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

Oleg V. Kovalenko, Claudia Wiese1 and David Schild1,*

Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, 02115, USA and 1Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA

Received July 25, 2006; Revised August 25, 2006; Accepted August 27, 2006

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 synopsis 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 recombinase 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.)
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 ectopic 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 specifically with human 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 vector pEG918, followed by the library DNA. Interacting 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 DMC1, and for Saccharomyces cerevisiae RAD51. Vector pGBK7 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 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′-CGTAACTGTCAGAGCATCTCTCGTGC-3′ was used in combination with the original 5′ primer, and 5′-GAAATGACTACCATCAAAGTACGAGTTCCGTTC-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 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 plBuescript 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′GATGACACAAGCTCTACCAGAGAC3′ and AP1-3′: 5′CTTGGCTTTACGCTAAGGACTGCG3′ and these amplify a 630 bp fragment. The primers for RAD51AP2 were AP2-5′: 5′CTGGTCTGAGTGAAGGAAATGATG3′ and AP2-3′: 5′GCGGTCTGACTCTTGGGAATGCCATG3′, 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-ΔC57) was cloned into pCGT, producing plasmid pOK90. Fortuitously, the vector pCGT used for cloning contained the sequence of human herpesvirus Vmw65

Downloaded from https://academic.oup.com/nar/article-abstract/34/18/5081/3112127 by guest on 17 February 2019
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 20 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 (Novagen) 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 antibody directed against the T7 tag (Novagen) was detected with alkaline phosphatase, and detection was carried out with anti-mouse or anti-rabbit antibodies were conjugated with 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. (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 hypothetic cDNA (XM_496545) and protein (XP_496545).

Sequence analysis of the mouse genome predicts a homologous protein of 976 amino acids (GenBank 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 sequence. Instead, this 154 amino acids region does not appear to be an extra unexcised intron, since it does not have a sequence resembling the consensus sequence. Instead, this 154 amino acids region does not appear to be an extra unexcised intron, since it does not have a sequence resembling the consensus sequence. Instead, this 154 amino acids region does not appear to be an extra unexcised intron, since it does not have a sequence resembling the consensus sequence. Instead, this 154 amino acids region does not appear to be an extra unexcised intron, since it does not have a sequence resembling the consensus sequence. Instead, this 154 amino acids region does not appear to be an extra unexcised intron, since it does not have a sequence resembling the consensus sequence. Instead, this 154 amino acids region does not appear to be an extra unexcised intron, since it does not have a sequence resembling the consensus sequence. Instead, this 154 amino acids region does not appear to be an extra unexcised intron, since it does not have a sequence resembling the consensus sequence. Instead, this 154 amino acids region does not appear to be an extra unexcised intron, since it does not have a sequence resembling the consensus sequence.
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 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 RAD51/AP1/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 testsis 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
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-D\(_{C57}\)) 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 \(\frac{48}{48}\%\) of the strength of the 321 amino acid fragment (Table 1). In light of our results on RAD51AP2-D\(_{C57}\), 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 \(\beta\)-galactosidase (3–5 units of \(\beta\)-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. 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

---

**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 \(\beta\)-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 \(\beta\)-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

<table>
<thead>
<tr>
<th>Gal4 DBD(^a) plasmid</th>
<th>Gal4 AD plasmid</th>
<th>Gal4 DBD protein fusion</th>
<th>Gal4 AD protein fusion</th>
<th>Units of (\beta)-galactosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEG918</td>
<td>pOK42</td>
<td>RAD51</td>
<td>RAD51AP2-C321</td>
<td>300.9</td>
</tr>
<tr>
<td>pOK21</td>
<td>pOK42</td>
<td>DMC1</td>
<td>RAD51AP2-C321</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>pEG978</td>
<td>pOK42</td>
<td>ScRAD51</td>
<td>RAD51AP2-C321</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>pOK31</td>
<td>pOK42</td>
<td>RAD51AP1</td>
<td>RAD51AP2-C321</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>pEG918</td>
<td>pOK77</td>
<td>RAD51</td>
<td>RAD51AP2-C57(^b)</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>pEG918</td>
<td>pOK40</td>
<td>RAD51</td>
<td>RAD51AP2-C92</td>
<td>143.4</td>
</tr>
</tbody>
</table>

\(^a\)DBD and AD stand for the DNA-binding and activation domain of Gal4, respectively. The units of \(\beta\)-galactosidase are average values from at least four liquid cultures. All inserts are human except ScRAD51, which is from yeast.

\(^b\)This plasmid actually only encodes the C321 fragment deleted for the last 57 amino acids, and similarly in Table 2.
Y2H vectors and tested for their interaction with human RAD51. All four interacted with RAD51, although more weakly then 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 then 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-AC57), 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-AC57 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 (Figure 4B). Results from three independent experiments indicate that co-precipitation of cells for co-IP analysis (Figure 4B). Results from three (MMS) or mitomycin C (MMC) 3 h before harvesting damage was introduced by adding methyl methanesulfonate overexpressed in HEK293 cells as described above, and DNA damage. To test this, RAD51AP2-C293 was transiently expressed in mitotic human cells it can associate with RAD51.

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 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...
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-DC57) 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 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
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. 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 ~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-terminus 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 it interacts 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 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, TKPFRVPYKNTHP) 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, RSTFPVKGYVRHSPLTLGMMKKTIPPEPRPLHDP) 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 are also 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 synopsis (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 RAD51AP1 and RAD51AP2 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.

ACKNOWLEDGEMENTS

This work was initiated when O.V.K. was in the laboratory of Dr Charles M. Radding at the Department of Genetics, Yale University, and his support and intellectual input for this research are greatly acknowledged. Part of this work was supported by NCI/NIH grant P01 CA92584 titled ‘Structural Biology of DNA Repair Machines’ (D.S.), and by an LBNL internal LDRD (Laboratory Directed Research and Development Program) grant (D.S.). Funding to pay the Open Access publication charges for this article was provided by NCI (CA92584) and by LBNL (LDRD).

Conflict of interest statement. None declared.

REFERENCES