Identification of Functional Domains within the RAD1-RAD10 Repair and Recombination Endonuclease of Saccharomyces cerevisiae*

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Saccharomyces cerevisiae rad1 and rad10 mutants are unable to carry out nucleotide excision repair and are also defective in a mitotic intrachromosomal recombination pathway. The products of these genes are subunits of an endonuclease which recognizes DNA duplex/single-strand junctions and specifically cleaves the 3' single-strand extension at or near the junction. It has been suggested that such junctions arise as a consequence of DNA lesion processing during nucleotide excision repair and the processing of double-strand breaks during intrachromosomal recombination. In this study we show that the RAD1-RAD10 complex also cleaves a more complex junction structure consisting of a duplex with a protruding 3' single-strand branch that resembles putative recombination intermediates in the RAD1-RAD10-mediated single-strand annealing pathway of mitotic recombination. Using monoclonal antibodies, we have identified two regions of RAD1 that are required for the cleavage of duplex/single-strand junctions. These reagents also inhibit nucleotide excision repair in vitro, confirming the essential role of the RAD1-RAD10 endonuclease in this pathway.

The RAD1 and RAD10 genes of Saccharomyces cerevisiae are indispensable for the process of nucleotide excision repair (NER) of DNA (1–3) and are additionally required for a specialized mitotic recombination pathway (4–10). Their requirement for recombination distinguishes the RAD1 and RAD10 genes from other members of the RAD3 epistasis group, which are also indispensable for NER but are not required for recombination (11). The demonstration of a specific stable interaction between the RAD1 and RAD10 proteins in vitro and in vivo (12–14) suggests that these proteins function as a complex during both NER and intrachromosomal mitotic recombination and provides an explanation for the similar phenotype of rad1 and rad10 mutants.

Physical analysis of recombination intermediates in a rad1 mutant (15) and more recently in a rad10 mutant (16) suggested that the products of the RAD1 and RAD10 genes may contribute to a nuclease activity that removes noncomplementary 3' single-strand extensions from duplex DNA molecules during the recombination process. In agreement with this prediction a nuclease activity was detected when purified RAD1 and RAD10 proteins were mixed together but not with either protein alone (17, 18). This endonuclease, which consists of one molecule each of RAD1 and RAD10 proteins, was initially shown to degrade single-stranded DNA circles and to nick supercoiled duplex DNA (17–19). Recently it has been demonstrated that the RAD1-RAD10 enzyme is a junction-specific DNA endonuclease that uniquely cleaves the 3' single-strand extension at the duplex/single-strand junction (20). Presumably the single-stranded circular and supercoiled duplex substrates were cleaved at such junctions randomly generated as a consequence of secondary structures in these DNA molecules.

This junction-cleaving activity is consistent with the predicted role of the RAD1 and RAD10 polypeptides in mitotic recombination (15, 16) and also suggests that the RAD1-RAD10 endonuclease catalyzes inversions 5' to sites of base damage during NER (20). Consistent with this notion, the RAD1-RAD10 complex has been shown to cleave synthetic duplex DNA "bubble" structures on the 5' side of the central looped out region (21). In combination with human XPG protein (the homolog of yeast RAD2 protein), a junction-specific endonuclease with the opposite polarity (22), the RAD1-RAD10 complex catalyzes the excision of the central looped out region of bubble structures as an oligonucleotide fragment (21). Thus, the combined action of these two junction-specific endonucleases mimics the dual incision reaction that is a characteristic of NER.

There is evidence for endonuclease complexes that are functionally homologous with S. cerevisiae RAD1-RAD10 in other eukaryotes. In Schizosaccharomyces pombe the products of the Swi10+ and Rad16+ genes (homologs of the S. cerevisiae RAD10 and RAD1 genes, respectively), have been shown to interact (23). In human cell extracts the protein of the ERCC1 gene, which is the human homolog of the S. cerevisiae RAD10 gene (11), is tightly associated with a protein that complements defective NER in extracts of mutant cells designated ERCC4 and XPF (24, 25). Since the polypeptide encoded by ERCC4 cDNA exhibits homology with RAD1 (26), it is almost certainly the human homolog of RAD1, and it is also likely that XPF and ERCC4 are in fact the same protein. The purified XPF-ERCC1 complex has single-strand DNA endonuclease activity (27) and generates the 5' incisions during NER in human cells (28).

Since both RAD1 and RAD10 proteins bind to single-stranded DNA, both polypeptides may contribute to DNA substrate recognition (17, 29). Similarly, the endonuclease active site may reside in one of the polypeptides or may be composed...
of residues from both subunits. The RAD1 and RAD10 polypeptides have been identified as components of a larger multiprotein complex (30) in extracts of yeast cells, designated the nucleotide excision repairosome (30). These observations suggest that interactions with other NER proteins are required to properly orient the RAD1-RAD10 endonuclease at the appropriate cleavage site near DNA lesions. In this report we describe the characterization of monoclonal antibodies raised against the purified RAD1 and RAD10 polypeptides. Using these reagents we have identified regions of RAD1 protein that are required for DNA cleavage at duplex/single-strand junctions. These RAD1 antibodies inhibited the NER reaction catalyzed by yeast nuclear extracts, confirming the role of this nuclease activity in NER. The RAD10 antibodies also inhibited the cleavage of junctions, probably by interfering with RAD1-RAD10 complex formation. However, these antibodies did not inhibit NER catalyzed by yeast nuclear extracts, consistent with the notion that the RAD1 protein is incorporated into a larger complex (repairosome) and hence may be inaccessible to some antibody probes. Additionally, we show that the RAD1-RAD10 complex cleaves more complex junction structures consisting of a DNA duplex with a protruding 3′ single-strand branch. The branched substrate with the protruding 3′ single-strand mimics a putative recombination intermediate in the RAD1-RAD10-mediated single-strand annealing pathway of mitotic recombination.

MATERIALS AND METHODS

Materials—Mouse IgG purified from serum and mouse IgM purified from the tissue culture supernatant of a mouse myeloma cell line were purchased from Pierce and Cappel, respectively. Tissue culture supernatants from IgG secreting monoclonal cell lines that recognize the human proteins RAD1 (31) and Syp-2 (32) were provided by Dr. Eva Lee and Dr. Montalvo, respectively.

Purification of RAD1 and RAD10 Proteins—RAD1 and RAD10 proteins were purified as described previously (19). Protein concentrations were determined by the method of Bradford (33).

Generation of Monoclonal Antibodies—Mice were immunized with either RAD1 or RAD10 proteins. After monitoring by immunoblotting, the mouse with the highest titer antiserum was sacrificed and its spleen removed. The spleen cells were fused with myeloma cells, and the resultant hybridomas were screened for secretion of specific antibodies by enzyme-linked immunosorbent assay. Antibodies that were positive in the enzyme-linked immunosorbent assay were analyzed further. Two RAD1 and two RAD10 hybridomas, which produced antibodies that fully reacted both in immunoblotting and immunoprecipitation experiments, were selected for further characterization. We have been unable to isolate a pure antibody-secreting cell population from the RAD10 hybridomas. Therefore we have continued to subculture from the original hybridomas, SBS and TH3, and have examined the properties of the secreted IgG molecules. The RAD1 monoclonal cell lines 2BS and 3E3 secrete IgM and IgG antibodies, respectively.

Purification of Mouse IgM from Tissue Culture Supernatant—Tissue culture supernatants (50 ml) and mouse IgG (5 mg) were dialyzed overnight at 4°C against phosphate-buffered saline (PBS) (pH 7.0) and then applied to a 1-ml GammaBind Plus Sepharose column (Pharmacia Biotech Inc.). After washing with PBS (pH 7.0) bound proteins were eluted with 100 mM glycine-HCl (pH 2.8). Fractions were immediately neutralized with 1 m Tris-HCl (pH 8.0) and their protein content was determined by the method of Bradford (33). Fractions containing protein were pooled, dialyzed against PBS (pH 7.0), concentrated by ultrafiltration (Centricon-50, Amicon, Inc.) and then stored in aliquots at 80°C. The antibody preparations were analyzed by Coomassie Blue staining after SDS-polyacrylamide gel electrophoresis and assayed for the presence of contaminating nuclease activities as described below.

Purification of Mouse IgG from Ascites—Ascites fluid was collected from mice infected with the IgG-secreting monoclonal cell line 2BS. IgG was partially purified from the ascites fluid (5 ml) using an E-Z-SEP kit (Pharmacia) according to the manufacturer's instructions. Partially purified IgG was dialyzed against 50 mM 2-(N-morpholino)ethanesul-
37°C. The enzyme/antibody samples were then added to reaction mixtures (final volume 20 μl) containing 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 5 mM dithiothreitol, and DNA substrate. Reactions were incubated at 37°C for 40 min. After deproteinization by incubation with 0.1% SDS and 100 μg of proteinase K for 10 min at 37°C, reaction products were electrophoresed through a 15% polyacrylamide gel. The gel was then dried and exposed to x-ray film.

**In Vitro NER**—Yeast whole cell extract (40 μg) containing overexpressed RAD2 and nuclear extract (200 μg) from SX46A cells (38) were mixed with or without antibodies as indicated. After incubation at 26°C for 30 min, NER assay components, 300 ngeach of AAF-treated pUC18 DNA and undamaged pGEM3Zf(+) DNA, 45 mM Hepes-KOH (pH 7.8), 7.4 mM MgCl₂, 5 mM dithiothreitol, and DNA substrate. Reactions were incubated at 37°C for 40 min. After deproteination by incubation with 0.1% SDS and 100 μg of proteinase K for 10 min at 37°C, reaction products were electrophoresed through a 15% polyacrylamide gel. The gel was then dried and exposed to x-ray film.

**RESULTS**

**Specificity of RAD1 and RAD10 Monoclonal Antibodies**—The RAD1-RAD10 endonuclease is the first known example of a eukaryotic nuclease that consists of more than one polypeptide. In order to investigate structure-function relationships within this complex, monoclonal antibodies were generated against purified RAD1 and RAD10 polypeptides. After initial screening by an enzyme-linked immunosorbent assay, two RAD1 hybridomas and two RAD10 hybridomas were selected for further study. After separation by denaturing gel electrophoresis, purified RAD1 and RAD10 proteins were stained with Coomassie Blue (Fig. 2A). In immunoblotting experiments identical samples of RAD1 and RAD10 were transferred to nitrocellulose membranes. As expected, the RAD1 antibodies 3E3 and 2B5 specifically recognized RAD1 protein but not RAD10 protein (Fig. 2B), whereas the RAD10 antibodies 5B5 and 7H3 specifically recognized RAD10 protein but not RAD1 protein (Fig. 2C).

The results of immunoprecipitation experiments demonstrated that both RAD1 antibodies interacted with in vitro-translated RAD1 protein (Fig. 3A, lanes 4 and 7). To map the epitopes recognized by these antibodies we performed immunoprecipitations with several truncated versions of RAD1. The IgM antibody 2B5 immunoprecipitated a RAD1 polypeptide lacking the C-terminal 610 residues (Fig. 3B, lane 5) but not RAD1 polypeptides with N-terminal deletions of more than 237 residues (Fig. 3A, lanes 8 and 9; Fig. 3B, lane 6). Hence, the epitope recognized by antibody 2B5 appears to reside within the N-terminal 237 amino acid residues. In contrast, the epitope recognized by the IgG antibody 3E3 appears to be...
located between residues 596–782, since RAD1 polypeptides with N-terminal deletions of up to 590 residues were immunoprecipitated by 3E3 (Fig. 3A, lane 6; Fig. 3B, lane 4), whereas RAD1 polypeptides lacking either the N-terminal 782 residues or the C-terminal 504 residues were not (Fig. 3A, lane 5; Fig. 3B, lane 3). Thus, both RAD1 antibodies recognize regions of RAD1 protein that are distinct from a region (amino acid residues 809–997) previously shown to be required for its interaction with RAD10 protein (13, 14).

In similar experiments with the RAD10 antibodies 5B5 and 7H3 both antibodies immunoprecipitated full-length RAD10 polypeptide (Fig. 4, lanes 5 and 7) as well as a RAD10 polypeptide lacking the N-terminal 74 residues (Fig. 4, lanes 6 and 8). The deleted version of RAD10 protein contains residues 90–210, which are required for its interaction with RAD1 protein (13, 14). This region of RAD10 is conserved in the functionally homologous human protein ERCC1 (40).

Effects of the Antibodies on the Nuclease Activity of the RAD1-RAD10 Complex—Having mapped antibody binding sites as a function of the linear amino acid sequences of the RAD1 and RAD10 polypeptides, we examined the effects of antibody binding on the DNA junction cleavage activity of the RAD1-RAD10 complex. As reported previously (20), RAD1 and RAD10 proteins in combination cleave DNA substrates with duplex/single-strand junctions (Figs. 5 and 6), whereas no nuclease activity was observed with either protein alone or with linear duplex molecules (data not shown). The RAD1 IgG antibody 3E3 and the RAD10 IgG antibodies 7H3 and 5B5 significantly inhibited the specific cleavage of Y-structures (Fig. 1) by the RAD1-RAD10 endonuclease activity (Fig. 5). Similar quantities of purified mouse serum IgG (Fig. 5) and IgG monoclonal antibodies 13D10 and 6C5 (data not shown), which are specific for unrelated proteins, had no such effect. Specific inhibition of the cleavage of Y-structures was also observed with the RAD1 IgM monodonal antibody 2B5 compared with purified mouse IgM protein (Fig. 6). Similar results were obtained in assays that measured nicking of supercoiled duplex DNA (data not shown).

The Y-structures used as substrate are postulated common intermediates generated during NER and the RAD1-RAD10-
mediated intrachromosomal recombination pathway (15, 16, 20). In the single-strand annealing mechanism proposed for the recombination pathway the junction recognized and cleaved by the RAD1-RAD10 endonuclease may be a duplex with a protruding 3′ single-strand branch rather than a duplex with noncomplementary 3′ and 5′ extensions at the same end (21).

We therefore examined the ability of the RAD1-RAD10 endonuclease to cleave duplex structures with protruding 5′ or 3′ single-strand branches (Figs. 1 and 7). In agreement with the substrate specificity observed in studies with Y-structures (20) the RAD1-RAD10 endonuclease cleaved duplexes with protruding 3′ single-strand branches, although about 2-fold less product was detected with the branched substrate (Fig. 7).

Cleavage of the duplexes with protruding 3′ single-strand branches was inhibited by the RAD10 antibody 7H3 and the RAD1 antibody 2B5 but not by unrelated IgG and IgM antibodies (data not shown). In similar assays with a duplex substrate containing a protruding 5′ single-strand branch the RAD1-RAD10 endonuclease was significantly less active, producing 5–10-fold less product when compared to reactions with the duplex substrate containing a protruding 3′ single-strand branch (Fig. 7).

Effects of the Antibodies on NER in Vitro—Cleavage of the 3′ single-strand extension at duplex/single-strand junctions by the RAD1-RAD10 endonuclease suggests that this enzyme catalyzes 5′ incisions at bubble structures presumably generated during the process of NER. To confirm the essential role of the RAD1-RAD10 endonuclease in NER we examined the effects of the RAD1 and RAD10 antibodies on yeast nuclear extracts which are known to support complete NER in vitro. In this assay, damaged and undamaged plasmid DNA substrates, which can be distinguished by a difference in size, are incubated with nuclear extract in the presence of deoxynucleoside triphosphates, one of which is labeled. DNA damage-dependent DNA synthesis is measured by autoradiography after separation of the DNA substrates by electrophoresis. Preincubation of yeast nuclear extracts with the RAD1 IgM monoclonal antibody 2B5 resulted in significant inhibition of NER, whereas similar quantities of mouse IgM had no effect on the reaction (Fig. 8A, lanes 3 and 5). Extensive inhibition of NER was also observed in experiments with the RAD1 IgG monoclonal antibody 3E3 (Fig. 8B, lanes 2 and 3). In contrast, preincubation of the extracts with similar quantities of the RAD10 antibody 5B5 resulted in only slight inhibition of NER (Fig. 8B, lanes 4 and 5), and preincubation with the RAD10 antibody 7H3 had no detectable effect (Fig. 8C, lanes 2 and 3).

The effects of the monoclonal antibodies on RAD1-RAD10 endonuclease activity and on NER in vitro are summarized in Table I. Our results indicate that epitopes recognized by both the RAD1 antibodies 2B5 and 3E3 and the RAD10 antibodies 7H3 and 5B5 are required for the junction-specific nuclease activity of the RAD1-RAD10 complex. Those recognized by the RAD1 antibodies 2B5 and 3E3 are apparently accessible in yeast nuclear extracts that support complete NER in vitro. In contrast, the epitopes recognized by the RAD10 antibodies 7H3 and 5B5 are not. The RAD1 antibodies recognize epitopes in regions of RAD1 protein other than those required for its interaction with RAD10 protein (13, 14). The observed inhibition of nuclease activity by the binding of RAD1 antibody therefore implicates these regions in substrate recognition and cleavage. In contrast, the RAD10 antibodies recognize epitopes in regions of the protein required for its interaction with RAD1 protein (13, 14). Thus, the inhibitory effect of the RAD10 antibodies on junction-specific endonuclease activity may derive from bind-
ing to a region of RAD10 protein required for nuclease activity or to a region of the protein required for complex formation with RAD1 protein. In an attempt to address this issue we compared the complex-specific nuclease activity of RAD1-RAD10 complexes formed prior to the addition of RAD10 antibodies with that observed when a complex between one of the polypeptides and an appropriate antibody was formed prior to the addition of the second polypeptide. We observed that the RAD10 antibody 7H3 was more effective in inhibiting nuclease activity when it was preincubated with RAD10 (Fig. 9), suggesting that the 7H3 antibody interferes with complex formation by its ability to bind to the region of RAD10 that interacts with RAD1. A similar effect was not observed with the 5B5 antibody (data not shown). This may reflect differences in the binding affinities of the antibodies for the region of RAD10 that interacts with RAD1.

**DISCUSSION**

The interaction of the yeast RAD1 and RAD10 polypeptides generates an endonuclease activity that cleaves DNA at duplex/single-strand junctions with 3' single-strand extensions (20). It has been reported that RAD1 protein alone specifically cleaves DNA substrates containing Holliday junctions (41). We have been unable to reproduce the latter observation with our preparations of homogeneous RAD1 protein and have only observed nuclease activity (but not cleavage of Holliday junctions) in combination with RAD10 protein (18–21). DNA molecules containing duplex/single-strand junctions have been postulated as intermediates in both the NER pathway and the intrachromosomal mitotic recombination pathway that requires RAD1 and RAD10 gene products (15, 16, 20). In contrast to the bubble structure postulated in NER, the junction-containing DNA substrate in the recombination pathway may be a branched molecule consisting of a duplex with a nonhomologous 3' single-strand protruding from the duplex (21). In the present study we have demonstrated that the RAD1-RAD10 complex recognizes and cleaves such a structure, whereas it exhibits very little cleavage activity with a similar structure containing a protruding 5' single-strand branch.

To investigate structure-function relationships within the RAD1-RAD10 endonuclease complex, monoclonal antibodies

| Antibody | Polypeptide recognized | Inhibition of nuclease activity | Inhibition of in vitro NER |
|----------|------------------------|---------------------------------|---------------------------|
| 2B5      | 2B5_1-237              | ++                             | Yes                       |
| 3E3      | 3E3_596-782            | ++                             | Yes                       |
| 5B5      | 5B5_74-210             | ++                             | No                        |
| 7H3      | 7H3_74-210             | ++                             | No                        |
specific for both RAD1 and RAD10 polypeptides were generated. Using these reagents we have identified regions of the polypeptides required for cleavage of DNA molecules containing duplex/single-strand junctions. The results of our study demonstrate that the N-terminal 237 residues and residues 596–782 of RAD1 polypeptide contribute (at least in part) to the nuclease active site. A comparison of these regions of RAD1 with homologous sequences encoded by the nuclease activesite. A comparison of these regions of RAD1 and RAD10 endonuclease activity in the RAD1 monodonal 285, whose cognate epitope resides (at least partly) within a region of RAD1 (residues 1–237) that contains seven of the nine leucine-rich motifs, demonstrates that this region of RAD1 is required for enzymatic activity but does not exclude its involvement in protein-protein interactions. The results with the RAD1 monoclonal antibody 3E3 suggest that the putative C-domain is also intimately involved in the nuclease activity of the RAD1-RAD10 complex in addition to being required for the interaction with RAD10. Monoclonal antibodies that bind to the N-terminal region of RAD1 also inhibit NER in a cell-free system, confirming the essential role of RAD1-RAD10 endonuclease activity in NER. In contrast, the RAD10 antibodies do not inhibit NER in this system. Since the RAD10 antibodies recognize the region of RAD10 protein that interacts with RAD1 (13, 14), our results are consistent with the notion that these antibodies inhibit nuclease activity by competing with RAD1 for the interacting site on RAD10 protein, thereby blocking RAD1-RAD10 complex formation. Although the RAD10 antibodies inhibited nuclease activity in the presence of an excess of a nonspecific protein, we cannot exclude the possibility that the RAD10 antibodies cannot effectively compete for the RAD1 interacting site in the complex mixture of proteins present in cell-free extracts. Alternatively and, in our view, more likely, we suggest that RAD10 may be inaccessible to the antibodies because the RAD1-RAD10 complex is itself a component of a larger multiprotein complex (repairosome) in such extracts.

Based on the specific DNA duplex/single-strand junction cleaving activity of the RAD1-RAD10 complex we predicted that this enzyme catalyzes the 5’ incision during NER in yeast (20). In agreement with this model, it has recently been demonstrated that the ERCC1-XPF complex, the human homolog of RAD1-RAD10, generates the 5’ incision during NER in human cells (28). Since neither of these functionally homologous enzymes nor the other junction-specific cleaving enzymes RAD2 and XPG recognize DNA damage directly (17–19, 43, 44), the putative bubble substrate will appear as a symmetrical structure to these enzymes. We presume that in vivo specific protein-protein interactions appropriately position the junction-specific endonucleases so that incisions are made only in the DNA strand containing the lesion being repaired.

There is genetic and biochemical evidence indicating that the 5’ and 3’ incisions are coordinated (28, 45–47). Our observation that the RAD1 antibodies completely inhibit NER in vitro are consistent with this idea. The simplest explanation of these results is that a functional incision complex is dependent on the correct positioning of both the 5’- and 3’-endonuclease activities. During human NER, the DNA damage recognition protein XPA is known to associate with ERCC1 (48–50), although an interaction between the homologous yeast proteins RAD14 and RAD10 does not appear to occur. Furthermore, XPA also interacts with RPA, which in turn interacts with XPG, linking the second endonuclease with the lesion-recognition protein (51). It is probable that the yeast incision endonucleases are positioned by different protein-protein interactions within the nuclease excision repairosome (30). The availability of the specific reagents generated in this study is expected to facilitate further analysis of the complex dual incision reaction of NER and the role of the RAD1-RAD10 complex in intrachromosomal mitotic recombination.

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