DNA damage activates cell cycle checkpoints that prevent progression through the cell cycle. In yeast, the DNA damage checkpoint response is regulated by a series of genes that have mammalian homologs, including rad1, rad9, hus1, and rad17. On the basis of sequence homology, yeast and human Rad1, Rad9, and Hus1 protein homologs are predicted to structurally resemble the sliding clamp PCNA. Likewise, Rad17 homologs have extensive homology with replication factor C (RFC) subunits (p36, p37, p38, p40, and p140), which form a clamp loader for PCNA. These observations predict that Rad1, Hus1, and Rad9 might interact with Rad17 as a clamp-clamp loader pair during the DNA damage response. In this report, we demonstrate that endogenous human Rad17 (hRad17) interacts with the PCNA-related checkpoint proteins hRad1, hRad9, and hHus1. Mutational analysis of hRad1 and hRad17 demonstrates that this interaction has properties similar to the interaction between RFC and PCNA, a well characterized clamp-clamp loader pair. Moreover, we show that DNA damage affects the association of hRad17 with the clamp-like checkpoint proteins. Collectively, these data provide the first experimental evidence that hRad17 interacts with the PCNA-like proteins hRad1, hHus1, and hRad9 in a manner similar to the interaction between RFC and PCNA.

In response to DNA damage, eukaryotic cells block cell cycle progression in a process commonly known as the DNA damage-induced checkpoint response. Studies in genetically tractable yeast model systems have identified a large number of genes, dubbed checkpoint genes, that are essential for DNA damage-inducible checkpoint activation (reviewed in Refs. 1–5). Epistasis and biochemical analyses in yeasts and humans have provisionally ordered the checkpoint proteins into a signaling pathway in which DNA damage relays activating signals through the phosphatidylinositol 3-kinase-related kinases, which include SpRad3, ScMec1, ATR, and ATM. The phosphatidylinositol 3-kinase-related kinases regulate activation of the serine-threonine protein kinases Chk1 and Chk2 (6–10), which phosphorylate the cell-cycle phosphatase Cdc25 (7, 9, 11, 12). Phosphorylation of Cdc25 inhibits its activity (13, 14) and its accumulation in the nucleus (15, 16), thereby preventing activation of the CyclinB-Cdc2 complex and blocking the G2/M transition after DNA damage.

Studies in Schizosaccharomyces pombe and Saccharomyces cerevisiae demonstrated that the checkpoint proteins spRad1, spHus1, spRad9, and spRad17 (using S. pombe nomenclature) or their homologs are essential for DNA damage-activated checkpoint responses (reviewed in Refs. 1 and 3–5). Furthermore, these studies suggest that all four proteins act early in the DNA damage-induced signaling pathway. Sequence analyses provide a few clues regarding potential functions of these proteins. Yeast, human, and fly Rad1 exhibit sequence homology with Ustilago maydis Rec1 (17–23), a checkpoint protein and a 3’-5’ exonuclease (24), suggesting that Rad1 may also be a nuclease. However, a highly conserved DEX motif from nucleases is poorly conserved among mammalian Rad1 homologs (22), and purified hRad1 has been variably reported to have nuclease activity (19, 20). Likewise, recombinant hRad9 has also been reported to possess nuclease activity (25). Recently, however, an alternative function for Rad1, Hus1, and Rad9 has been postulated. All three proteins exhibit sequence and possible structural homology with the sliding clamp protein PCNA (26, 27). Homotrimers of PCNA form a toroidal structure that encircles DNA and tethers DNA polymerase δ to DNA during replication (reviewed in Refs. 28 and 29). This homology raises the possibility that Rad1, Rad9, and Hus1 may also form clamp-like structures that participate in the recognition or processing of damaged DNA. Consistent with this idea, Rad1, Rad9, and Hus1 have all been shown to interact with one another in cell lysates (26, 30, 31) and by yeast two-hybrid analyses (30, 32). Additionally, we have recently shown that hRad1, hHus1, and hRad9 are converted to extraction-resistant nuclear foci following DNA damage (33).

Because clamp proteins encircle the DNA and are topologically linked with the DNA, they must be loaded onto the DNA by clamp loaders. The clamp loader for PCNA is replication factor C (RFC), a structure-specific heteropentameric complex (p36, p37, p38, p40, and p140) that recognizes primer-template junctions (28, 29). RFC, which is only fully functional as a pentamer (34–38), cracks the PCNA clamp and loads the clamp around the DNA in an ATP-dependent manner. If Rad1, Rad9, and Hus1 also form clamps, a clamp loader would likewise be required to load them onto the DNA. One potential clamp loader is the checkpoint protein Rad17, which exhibits significant homology with all five RFC subunits (39–43). The homology appears related to function. The S. cerevisiae homolog of Rad17 (scRad24) interacts with four of the five RFC subunits, forming a distinct multimeric structure in which scRad17 (scRad24) replaces the large RFC subunit in the complex (44).

Taken together, these observations suggest a tentative...
hRad17 Interacts with hRad1, hHus1, and hRad9

RESULTS

hRad17 Interacts with hRad1, hHus1, and hRad9—Yeast two-hybrid analyses demonstrated that hRad17 interacted with hRad1 (26, 43). However, several groups, including ours, were unable to observe an interaction between Rad1 and Rad1 homologs when looking for interactions among endogenous proteins in cell lysates (26, 31, 44, 45). Thus, we considered the possibility that lysis buffer conditions might affect interactions between these proteins and reevaluated the possibility that hRad17 interacts with hRad1. Our initial experiments were performed using a relatively harsh lysis buffer that had a total ionic strength of approximately 250 mN and contained 1% Triton X-100. When we lysed K562 cells in a milder, but still isotonic, buffer (150 mM KCl, 5 mM MgCl₂, and 50 µg/ml digitonin), hRad17 immunoprecipitates contained high levels of hRad1, hHus1, and hRad9, whereas control precipitations with protein A-Sepharose or preimmune sera did not (Fig. 1). In reciprocal hHus1, hRad1, and hRad9 immunoprecipitations, hRad17 was readily detected in each immunoprecipitation. The same interactions were observed if 0.1% Triton X-100 was substituted for digitonin (data not shown). Moreover, addition of 50 µg/ml ethidium bromide to the cell lysates, immunoprecipitation reactions, and washes had no effect on the interactions of hRad1, hHus1, and hRad9 with hRad17, suggesting that the observed interactions are not mediated by DNA (data not shown). Collectively, these results demonstrate that hRad17 interacts with the hRad1, hHus1, and hHus1 checkpoint protein complex.

The Nucleotide-binding Region of hRad17 Is Required for Interaction with hRad1, hHus1, and hRad9—hRad17 and RFC have consensus nucleotide-binding domains (40, 46). Mutation of a conserved lysine to glutamic acid (K118E) in the conserved P-loop of the nucleotide-binding domain of yeast spRad17 resulted in a nearly null phenotype (40). In human cells, overexpression of hRad17 with Lys-142 mutated to glutamate (K142E) disrupted the DNA damage-induced G2/M checkpoint arrest (47). Additionally, mutation of the analogous lysine in four of the five RFC subunits disrupted PCNA clamp-loading activity (34, 38). Taken together, these results demonstrated that an intact nucleotide-binding domain is essential for Rad17 and RFC functions. Previous work with purified components showed that the RFC heteropentamer binds PCNA much more tightly in the presence of ATP and Mg²⁺ than in the absence of these cofactors (35). Thus, we asked whether an intact nucleotide-binding domain was required for stable interaction of hRad17 with hRad1, hHus1, and hRad9. We mutated the highly conserved Lys-142 in nucleotide-binding P-loop of the Walker A motif of hRad17 to either glycine (K142G) or glutamate (K142E). We co-expressed either wild-type or mutant AU1-hRad17 in K562 cells and immunoprecipitated hRad17 (anti-AU1). Wild-type hRad17 strongly associated with three endogenous checkpoint proteins (Fig. 2A). hRad17 also interacted only with fully modified hRad9, which was consistent with previous work showing that hRad1 and hRad9 only interacted with fully modified hRad9 (31). In contrast, conversion of the positively charged lysine to a neutral glycine (K142G) reduced interaction, whereas charge reversal (K142E) completely abolished binding.

model in which a Rad17 clamp loader may interact with the Rad1, Hus1, and Rad9 clamp-like proteins during recognition or processing of DNA damage. This model predicts that Rad17 should interact with Rad1, Hus1, or Rad9. To date, this model has been largely untested. Although an interaction between Rad1 and Rad17 homologs has been documented by yeast two-hybrid analyses (26, 43), several groups have reported that endogenous Rad17 does not interact with Rad1 (26, 31, 44, 45). We now report that hRad17 does indeed interact with all three PCNA-like proteins, hRad1, hRad9, and hHus1, in cell lysates. Furthermore, by mutational analysis, we demonstrate that the interaction between hRad17 and hRad1 has biochemical features similar to the RFC-PCNA interaction. Finally, we show that DNA damage alters the interaction of hRad17 with hHus1, hRad1, and hRad9.

EXPERIMENTAL PROCEDURES

Cell Growth, Transformations, and Antibodies—K562 cells were cultured and transfected as described previously (31). All transfections contained 40 µg of DNA, which was adjusted by addition of empty vector if required. Monoclonal antibodies to the epitope tags hemagglutinin and AU1 were from Babco. The FLAG-M2 monoclonal antibody was from Sigma. Anti-GFP rabbit serum was from Molecular Probes. The FLAG-M2 monoclonal antibody and AU1 were from Babco. The FLAG-M2 monoclonal antibody and AU1 were from Babco. The FLAG-M2 monoclonal antibody and AU1 were from Babco. The FLAG-M2 monoclonal antibody and AU1 were from Babco. The FLAG-M2 monoclonal antibody and AU1 were from Babco.

Plasmid Construction and Mutagenesis—Expression vectors for FLAG-hRad1 and hemagglutinin (HA)-hHus1 have been described previously (31). AU1-hRad17 was constructed by appending an in-frame AU1 epitope tag to the N terminus of hRad17 using the polymerase chain reaction (PCR). The PCR product was cloned into pcDNA3 (Invitrogen). An in-frame S-TAG™ (Novagen Inc.) was added to the N terminus of RFC p38 using a PCR strategy. The PCR product was cloned into pcDNA3. All cloned PCR products and mutants were sequenced to assure fidelity of the amplification and mutagenesis, respectively. Furthermore, by mutational analysis, we demonstrate that the interaction between hRad17 and hRad1 has biochemical features similar to the RFC-PCNA interaction. Finally, we show that DNA damage alters the interaction of hRad17 with hHus1, hRad1, and hRad9.

FIG. 1. hRad17 interacts with hRad1, hHus1, and hRad9. K562 cell lysates were precipitated with protein A-Sepharose (PAS) only, preimmune, or immune sera as indicated. Washed precipitates were resolved by SDS-polyacrylamide gel electrophoresis and immunoblotted as indicated. IP, immunoprecipitation.
To determine whether mutation of Lys-142 in hRad17 affected interactions with other potential partners of hRad17, we co-expressed wild-type and mutant hRad17 with RFC p38 (Fig. 2B). RFC p38 is one of the four small subunits of the heteropentameric RFC complex. Previous work in yeast showed that the S. cerevisiae Rad17 homolog (scRad24) interacted with the four small subunits of yeast RFC but not the large subunit (44), suggesting that a similar interaction might also occur in humans. Wild-type hRad17 co-precipitated S-Tag™ RFC p38, demonstrating that hRad17, like its S. cerevisiae counterpart (scRad24) interacted with the four small subunits of yeast RFC but not the large subunit (44), suggesting that a similar interaction might also occur in humans. Wild-type hRad17 co-precipitated S-Tag™ RFC p38, demonstrating that hRad17, like its S. cerevisiae counterpart (scRad24), interacts with RFC p38. Both hRad17 mutants interacted with RFC p38, although at a slightly reduced level, indicating that mutation of the nucleotide-binding motif does not globally disrupt interactions with other binding partners. In summary, these results demonstrate that, as in yeast, hRad17 interacts with human RFC p38 (endogenous hRad17 and RFC p38 also interact, data not shown). Additionally, they suggest that an intact hRad17 nucleotide-binding domain is essential for interaction of hRad17 with hRad1, hHus1, and hRad9.

An hRad17 Mutant Associates with hRad9 and hHus1 but Cannot Bind hRad17—To characterize the interaction of hRad17 with hRad1 further, we examined the proposed structural homology between hRad1 and PCNA (26, 27). In PCNA, a peptide corresponding to the C-terminal α-helix is required for functional interactions between PCNA and RFC (48). Consequently, we mutated the analogous region of hRad1 by replacing an eight-amino acid stretch with alanines (M3, amino acids 226–233). We then examined interaction of the polyalanine-substituted hRad1 (M3) mutant with all members of the complex (Fig. 3). Wild-type and mutant (M3) hRad1 (FLAG-tagged) constructs were transfected into K562 cells, and hRad1 was immunoprecipitated (IP) and blotted with horseradish peroxidase-conjugated S-protein (middle panel) to detect RFC p38 followed by anti-AU1 to detect hRad17 (top panel).

hRad17 Interacts with hRad1, hHus1, and hRad9

Fig. 2. The nucleotide-binding region of hRad17 is required for interaction with hRad1, hHus1, and hRad9 but not Rfc38. A, K562 cells were cotransfected with GFP-hRad9 (10 μg), FLAG-hRad1 (10 μg), hemagglutinin (HA)-hHus1 (10 μg), and 10 μg of AU1-tagged wild-type hRad17 (R17), hRad17 (K142G), or hRad17 (K142E). A portion of each lysate (cell lysate) was fractionated by SDS-polyacrylamide gel electrophoresis to assess protein expression. The remainder of each lysate was immunoprecipitated (IP) with anti-AU1 (α-AU1 [hRad17]), and the washed immunoprecipitates were sequentially immunoblotted for tagged hHus1, hRad1, and hRad9. B, K562 cells were co-transfected with 10 μg of AU1-tagged wild-type (wt) hRad17 (R17) and hRad17 mutants (K142G and K142E) along with 5 μg of S-TAG™-RFC38. A portion of the lysate was precipitated with S-protein agarose to demonstrate equal protein expression of S-TAG™-RFC p38 (bottom panel). The remainder of the lysate was immunoprecipitated with anti-AU1 (hRad17) and blotted with horseradish peroxidase-conjugated S-protein (middle panel) to detect RFC p38 followed by anti-AU1 to detect hRad17 (top panel).

Fig. 3. Mutant hRad1 associates with hRad9 and hHus1 but not hRad17. K562 cells were transfected with empty vector (EV) or 5 μg of FLAG-tagged wild-type hRad1 (WT) and mutant hRad1 (M3). Anti-FLAG immunoprecipitates (IP) were sequentially immunoblotted with hHus1, hRad9, hRad17, and FLAG (detects hRad1).

Fig. 4. hRad17 does not interact with extraction-resistant hRad1, hHus1, and hRad9. K562 cells were treated with vehicle (–) or 2 μg/ml 4-nitroquinoline oxide (4-NQO) for 60 min. Low and high salt extracts were then prepared, and the ionic strength of the low salt extracts was adjusted to equal that of the high salt extracts (250 mM NaCl). Parallel salt-equalized lysates were precipitated with anti-hRad9 and anti-hRad17. The immunoprecipitates (IP) were immunoblotted sequentially as indicated. The bands present in the anti-hRad1 immunoblot in the high salt extract are nonspecific.
ever, the M3 mutant did not interact with hRad17, suggesting that this portion of hRad1 is required for interaction with hRad17.

hRad17 Does Not Interact with hRad9-hHus1-hRad1 Complex That Associates with DNA after DNA Damage—In undamaged cells, the hRad9-hHus1-hRad1 complex is readily extracted by cell permeabilization in low salt buffers (33). Under identical conditions, hRad17 is also quantitatively extracted from nuclei, indicating that all four checkpoint proteins are only loosely associated with nuclear components. However, DNA damage converts the hRad9-hHus1-hRad1 complex to a DNA-bound, extraction-resistant form that can be recovered by high salt extraction. In sharp contrast, hRad17 was not converted to an extraction-resistant complex after DNA damage (33), suggesting that DNA damage does not induce stable association of hRad17 with nuclear components after DNA damage under these extraction conditions. This result suggests that hRad17 might interact selectively with the readily extractable hRad9 complexes but not with the extraction-resistant complexes. To test this possibility, K562 cells were treated with the DNA-damaging agent 4-nitroquinoline oxide, which potently converts the hRad9 complex to the extraction-resistant form (Fig. 4 and Ref. 33). After preparation of the low and high salt extracts, the low salt extracts were adjusted to the same ionic strength as the high salt extracts (250 mM) and hRad9 and hRad17 were then immunoprecipitated from parallel sets of salt-equalized extracts. As previously reported, 4-nitroquinoline oxide converted a large fraction of the hRad9 pool to a less-extractable form (Fig. 4). Reprobing the hRad9 immunoprecipitates revealed that hRad17 associated with the readily extractable hRad9 complex but not the less-extractable form of hRad9. Immunoblotting of hRad17 immunoprecipitates confirmed that hRad17 was not converted to an extraction-resistant form following DNA damage, although we have occasionally observed very low levels of hRad17 association after DNA damage. Consistent with these results, neither hRad9, hHus1, nor hRad1 was associated with hRad17 in the high salt extracts. The differential interactions of hRad17 with the PCNA-like proteins in the high and low salt extracts after DNA damage are not due to disruption by the increased ionic strength of the high salt extracts because hRad17 interacted with all three proteins in the salt-adjusted (250 mM NaCl) low salt extracts (Fig. 4). Taken together, these results indicate that DNA damage triggers the formation of extraction-resistant hRad1, hHus1, and hRad9 complexes, which no longer associate with hRad17.

DISCUSSION

The checkpoint proteins Rad1, Hus1, Rad9, and Rad17 are required for early events in the activation of the DNA damage checkpoint-signaling pathway (1, 3–5). Initially, there were few clues regarding their functions. Recently, however, Rad1, Hus1, and Rad9 have been predicted to be PCNA-like clamp proteins (26, 27), and S. cerevisiae Rad17 (scRad24), which is homologous with RFC subunits, was found to interact with four of the five RFC subunits, suggesting that it may be a clamp loader (44). Collectively, these observations suggest that DNA damage-induced checkpoint activation requires a clamp and a clamp loader. One prediction from this model is that the components of the clamp (Rad1) and clamp loader (Rad17) interact. Although several groups showed that Rad17 and Rad1 homologs interacted in yeast two-hybrid analyses (26, 43), initially, we and others could not demonstrate an interaction between endogenous Rad17 and Rad1 homologs (26, 31, 44, 45). Here, for the first time, we demonstrate that endogenous hRad17 and hRad1 interact, and we also show that hRad17 interacts with hRad9 and hHus1, two other PCNA-like checkpoint proteins. One possible organization for this complex is with hRad1, hHus1, and hRad9 forming a clamp-like complex, with hRad1 linking an hRad1-hHus1-hRad9 complex to hRad17 (Fig. 5). This idea is supported by the finding that yeast and human Rad17 interacts with Rad1 but not Rad9 or Hus1 in two-hybrid analyses (26, 43) and by our present observation that the hRad1 M3 mutant disrupts the interaction with hRad17 but not hRad9 and hHus1.

Additionally, our studies with mutant hRad17 and hRad1 suggest that the interactions between hRad17 and hRad1 may be similar to those between RFC and PCNA. First, mutations that disrupt the nucleotide-binding domain of hRad17 prevent interaction with hRad1 but not RFC p38, suggesting that nucleotide binding is important for stable association with hRad1. A similar requirement for nucleotide binding has been noted for RFC-PCNA interactions: in the presence of ATP and Mg2+, RFC interacted more tightly with PCNA (35). Second, the hRad1 M3 mutation selectively disrupted interaction between hRad1 and hRad17. The analogous region in PCNA is also required for productive interactions between PCNA and RFC (48), suggesting that parallel regions of hRad1 and PCNA participate in interactions with clamp loaders.

Several lines of investigation now suggest a provocative model regarding the roles of hRad1, hHus1, hRad9, and hRad17 in DNA damage-activated responses. In this model, an hRad17-containing clamp would recognize structural alterations induced by DNA damage (Fig. 5), much as classical RFC complexes recognize primer-template junctions. The hRad17-containing clamp loader would then load hRad1, hHus1, and hRad9 clamps onto DNA, thus converting them from readily extractable nuclear proteins to extraction-resistant nuclear foci (33). However, the results presented in Fig. 4 demonstrate that even after DNA damage, hRad17 does not stably associate with nuclear elements, suggesting that hRad17 may interact only transiently with DNA. Once hRad9, hHus1, and hRad1 are loaded onto DNA, they may then recruit proteins that participate in DNA processing or activation of the downstream checkpoint signaling machinery.

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