The XPF/ERCC1 heterodimer is a DNA structure-specific endonuclease that participates in nucleotide excision repair and homology-dependent recombination reactions, including DNA single strand annealing and gene targeting. Here we show that XPF/ERCC1 is stabilly associated with hRad52, a recombinational repair protein, in human cell-free extracts and that these factors interact directly via the N-terminal domain of hRad52 and the XPF protein. Complex formation between hRad52 and XPF/ERCC1 concomitantly stimulates the DNA structure-specific endonuclease activity of XPF/ERCC1 and attenuates the DNA strand annealing activity of hRad52. Our results reveal a novel role for hRad52 as a subunit of a DNA structure-specific endonuclease and are congruent with evidence implicating both hRad52 and XPF/ERCC1 in a number of homologous recombination reactions. We propose that the ternary complex of hRad52 and XPF/ERCC1 is the active species that processes recombination intermediates generated during the repair of DNA double strand breaks and in homology-dependent gene targeting events.

In the yeast Saccharomyces cerevisiae, Rad1 and Rad10 proteins form a stable complex with DNA structure-specific endonuclease activity (1, 2). During the removal of DNA lesions by nucleotide excision repair (NER), Rad1-Rad10 complex makes the 5’ incision in a bubble structure generated as a result of localized unwinding of the damaged DNA (2, 3). A stable interaction with the DNA damage recognition proteinRad14 appears to mediate the specific recruitment of Rad1/Rad10 to the damaged strand (4). Interestingly, unlike the other members of the RAD3 epistasis group that constitute the yeast NER pathway, the RAD1 and RAD10 genes also participate in specialized forms of mitotic recombination including the single strand annealing (SSA) pathway of recombination between direct sequence repeats and the integration of plasmid DNA into homologous chromosomal sequences (reviewed in Ref. 5).

In mammalian NER, XPF/ERCC1, the equivalent of Rad1/Rad10, interacts with XPA, the equivalent of Rad14, and makes the 5’ incision in the damaged strand (6–12). Similar to its yeast counterpart, XPF/ERCC1 is multifunctional, participating in intrachromosomal recombination between direct sequence repeats, the repair of DNA interstrand cross-links, and gene targeting (13–17). Although the XPF/ERCC1 endonuclease removes non-homologous single strand tails during targeted homologous recombination and SSA (17), a recent study (13) has revealed that XPF/ERCC1 is still required for gene targeting even when the targeting construct is homologous with the genomic locus. At the present time it is not known how XPF/ERCC1 is recruited to the specific recombination intermediates generated during SSA and targeted homologous recombination.

In yeast, the majority of DNA double strand breaks are repaired by recombinational repair pathways mediated by the products of genes in the RAD52 epistasis group (18, 19). Within this epistasis group, inactivation of the RAD52 gene results in the most severe phenotype (18, 19). Interestingly, genetic studies have implicated Rad52 in the same types of specialized mitotic recombination, SSA and homology-dependent integration of plasmid DNA, as the Rad1/Rad10 endonuclease (20, 21). Although inactivation of vertebrate RAD52 homologs does not significantly affect cellular sensitivity to agents that cause DNA double-strand breaks, these mutant cell lines are defective in the targeting of DNA molecules to homologous chromosomal loci, although to a lesser extent than that observed in yeast rad52 mutants (22, 23).

Eukaryotic Rad52 protein contains a conserved N-terminal domain that binds to DNA and self-associates to form a heptameric ring structure (24–27). Consistent with their role in SSA, both human (h) and yeast (y) Rad52 proteins promote the renaturation of complementary DNA single strands (28, 29). In this study, we provide evidence that the function of the XPF/ERCC1 DNA structure-specific endonuclease is modulated by a direct interaction with hRad52. These results reveal a novel role for hRad52 in recombination and suggest a mechanism for the targeting and activation of the XPF/ERCC1 endonuclease in recombination reactions.

**EXPERIMENTAL PROCEDURES**

**Fractionation of HeLa Extract by Immunoaffinity Chromatography with XPF Antibody—XPF antibodies (NeoMarkers, 50 μg) and anti-
Recombinant Repair by XPF

Recombinant hRad52—Escherichia coli BL21(DE3) cells harboring pET28b-hRad52 that encodes hRad52 with a C-terminal His$_6$ tag were grown in Terrific Broth (33) and induced with 100 µg/ml of IPTG for 18 h. Cells were collected by centrifugation and then washed extensively with Buffer A (25 mM HEPES-KOH, pH 7.5, 300 mM NaCl, 2 mM EDTA, and 1% Nonidet P-40) containing a mixture of protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.4 mM aprotinin, 0.5 mM leupeptin, 0.7 mM ethidium bromide, 1 mM sodium orthovanadate, and 1 mM β-glycerophosphate) and a final concentration of 20 mM EDTA.

Cloned hRad52 (0.4 pmol) was pre-incubated with 0.4 pmol of hRad52 fusion proteins were introduced into BL21(DE3) cells. When the $A_{600}$ reached 0.8, 1 mM isopropylthiogalactoside was added, and growth continued for 4 h. Cells were harvested by centrifugation, resuspended in Buffer C (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 1 mM EDTA, and 1% Nonidet P-40) containing a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.4 mM aprotinin, 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin) using a French press. After centrifugation (100,000 × g, 60 min), ammonium sulfate (0.32 g/ml) was added to the cleared lysate. The resulting precipitate was collected by centrifugation (18,000 × g, 30 min) and then resuspended in Buffer D (50 mM Tris-HCl, pH 7.5, 10% glycerol, 1 mM EDTA, 1 mM β-mercaptoethanol) containing the mixture of protease inhibitors. The conductivity of the protein solution was adjusted to that of 100 mM KCl with Buffer C and then mixed with nickel nitrilotriacetic acid-agarose beads (Qiagen) pre-equilibrated with Buffer C containing 100 mM KCl and 50 mM imidazole. After being poured into a column, the beads were washed extensively with the same buffer, followed by the sequential elution of bound hRad52 with 0.2 and 0.5 M KCl, and 50 mM imidazole in Buffer C. Fractions containing hRad52 were loaded onto an 8-ml Source S column (Amersham Biosciences) and eluted with a linear gradient from 100 to 600 mM KCl in Buffer C. Nearly homogenous hRad52 (~11 mg from a 1-liter culture) was stored in small aliquots at -80 °C.

Purification of Recombinant yRad52—His-tagged yRad52 was purified from E. coli as described previously (35).

Specific Association between XPF/ERCC1 and hRad52 in Human Cell Extracts—To identify proteins that associate with XPF/ERCC1, we fractionated a HeLa cell extract by immuno-affinity chromatography with either anti-XPF antibodies or anti-His$_6$ antibodies as the ligand. As expected, both XPF and ERCC1 were specifically retained by the anti-XPF beads and detected in the 1 M KCl and 0.2 M glycine eluates by immunoblotting (Fig. 1A, compare lanes 3 and 4 with lanes 7 and 8). Interestingly, hRad52 was detected in the 1 M KCl eluate from the anti-XPF beads (Fig. 1A, lane 3) but not in an equivalent fraction from either the anti-His$_6$ beads (Fig. 1A, lane 7) or protein A-agarose beads alone (data not shown). To provide further evidence for a specific association between XPF/ERCC1 and hRad52, we fractionated a HeLa nuclear extract by hRad52 affinity chromatography. ERCC1 specifically bound to the hRad52 beads and was eluted by the same ionic conditions that disrupted the association between hRad52 and the anti-XPF beads (data not shown).

Because both the XPF/ERCC1 complex and hRad52 bind to DNA, their association in the affinity chromatography experiments may have been mediated by DNA in the extract. To examine this issue, proteins were immunoprecipitated from a HeLa cell extract in the presence of ethidium bromide to disrupt DNA-protein interactions (Fig. 1B). As expected, XPF was immunoprecipitated by XPF antibodies and by antibodies specific to hRad52. Because both the XPF/ERCC1 complex and hRad52 bind to DNA, their association in the affinity chromatography experiments may have been mediated by DNA in the extract.
specific for its partner protein, ERCC1 (Fig. 1B). In accord with the affinity chromatography experiments, XPF was specifically immunoprecipitated by hRad52 antibody (Fig. 1B). Together these results strongly suggest that XPF/ERCC1 and hRad52 stably associate under physiological conditions.

**Direct Physical Interaction between the DNA Binding Domains of hRad52 and the XPF Subunit of the XPF-ERCC1 Complex**—To determine whether there is a direct interaction between hRad52 and XPF/ERCC1, we performed pull-down assays with purified recombinant XPF/ERCC1 (Fig. 2A, lane 2) and GST-hRad52. XPF/ERCC1 bound to glutathione- Sepharose beads liganded by GST-hRad52 but not to beads liganded by GST (Fig. 2B, compare lanes 2 and 3). A similar result was obtained when the pull-down assays were carried out in the presence of ethidium bromide (Fig. 2B, lanes 4 and 5) confirming that the association between XPF/ERCC1 and hRad52 is not mediated by DNA.

The interaction of hRad52 with the subunits of the XPF-ERCC1 complex was examined in pull-down assays using *in vitro* translated polypeptides. Labeled XPF bound to GST-hRad52 beads but not to either GST-yRad52 or GST beads alone (Fig. 2C, lanes 2–4). In contrast, no specific binding of *in vitro* translated ERCC1 to GST-hRad52 beads was observed (data not shown). To map the region of hRad52 that interacts with XPF, we expressed and purified the N- and C-terminal domains of hRad52 as GST fusion proteins. XPF bound to full-length hRad52 and its N-terminal domain but not to the C-terminal domain (Fig. 2D). Together, these results demonstrate that XPF/ERCC1 and hRad52 physically interact in a species-specific reaction that is mediated by the XPF subunit of the XPF-ERCC1 complex and the N-terminal DNA binding domain of hRad52.

**hRad52 Stimulates the DNA Structure-specific Endonuclease Activity of XPF/ERCC1**—To elucidate the functional consequences of the interaction between hRad52 and XPF/ERCC1, the effect of purified hRad52 (Fig. 3A, lane 2) on the nuclease activity of XPF/ERCC1 was examined. In these assays we used the preferred DNA substrate of XPF/ERCC1, a splayed arm structure formed that is cleaved by XPF/ERCC1 at the duplex/single strand junction releasing the 3’ single-stranded tail (3, 8, 10–12, 38). Pre-incubation of the DNA substrate with increasing amounts of hRad52 progressively inhibited XPF/ERCC1 nuclease activity (Fig. 3B, lanes 4–7). In contrast, pre-incubation of XPF/ERCC1 with the same amounts of hRad52 before mixing with the DNA substrate stimulated nuclease activity (Fig. 3B, lanes 10–13). Maximal stimulation occurred at a ratio of about 6 hRad52 molecules to 1 XPF/ERCC1 complex (Fig. 3B). Further increases in the amount of hRad52 progressively reduced nuclease activity (data not shown). At the optimum ratio, hRad52 enhanced the initial rate of endonucleolytic cleavage catalyzed by XPF/ERCC1 about 3-fold (Fig. 3C). Because hRad52 forms a heptameric ring (24, 25), these results suggest that a single XPF/ERCC1 heterodimer interacts with the hRad52 heptamer to yield a ternary complex that has increased nuclease activity. To provide further support for this model, we examined the effect of yRad52, which has similar biochemical properties to hRad52 but does not appear to bind XPF (Fig. 2C). In contrast to hRad52 (Fig. 3B, lanes 10–13), pre-incubation of XPF/ERCC1 with increasing amounts of yRad52 inhibited nuclease activity (Fig. 3D, lanes 5–8). Thus, the increased endonuclease activity of XPF/ERCC1 is dependent upon a specific physical interaction with hRad52.

Because Rad52 is a DNA-binding protein, we considered the possibility that the interaction of hRad52 with XPF/ERCC1 may change the DNA substrate specificity of this endonuclease.
A duplex Y structure was not cleaved by XPF/ERCC1 either with or without hRad52 (data not shown). In contrast, hRad52 did stimulate the weak cleavage activity of XPF/ERCC1 on a duplex substrate with a 3′ single strand flap, but the degree of stimulation was similar to that observed with the preferred splayed arm substrate (data not shown). Thus, under the reaction conditions used, hRad52 does not seem to alter DNA substrate specificity of XPF/ERCC1.

XPF/ERCC1 Attenuates the DNA Strand Annealing Activity of hRad52

The ability of eukaryotic Rad52 to bind to single strand DNA and to promote the annealing of complementary DNA single strands (25, 28) is consistent with the involvement
of this factor in SSA (21, 28, 29–43). Because XPF/ERCC1 interacts with the DNA binding domain of hRad52, we examined whether this interaction modulates the ability of hRad52 to anneal complementary DNA single strands. As expected (28, 29), both hRad52 and yRad52 promoted the annealing of complementary DNA single strands, whereas XPF/ERCC1 had no significant annealing activity (Fig. 4). Interestingly, pre-incubation with XPF/ERCC1 markedly attenuated the strand annealing activity of hRad52 (Fig. 4) but had no effect on strand annealing by yRad52 (Fig. 4). Thus, inhibition of hRad52-mediated strand annealing is dependent upon a specific physical interaction with XPF/ERCC1, suggesting that the binding of hRad52 to DNA and to XPF/ERCC1 are mutually exclusive. Taken together, our results provide evidence that hRad52 and XPF/ERCC1 form a stable ternary complex that cleaves specific recombination intermediates.

**DISCUSSION**

During NER, protein-protein interactions with XPA and RPA position XPF/ERCC1 to make the 5’ incision in the damaged DNA strand (6–12). In contrast, the molecular mechanisms that underlie the recruitment and activation of XPF/ERCC1 in homology-dependent recombination reactions have not been identified. Here we have shown an association between XPF/ERCC1 and hRad52 in human cell extracts and demonstrated a direct interaction between XPF and the N-terminal DNA binding domain of hRad52. The physical link between XPF/ERCC1 and hRad52 is congruent with results from genetic studies in mammalian cells (21–23, 39, 44–48) and *S. cerevisiae* (21–23, 39, 44–48), implicating these proteins in mitotic recombination pathways that include SSA and the integration of DNA molecules into homologous chromosomal sequences.

The yeast Rad1/Rad10 endonuclease removes non-homologous 3’ single strand tails from recombination intermediates that would otherwise prevent completion of the recombination event (39, 48). Similar studies with Chinese hamster ovary ercc1 mutant cell lines have provided evidence that the XPF/ERCC1 complex also participates in intrachromosomal recombination between direct sequence repeats and removes non-homologous single strand tails in homology-mediated gene targeting events (15, 17). However, more recently, it was found that ERCC1 is also essential for targeted gene replacement in mouse ES cells even when the ends of the targeting constructs are homologous with the genomic locus (13). Thus, it appears that XPF/ERCC1 plays a critical role in the processing of a different type of recombination intermediate that does not have a non-homologous single strand tail, namely the heteroduplex intermediate that is generated as a result of stalled branch migration during gene targeting (13).

Based on the biochemical properties of eukaryotic Rad52 (28, 29), it has been assumed that it would be involved in the annealing of complementary DNA single strands to generate the DNA structures that are subsequently recognized and cleaved by Rad1/Rad10 in yeast and XPF/ERCC1 in mammalian cells. Although our studies do not exclude the involvement of Rad52 in the strand annealing reaction, they have revealed a novel and unexpected role for Rad52 at a different and later stage in these pathways, the cleavage of recombination intermediates. Specifically, we have shown that hRad52 and XPF/ERCC1 form a stable complex in human cell extracts. Because formation of this ternary complex not only enhances the structure-specific endonuclease activity of XPF/ERCC1 but also inhibits DNA binding by hRad52, we suggest that the role of hRad52 in the ternary complex is to recruit, via protein-protein interactions, the DNA structure-specific endonuclease to specific recombination intermediates generated during SSA and in gene targeting.

**Acknowledgments**—We thank Dr. Rick Wood for the XPF/ERCC1 expression plasmid and purification protocol, Dr. Eva Lee for hRad52.
antibodies, and Wendy Bussen for assistance with the strand annealing assay. We are grateful to Dr. Sang Eun Lee for discussions and critical review of the manuscript.

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Physical and Functional Interaction between the XPF/ERCC1 Endonuclease and hRad52

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J. Biol. Chem. 2004, 279:13634-13639.
doi: 10.1074/jbc.M313779200 originally published online January 20, 2004

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