 50% homology (six identical residues and two conserved residues) (Figure 6A). We have demonstrated previously that the human RAD54 protein interacts with human RAD51 through sequences located within first 142 residues of RAD54 (16). The region of RAD54 that shares homology with RAD51AP1-CTD and RAD51AP2-CTD is within this previously defined RAD51-interacting region.

The homology of human RAD54 96–111 with the C-termini of RAD51AP2 and RAD51AP1 that facilitate the interaction with RAD51 (Figure 6A) suggests that these residues within RAD54-N142 may be critical for the interaction with RAD51. To test this, truncation mutations were made from RAD54-N142. When tested in the Y2H system for interaction with RAD51, RAD54-N89 gave between 25 and 32% as strong an interaction as full length RAD54-N142 (Figure 6B and C). Since RAD54-N89 does not contain any of the residues shared in common with RAD51AP1 and RAD51AP2, it suggests that the conserved region is not required to obtain significant interaction between RAD54 and RAD51. In addition, RAD54 90–142 that contains the complete conserved region, failed to interact with RAD51 (Figure 6B). On the other hand, there is some evidence that this conserved region may play a role in increasing the interaction with RAD51. e.g. RAD54-N118 fragment gave a much stronger interaction than RAD54-N89. In addition, a 40 amino acids fragment (RAD54 79–118) that completely contains the conserved region gave \sim 43% of the strength of the RAD51 interaction with RAD54-N142. Two larger fragments, each containing RAD54 79–118, but each also including additional residues on one side or the other, both give $>$ 70%, as compared to RAD54-N142. These results

suggest that the core RAD51 interacting region is RAD54 79–118, but that there are additional residues on each side that independently and additively increase the strength of the interaction.

In addition, point mutations were introduced in RAD54 region 79–118, and analyzed in the two-hybrid system (Figure 6C). Amino acid substitutions (RAD54-L109Q and -H110A) in the very conserved Leu-His motif did not affect the RAD54–RAD51 interaction at all, nor did a P112A substitution (Figure 6C). However, we found that residues N-terminal to the RAD51AP1/RAD51AP2 homology region in RAD54 are important for the interaction with RAD51, since RAD54-F82S and -P85A abolish the RAD51 interaction completely (Figure 6C).

In summary, both RAD51AP2 and RAD51AP1 use a common sequence motif located near their C-termini for RAD51-binding. In contrast, a similar sequence motif present in the amino-terminus of RAD54 protein is not completely critical for the interaction with RAD51. Instead, residues closer to the amino-terminus of RAD54, such as F82 and P85 located outside of the homologous region, seem to be essential for the interaction with RAD51.

DISCUSSION

RAD51AP2 shares a conserved RAD51-binding motif with RAD51AP1

We isolated a fragment of a previously unknown protein, RAD51AP2, via its interaction with human RAD51 in a Y2H library screen (Figure 1). The full length *RAD51AP2* ORF was cloned and sequenced, and the sequence suggests that the full-length protein is 1159 amino acid residues with no significant homology to any other protein, apart from its C-terminal RAD51-interacting region. We provided evidence that the interaction between RAD51AP2 and RAD51 is facilitated by a short sequence at the C-terminus of RAD51AP2 (Table 1), which displays strong homology (81% in a 16 amino acids region) to the C-terminal region of RAD51AP1 (Figure 5A), a previously identified 335 amino acids RAD51-interacting protein (19,37). As with RAD51AP2, this conserved motif in RAD51AP1 also comprises part of its interacting region with RAD51 (Table 2). Among the 16 amino acids spanning the highly homologous sequence motif between human RAD51AP2 and RAD51AP1, 11 residues are identical and two are highly conserved. We have not tested yet whether this highly homologous sequence motif (16 amino acids) is sufficient for either the RAD51AP2–RAD51 or the RAD51AP1–RAD51 interaction. However, our Y2H results show that the longer 25 residue-encompassing C-terminal region of RAD51AP1 and the last 33 residues in RAD51AP2 (inclusive of a stretch of residues with no homology to RAD51AP1 at the very C-terminus—see Figure 5A) are fully capable of interaction with RAD51. Interestingly, in each gene, a relatively short C-terminal exon completely encodes the RAD51-interacting region, and RAD51AP1 and RAD51AP2 share no other region of homology.

Our Y2H results on site-specific mutations in both RAD51AP1 and RAD51AP2 demonstrate that for both proteins several of the highly conserved residues are crucially important to maintain the interaction with RAD51. To the

best of our knowledge, RAD51AP1 and RAD51AP2 carry the first binding-motif for RAD51 that is commonly shared by more than one of RAD51's interacting partners. A somewhat similar situation exists for BRCA2 and its multiple BRC repeats, since these repeats also bind to RAD51 via a conserved sequence motif (20). However, the BRCA2-BRC repeat sequence does not appear to be present in any additional RAD51-interacting partner, although it does share homology with a RAD51 self-interacting region (22).

We speculate that both RAD51AP1 and RAD51AP2 may interact with the same region on RAD51 via their commonly shared homologous sequence motifs. If this were the case, their simultaneous binding to a single RAD51 monomer would be very unlikely. Since RAD51 forms both multimers and filaments, it is still possible that RAD51AP1 and RAD51AP2 could potentially bind to adjacent or nearby RAD51 monomers. As discussed below, *RAD51AP2* appears not to be expressed in mitotic cells, but since *RAD51*, *RAD51AP1* and *RAD51AP2* are all expressed in meiosis simultaneous interactions could still occur in meiotic cells.

At least 12 of the DNA replication- and repair-related protein partners that interact with PCNA (proliferating cell nuclear antigen) also utilize a conserved motif, but different from the RAD51-binding motif described here (41). In our study, a highly conserved RAD51-binding motif has been identified, but it appears to be present in only two different human proteins (RAD51AP1 and RAD51AP2), with the possible exception of RAD54 (discussed below). Unlike the RAD51-binding motif, the PCNA-binding motif is ancient and conserved to archaea and bacteriophages (41). Nonetheless, it still is possible that an ancestral RAD51- or RecA-binding motif may exist that is related to the motif described here, but that the ancestral motif is insufficiently conserved to be recognizable.

RAD54 shares limited homology with the identified RAD51-binding motif

While attempting to identify other previously known partners of RAD51 that may carry a sequence motif with some homology to the conserved motif in RAD51AP1/RAD51AP2, we identified RAD54, which shares 50% homology within the 16 amino acids core region. Previously, we already reported that the N-terminal region of human RAD54 (amino acids 1–142) facilitates binding to RAD51 (16). Our sequence alignment (Figure 6A) shows that amino acids 96–111, embedded within this N-terminal region of RAD54, display significant homology to the conserved binding-motif in RAD51AP1/RAD51AP2. However, the results from the Y2H experiments also suggest that in RAD54 amino acids 1–89 are sufficient to convey a strong interaction with RAD51 (Figure 6B and C), indicating that the residues with homology (amino acids 96–111) to the conserved RAD51-interacting motif in RAD51AP1/RAD51AP2 are not required for the RAD54–RAD51 interaction. In addition, two key residues in the N-terminal region of RAD54 located upstream of the homologous region appear to be important for its interaction with RAD51 (Figure 6C). Conversely, several residues within the amino acids 96–111 region appear not to be involved in the RAD54–RAD51 interaction. However,

when we analyzed truncated forms of RAD54 in the Y2H system we obtained evidence that amino acids 96–111 may play a secondary role in enhancing the interaction between RAD54 and RAD51 (Figure 6B and C). Additionally, an extensive N-terminal domain of RAD54 appears to be required to obtain full interaction strength with RAD51 in the Y2H system (Figure 6B). Our results suggest that the interaction between RAD54 and RAD51 may involve either a large interface or a series of multiple shorter interfaces. RAD54 has been postulated to play an important role in many stages of HRR (42), and its interaction with RAD51 in human cells is very likely to be required for at least some of these activities.

The interaction between RAD51 and the N-terminal domain of RAD54 has also been observed in the yeast *S.cerevisiae* (43,44). In recent *in vitro* experiments, the direct RAD51–RAD54 interaction has been shown to be important for RAD54 enhancing the DNA pairing reaction mediated by RAD51 (45). This paper also reported that RAD51 stimulates both the RAD54 ATPase activity and the ability of RAD54 to introduce superhelical tension into circular plasmid DNA, and that these activities also depend on the RAD51–RAD54 direct interaction. This recent study also showed that there are separable epitopes in RAD54 that interact with RAD51, in accord with our speculations for an extensive RAD51–RAD54 interface (see above). In our study however, the region of homology between human RAD54 and both RAD51AP1 and RAD51AP2 does not appear to be a separable interacting epitope in RAD54, since RAD54 amino acids 90–142 includes the entire homologous region but does not by itself interact with human RAD51 (Figure 6B). It is worth noting that the yeast RAD54 protein actually shares two different regions with homology to the RAD51-interacting region of human RAD54. One region of ScRAD54 (amino acids 37–50, TKPFRVPYKNT^HIP) shares homology only with the HsRAD54 region that includes F82 and P85 (underlined F40 and P43 in ScRAD54), while the second region of ScRAD54 (amino acids 129–164, RSFTVPIKGYVQRHSLPLTLGMKKKITPEPRPL^HDP) shares homology with both the F82/P85 region and the region with homology with RAD51AP1 and RAD51AP2 (our unpublished analysis).

The RAD51AP2 protein appears to be a meiotic partner of RAD51

Among the 16 adult and 5 fetal tissues tested, we found that the *RAD51AP2* gene is expressed only in adult testis and fetal ovary (Figures 2 and 3), indicating that RAD51AP2 is a likely meiosis-specific partner of RAD51. No expression was observed in adult ovary, where cells are arrested in meiosis after recombination has been completed, nor was expression observed in fetal testis, where cells have not yet initiated meiosis. In addition, analyzing the enormous human EST database, no human *RAD51AP2* EST clones have been identified from non-meiotic tissue, confirming our observation that *RAD51AP2* seems to be expressed only in cells undergoing meiosis. We also tested if RAD51AP2 might be expressed in mitotic cells after DNA damage, but we found no such expression (Figure 3F). This was not unexpected, since even those DNA repair genes induced by

DNA damage are normally constitutively expressed at a detectable level. It is virtually impossible to prove that *RAD51AP2*, or any gene for that matter, is never expressed in any mitotic human cells, but our data strongly supports this view.

Although RAD51AP2 does not appear to be expressed in mitotic cells, our data show that an ectopically expressed recombinant RAD51AP2 fragment is capable of interacting with endogenous RAD51 *in vivo* (Figure 4). In addition, this interaction is stimulated ~7- and ~17-fold after exposure to the DNA-damaging agents MMC and MMS, respectively. Both exposure to MMC and to MMS produces DSBs that are repaired by HRR mediated by RAD51, and DSBs also are produced endogenously during DNA replication and during meiosis (7,8,12,36). The increase in the RAD51AP2–RAD51 association after DNA damage may occur if both proteins are targeted to the same locations within the nucleus, e.g. DSBs and/or RAD51 foci. Alternatively, or concomitantly, the RAD51–RAD51AP2 interaction may also be stabilized via posttranslational protein modification in response to high levels of DNA damage. If *RAD51AP2* is expressed only during meiosis, as seems likely from our data, DNA damage may mimic some aspect of meiosis such as the formation of DSBs, and if this is the case, then RAD51AP2 may preferentially interact with RAD51 when DSBs are present during pachytene (6–8). Studying the interactions of an ectopically overexpressed protein in mammalian cells can sometimes lead to false positives, but we feel that this is not likely to be the case here. Firstly, the *in vivo* interaction studies confirm a protein–protein interaction observed in the Y2H system that is very strong and that occurs when the inserts are in both orientations. Secondly, it seems rather unlikely that a false-positive protein–protein interaction would be stabilized by DNA damage, as was observed here.

Interestingly, the isolated RAD51AP2 amino acid sequence does not interact in the Y2H system with DMC1, a homolog of RAD51 that is meiotic specific (6–8) and required for meiotic chromosome synapsis (46,47). The interaction of RAD51AP2 with other meiotic-specific proteins, such as the SPO11 endonuclease and proteins involved in the synaptonemal complex, has not been tested, but would be worth testing.

RAD51-associated proteins frequently play an important role in recombination

Much has been learned recently about RAD51 function and regulation by studying its interacting partners. For example, the interaction of RAD51 with TP53 has been shown to control the specificity of RAD51 in HRR (23). The interaction of RAD51 with the BRCA2-BRC repeats have been postulated to play a role in aligning multiple monomers of RAD51 and then polymerizing them into a filament on ssDNA (48). RAD51 interacts with a DNA-binding domain of RPA, and this interaction has been postulated to help RAD51 displace RPA from ssDNA (33). Eventually understanding the function and structure of the interactions of RAD51 with RAD51AP1 and with RAD51AP2 should give us important information about HRR, meiotic recombination, and the role of these new proteins. For example, while this manuscript was in final review, a study was published showing

a role for RAD51AP1 in DNA repair and genomic stability (49), findings that are in accord with our own unpublished results (C. Wiese and D. Schild, manuscript in preparation).

In conclusion, RAD51AP2 has been identified as a novel meiotic-specific RAD51-interacting protein, and RAD51AP2 and RAD51AP1 share a common motif important for RAD51-binding. The fact that RAD51AP1 and RAD51AP2 are both vertebrate-specific suggest that these proteins may potentially play a regulatory role in recombination.

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Conflict of interest statement. None declared.

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