Reconstitution and Molecular Analysis of the hRad9-hHus1-hRad1 (9-1-1) DNA Damage Responsive Checkpoint Complex*

Matthew A. Burtelow‡§, Pia M. K. Roos-Mattjusˌ§, Matthew Rauen‡, Jeremy R. Babendure‡, and Larry M. Karnitz‡§**

From the Divisions of §Developmental Oncology Research and Radiation Oncology and the Departments of ¶Biochemistry and Molecular Biology and §Molecular Pharmacology, Mayo Clinic, Rochester, Minnesota 55905

DNA damage provokes multiple cellular responses, including the activation of DNA damage checkpoint signaling pathways, which arrest cell cycle progression in G₁, S, and G₂/M and possibly coordinate repair of the damage. Loss of the DNA damage checkpoint response leads to increased sensitivity to DNA-damaging agents, demonstrating that these pathways are critical for efficient recovery from DNA damage (reviewed in Refs. 1–6). The components of the checkpoint signaling pathways were initially identified by genetic analyses in the yeast, Schizosaccharomyces pombe and Saccharomyces cerevisiae, and recent studies demonstrated that the checkpoint genes are conserved between yeasts and humans (7–23). Collectively, these studies have given rise to a conserved model in which DNA damage activates members of the ATM (ataxia telangiectasia-mutated) family of phosphatidylinositol 3-kinase-related kinases. These protein kinases are then required to activate the downstream protein kinases Chk1 and Chk2 (20, 22–25), which regulate cell cycle arrest and possibly other DNA damage-induced responses.

Despite the recent progress in the dissection of the downstream protein kinase-mediated portions of the checkpoint signaling pathways, yeast genetic studies demonstrated that additional checkpoint genes also regulate the DNA damage response (reviewed in Refs. 2–6). For example, in S. pombe, spRad1, spHus1, spRad9, and spRad17 (using S. pombe nomenclature) are all essential for DNA damage-induced cell cycle arrest and activation of the spChk1 protein kinase after DNA damage (4–6). Thus, these results suggest that Rad1, Hus1, Rad9, and Rad17 function upstream of Chk1 activation in the DNA damage-signaling pathway.

Recently, molecular modeling studies were used to propose functions for Rad1, Hus1, Rad9, and Rad17 (26–28). In this model Rad9, Hus1, and Rad1 form a PCNA-like clamp. PCNA is homotrimeric doughnut-shaped structure that is loaded onto DNA by the clamp loader, replication factor C (RFC) (29, 30). Once loaded, PCNA encircles the DNA as a sliding clamp, tethering replication proteins, including DNA polymerase δ, to the replicating DNA. RFC is composed of four small subunits (p36, p37, p38, and p40) and one large subunit (p140), and all the subunits contain consensus nucleotide-binding Walker A and B motifs (31). RFC binds to primer-template junctions that are generated during replication, opens the PCNA clamp, and loads the clamp around the DNA in an ATP-dependent manner. Thus, the molecular modeling studies suggest that Rad9, Hus1, Rad1, and Rad17 may have functions analogous to the functions of PCNA and RFC.

In addition to the molecular modeling predictions, limited biochemical data also support this model. First, the S. cerevisiae homolog of hRad17 interacts with the four small subunits of RFC in a stable complex, suggesting that the Rad17-RFC complex may indeed be a clamp loader (32). However, no data currently support this contention. Second, yeast and mammalian Rad9, Hus1, and Rad1 interact in immunoprecipitation and yeast two-hybrid analyses (27, 33–35). However, the nature of the complex has not been explored. Third, hRad1, hHus1, and hRad9 interact with hRad17 in a manner that requires an intact hRad17 ATP-binding domain (36). Fourth, following DNA damage, the Rad9-Hus1-Rad1 complex is converted to an extraction-resistant, chromatin-bound complex, which may reflect clamping of the complex onto sites of damage (37). Collectively, these observations suggest a model in which the Rad17-RFC clamp loader recognizes DNA damage and then
loads a Rad9-Hus1-Rad1 clamp around the DNA. Once loaded around DNA, the Rad9-Hus1-Rad1 complex may tether signaling and repair molecules to the damaged site.

Despite the data supporting the clamp model, there are several experimental inconsistencies with the model. First, studies in S. pombe demonstrated that spHus1 and spRad1 could not interact in the absence of spRad9 (10, 27), which suggested that spRad9 linked the two proteins. In contrast, the clamp model predicts that spHus1 and spRad1 should interact even in the absence of spRad9. Second, additional studies in S. pombe indicated that spRad9, spHus1, and spRad1 interacted at very low stoichiometry, calling into question whether these proteins do indeed form a stable clamp (27). Third, there are phenotypic differences between sprad1 and sphus1 null mutants, which would be unexpected if the two proteins formed a portion of the clamp (38). To address these questions with the mammalian proteins and to characterize further the biochemical nature of hRad9, hHus1, and hRad1 complex, we examined the complex in human cell lysates using size-exclusion chromatography. We also reconstituted the complex in an insect expression system and mapped the interaction domains among the three proteins. These studies revealed that hRad9, hHus1, and hRad1 form a stable protein complex, which we dubbed the 9-1-1 complex. Our data, which are consistent with a circular head-to-tail organization for the three-member complex, also support the proposed sliding clamp model. Additionally, these studies identified a pool of monomeric hRad1, which may have functions separate from the 9-1-1 complex.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfections, and Antibodies—K562 cells were cultured as previously described (34). All transfections contained 40 μg of DNA, which was adjusted with empty vector if required, and were performed as described (34). Rabbit antisera against hRad1, hHus1, and hRad9, and the monoclonal antibody against hRad9 have been described previously (34, 37). Monoclonal antibodies to Myc, HA, AU1, and AU5 were from Covance. Monoclonal antibodies to FLAG were from Sigma. Rabbit antisera to green fluorescent protein (GFP) were from Molecular Probes. S-protein-agarose and S-protein-coupled to horseradish Peroxidase were from Novagen and used according to the manufacturers recommendations.

Plasmid and Baculovirus Construction—FLAG-tagged hRad1, AU1-tagged hRad9, and HA-tagged hHus1 have been described previously (34). To generate “half-mutants” of hRad1, hHus1, and hRad9, we examined the molecular modeling structures for these proteins (26–28) and constructed expression vectors that truncated the proteins in the putative loop that connects the two lobes. Polymerase chain reaction (PCR)-based strategies were used to generate the amino-terminal half-mutants of hRad1, hHus1, and hRad9. The hRad1 amino-terminal half-mutant contained amino acids 1–146 followed by four copies of the AU1 epitope tag. The hRad9 amino-terminal half-mutant extended from amino acids 1–129 and tandem HA epitope tags were added to the carboxyl terminus. The hHus1 amino-terminal half-mutant extended from amino acids 1–148 followed by tandem Myc epitope tags. The hRad9 carboxyl-terminal half-mutant fused S-Tag peptide (MKETAKAAKFERHNMDHSA) to amino acids 135–265 of hRad9. All epitope-tagged half-mutants were cloned into pcDNA3, and the cloned constructs were sequenced to verify fidelity of PCR amplification.

Recombinant baculoviruses encoding His6-tagged hRad9 and untagged hRad1 and hHus1 were generated using the Bac-to-Bac (Life Technologies, Inc.) expression system. The coding region of hRad9 was PCR amplified and cloned into-frame with the His6 tag of the pFastBacHTa baculoviral transfer vector. Similarly, untagged hRad1 and hHus1 were amplified by PCR and cloned into the pFastBac-1 transfer vector. All cloned PCR products were sequenced to assure accurate amplification. Transfer vectors were then transformed into DH10Bac E. coli and used for recombinant baculovirus production. The recombinant baculoviruses were amplified in Sf9 cells using CellFectin reagent (Life Technologies, Inc.) and viral supernatants were harvested after 3 days. hRad9, hHus1, and hRad1 protein expression was verified by immunoblotting cell lysates from infected Sf9 cells.

Immunoprecipitation Studies—Exponentially growing K562 cells (1 × 10⁶ per assay point) were washed in phosphate-buffered saline. Cells were lysed in buffer (10 mM HEPES, pH 7.4, 150 mM KCl, 10 mM MgCl₂, and 0.1% Triton X-100) supplemented with freshly added 10 mM β-glycerophosphate, 1 mM Na₃VO₄, 20 μg/ml pepstatin A, 10 μg/ml aprotinin, 20 μg/ml leupeptin, and 20 ng/ml microcin-LR for 10 min on ice. Cleared lysates (21,000 g for 5 min) were immunoprecipitated with the indicated mouse monoclonal antibodies and protein G-Sepharose (Sigma) or rabbit antisera and protein A-Sepharose (Sigma) at 4 °C for 1 h.

Immunoprecipitates were washed three times with lysis buffer solubilized in SDS-polyacrylamide gel electrophoresis (PAGE) buffer, and fractionated by SDS-PAGE. Proteins were transferred to Immobilon P membrane and immunoblotted as described (34).

Size-exclusion Chromatography—Exponentially growing K562 cells (2 × 10⁶ per fractionation) were washed in phosphate-buffered saline and lysed in 0.5 ml of lysis buffer supplemented with freshly added 10 mM β-glycerophosphate, 1 mM Na₃VO₄, 80 μg/ml pepstatin A, 40 μg/ml aprotinin, 80 μg/ml leupeptin, and 80 ng/ml microcin-LR for 10 min on ice. Cleared lysates (21,000 g for 5 min) were immunoprecipitated with the indicated mouse monoclonal antibodies and protein G-Sepharose (Sigma) or rabbit antisera and protein A-Sepharose (Sigma) at 4 °C for 1 h. Immunoprecipitates were washed three times with lysis buffer, calibrated with protein standards, and were incubated on ice for 1 h at 4 °C. The resin was washed with lysis buffer containing 5 mM imidazole. The complex was eluted in lysis buffer containing 300 mM...
RESULTS

Size-exclusion Chromatographic Analysis of hRad9, hHus1, and hRad1 in K562 Cell Lysates—Previous immunoprecipitation and yeast two-hybrid analyses indicated that hRad9, hHus1, and hRad1 interacted. To examine further the interactions among hRad9, hHus1, and hRad1, we analyzed these proteins by size-exclusion chromatography. K562 cells were lysed and clarified lysates were fractionated using a Superdex 200 column (Fig. 1A). Lysates and column fractions were immunoprecipitated and immunoblotted for hRad1. These studies revealed that hRad1, which has a predicted molecular mass of 31 kDa, fractionated into two separate pools. One pool eluted with an apparent molecular mass of ~40 kDa, suggesting that approximately half of the hRad1 pool exists in monomeric form in cell lysates. The remaining hRad1 eluted with an apparent molecular mass of 160 kDa, suggesting that this portion of the hRad1 pool is part of a larger protein complex.

To determine whether hHus1 and hRad9 exhibited similar elution profiles, we analyzed the remainder of the column fractions for hRad9 and hHus1. hRad9 has a predicted molecular mass of 43 kDa and hHus1 has a predicted molecular mass of 31 kDa, and yet both proteins co-eluted with the fast-eluting form of hRad1, suggesting that they are also part of larger protein complexes. Unlike hRad1, however, no monomeric hRad9 and hHus1 were present in the cell lysates. To assess whether the fast-eluting, 160-kDa complex contains interacting hRad1, hHus1, and hRad9, we immunoblotted the hRad9 immunoprecipitates for hRad1 and hHus1. Fig. 1B shows that the hRad9 immunoprecipitates contained both hRad1 and hHus1. Taken together, these results suggest that hRad9 and hHus1, and much of the hRad1 pool, are part of a larger, interacting protein complex that elutes from a size-exclusion column with an apparent molecular mass of 160 kDa. Because the predicted additive molecular mass of the complex is 105 kDa, this suggests that the complex is either non-spherical or associates with additional proteins.

hRad9, hHus1, and hRad1 Do Not Self-multimerize—We then addressed the possibility that self-multimerization of hRad1, hHus1, and hRad9 may contribute to complex formation. We co-overexpressed FLAG-tagged hRad1 with AU5-tagged hRad1, and, as a positive control, we also co-expressed FLAG-tagged hRad1 with HA-tagged hHus1 (Fig. 2A). Immunoblotting for FLAG revealed that although FLAG-tagged hRad1 interacted with hHus1 it did not interact with itself (AU5-tagged hRad1), even when the immunoblot was overexposed. An analysis examining hHus1 self-oligomerization showed that HA-tagged hHus1 interacted with Rad1 but did not associate with itself (Myc-tagged hHus1) (Fig. 2B).

We performed a similar analysis for hRad9. For this analysis, we generated differentially tagged hRad9 expression vectors that expressed carboxyl-terminally truncated hRad9 (amino acids 1–270). This truncation contains the predicted PCNA-like folds (amino acids 1–270) but removes a carboxyl-terminal extension (amino acids 271–391) of hRad9 that is not essential for interaction with hRad1 or hHus1 (data not shown and Fig. 2C). We used this portion of hRad9 for self-oligomerization analysis because we previously observed that when overexpressed alone in insect cells and K562 cells, full-length hRad9 elutes as a broad peak (~600 kDa to monomeric size) from Superdex 200. These results suggest that, unlike hRad1 and hHus1, full-length hRad9 aggregates when overexpressed (data not shown). Using the truncated hRad9 constructs, we found that GFP-tagged hRad9–270 (amino acids 1–270) interacted with hHus1 but did not interact with itself (AU1-tagged hRad9–270) (Fig. 2C). Taken together, these studies suggest
that self-oligomerization of hRad1, hHus1, and hRad9 does not likely contribute to 9-1-1 complex formation.

The hRad9-hHus1-hRad1 Complex Is Reconstituted in an Insect Cell Expression System—To analyze further the hRad9-hHus1-hRad1 complex, we generated recombinant baculovirus expression constructs for His6-tagged hRad9 and untagged hRad1 and hHus1. hRad9 undergoes extensive phosphorylation in undamaged mammalian cells (basal phosphorylation), and in response to DNA damage hRad9 is additionally phosphorylated (hyperphosphorylation) (34). Therefore, we examined whether hRad9 is basally phosphorylated when expressed in insect cells. Because basal hRad9 phosphorylation dramatically affects protein mobility when analyzed by SDS-PAGE, we examined the mobility of hRad9 in cell lysates derived from baculovirus-infected Sf9 cells. Unmodified hRad9 migrates with an apparent molecular mass of 43 kDa, equivalent to its predicted molecular mass, whereas fully phosphorylated hRad9 migrates with an apparent molecular mass of 66–68 kDa.

To verify that hRad9 phosphorylation patterns were similar when expressed in either insect or K562 cells, we overexpressed AU1-tagged hRad9, along with hRad1 (FLAG-tagged) and hHus1 (HA-tagged), in K562 cells (Fig. 3). In parallel, we co-infected insect cells with His6-tagged hRad9 and untagged hHus1 and hRad1. Transfected hRad9 (AU1), hHus1 (HA), and hRad1 (FLAG) were precipitated from K562 cells lysates using the indicated epitope tags. Lysates from infected Sf9 cells were immunoprecipitated with anti-hRad9, anti-hHus1, and anti-hRad1. The precipitates were fractionated by SDS-PAGE in parallel and immunoblotted for hRad9. As in K562 cells, insect cell-expressed hRad9 migrated as a series of modified forms ranging from partially to fully modified. Consistent with the results obtained in human cells, hRad1 and hHus1 interacted more efficiently with the highly modified forms of hRad9. Collectively, these results indicated that hRad9 undergoes physiologically relevant phosphorylations, and that the complex can be reconstituted in an insect expression system.

To characterize further the insect cell-expressed hRad9-hHus1-hRad1 complex, we fractionated the complex by size-exclusion chromatography using a Superdex 200 column (Fig. 4). We co-infected insect cells with excess hRad1 and hHus1 virus and a limiting amount of His6-hRad9 virus (data not shown) and purified the complex using Ni2+-chelate chromatography prior to size-exclusion chromatography. These experiments revealed that all three proteins co-eluted as a single peak at precisely the same position as the peak observed for hRad9, hHus1, and hRad1 in K562 cells. Thus, co-expression of recombinant hRad9, hHus1, and hRad1 reconstituted this checkpoint complex in an insect system. This result suggests that the slightly anomalous apparent molecular mass of the complex may be intrinsic to the complex. Alternatively, an insect cell-derived protein might associate with the complex, which increases the apparent chromatographic size of the 9-1-1 complex.

hRad9, hHus1, and hRad9 Interact in a Pair-wise Manner—The results presented in Figs. 1–4 demonstrated that all three proteins interacted and formed a trimolecular complex.
Insect cells were infected with the indicated virus combinations (Fig. 5) and immunoprecipitated either for hRad1, hHus1, or hRad9. The immunoprecipitates were then immunoblotted for the indicated proteins. As positive controls for the experiments, we also infected insect cells with all three viruses to generate the trimolecular complex. These studies revealed that in reciprocal immunoprecipitates hRad1 interacted with hHus1 in the absence of hRad9 (Fig. 5, A and C, left arrows), and that hRad1 interacted with hRad9 in the absence of hHus1 (Fig. 5, A, right arrow; Fig. 5B, left arrow). Additional analyses revealed that hRad9 interacted with hHus1 in the absence of hRad1 (Fig. 5A, right arrow; Fig. 5B, left arrow). Because the proteins are overexpressed in a heterologous system, these results suggest that the proteins interact directly in a pair-wise manner; however, it is possible that an insect homolog may participate in these interactions.

Mapping the Interaction Domains between hRad9, hHus1, and hRad1—Molecular modeling studies predict that Rad9, Hus1, and Rad1 fold into PCNA-like structures (26–28). Each PCNA subunit is composed of two structurally identical lobes joined by an interdomain connector loop (39). Using the molecular models, we constructed expression vectors that divided each protein within the linker domain connecting the lobes. We expressed the predicted amino-terminal lobe of hRad1 (amino acids 1–146) and hHus1 (amino acids 1–148), which we called amino-terminal half-mutants. Because the predicted PCNA-like fold of hRad9 extends only to approximately amino acid 270, we called the hRad9 mutant (amino acids 1–129) an amino-terminal half-mutant as well, even though it only represents one-third of the entire protein. Given that we were able to detect pair-wise interactions among all three proteins, we reasoned that these amino-terminal half-mutants should associate with one binding partner but not the other. We tested this hypothesis by transiently transfecting K562 cells with epitope-tagged full-length protein or the corresponding amino-terminal half-mutants along with epitope-tagged versions of the two potential binding partners and asked whether the overexpressed amino-terminal half-mutant associated with one or both partners (Fig. 6). The tagged half-mutant or corresponding full-length protein was then immunoprecipitated and the immunoprecipitates were immunoblotted for the co-transfected binding partners. These studies verified that full-length hRad9 interacted with both hRad1 and with hHus1. In contrast, the amino-terminal hRad9 half-mutant interacted with hRad1 but...
Reconstitution of the hRad9-hHus1-hRad1 Complex

Studies in the yeast *S. pombe* demonstrated that Rad1, Hus1, and Rad9 are required for early events in the activation of the DNA damage checkpoint-signaling pathway. Initially, there were few clues regarding their functions, although the proteins had been shown to interact. To further our understanding of how hRad1, hHus1, and hRad9 interact to form a checkpoint complex, we examined the endogenous complex, reconstituted the complex in a heterologous expression system, and mapped interactions among the subunits. In this report we show that the majority of human hRad9, hHus1, and hRad1 exist in a heterotrimeric complex. We also found that the PCNA-like portions of hHus1 (full-length), hRad1 (full-length), and hRad9 (amino acids 1–270) do not self-multimerize. Additionally, we reconstituted the 9-1-1 complex in an insect cell expression system and demonstrated that it had identical chromatographic behavior. Finally, our mapping studies demonstrated that each member of the complex interacted in a pair-wise fashion, and these studies also mapped the interactions among the three 9-1-1 complex members. Collectively, these results provide novel insights into the assembly, structure, and potential functions of the 9-1-1 DNA damage-responsive checkpoint complex.

The self-oligomerization analysis suggests that hRad9, hHus1, and hRad1 do not homomultimerize. This is in contrast to a study by Hang and Lieberman (35), which demonstrated that hHus1 and hRad9 self-oligomerize. One possible explanation for this discrepancy stems from our observation that full-length hRad9 forms large, higher-order aggregates when overexpressed without its partners hRad1 and hHus1. These higher-order aggregates may be physiologically relevant. However, analysis of endogenous hRad9, hHus1, and hRad1 (Fig. 1) did not reveal such complexes. Thus, we propose that hRad9, hHus1, and hRad1 form a heterotrimeric 9-1-1 complex that does not include self-multimers of any individual component.

Examination of the interactions within the 9-1-1 complex revealed that each member interacted in a pair-wise manner. We also mapped the pair-wise interactions using amino- and carboxy-terminal expression constructs. These results showed that: 1) the amino terminus of hRad9 interacted selectively with hRad1, 2) the amino terminus of hRad1 interacted with hHus1, and 3) the amino terminus of hHus1 interacted with hRad9. Further mapping, using the carboxy-terminal fragment of hRad9 predicted PCNA fold, demonstrated that this region associated with the amino terminus of hHus1. Thus, the PCNA-like portion of hRad9 is flanked by hRad1 on its amino terminus and by hHus1 on its carboxy terminus. Although we were unable to fully map the interactions among the subunits, our studies support a PCNA-like clamp model (Fig. 8) and define the orientation of the three proteins in the complex. It is

not with hHus1 (Fig. 6A). Thus, we concluded that the amino-terminal portion of hRad9 associates with hRad1. We repeated the analogous interaction studies with the hHus1 and hRad1 amino-terminal half-mutants, which revealed that the amino terminus of hHus1 interacted with hRad9 (Fig. 6B) and that the amino terminus of hRad1 associated with hHus1 (Fig. 6C).

To further map these interactions, we also generated expression vectors for the carboxy-terminal, PCNA-like lobes of hHus1 (amino acids 135–280) and hRad1 (amino acids 135–282). Despite generating several different constructs with a variety of tags (including large tags such as glutathione S-transferase), the carboxy-terminal hHus1 and hRad1 fragments were expressed very poorly compared with other half-mutants, suggesting that they did not fold appropriately. Consistent with these observations, these carboxy-terminal half-mutants were unable to interact with any other members of the 9-1-1 complex (data not shown). In contrast, an expression vector for the carboxy-terminal, PCNA-like lobe of hRad9 (amino acids 135–270) was expressed well. We then asked whether the Rad9 carboxy-terminal half-mutant could interact selectively with either the amino-terminal hHus1 or hRad1 half-mutants. These studies revealed that the carboxy-terminal hRad9 (R9-C) interacted exclusively with the amino-terminal hHus1 half-mutant (Fig. 7A) but not with the amino-terminal hRad1 half-mutant (Fig. 7B). Taken together with the results in Fig. 6, these results suggest that the PCNA-like portion of hRad9 (amino acids 1–270) is flanked on its amino terminus by hRad1 and on its carboxy terminus by the amino terminus of hHus1.

**DISCUSSION**

Studies in the yeast *S. pombe* demonstrated that Rad1, Hus1, and Rad9 are required for early events in the activation of the DNA damage checkpoint-signaling pathway. Initially, there were few clues regarding their functions, although the proteins had been shown to interact. To further our understanding of how hRad1, hHus1, and hRad9 interact to form a checkpoint complex, we examined the endogenous complex, reconstituted the complex in a heterologous expression system, and mapped interactions among the subunits. In this report we show that the majority of human hRad9, hHus1, and hRad1 exist in a heterotrimeric complex. We also found that the PCNA-like portions of hHus1 (full-length), hRad1 (full-length), and hRad9 (amino acids 1–270) do not self-multimerize. Additionally, we reconstituted the 9-1-1 complex in an insect cell expression system and demonstrated that it had identical chromatographic behavior. Finally, our mapping studies demonstrated that each member of the complex interacted in a pair-wise fashion, and these studies also mapped the interactions among the three 9-1-1 complex members. Collectively, these results provide novel insights into the assembly, structure, and potential functions of the 9-1-1 DNA damage-responsive checkpoint complex.

The self-oligomerization analysis suggests that hRad9, hHus1, and hRad1 do not homomultimerize. This is in contrast to a study by Hang and Lieberman (35), which demonstrated that hHus1 and hRad9 self-oligomerize. One possible explanation for this discrepancy stems from our observation that full-length hRad9 forms large, higher-order aggregates when overexpressed without its partners hRad1 and hHus1. These higher-order aggregates may be physiologically relevant. However, analysis of endogenous hRad9, hHus1, and hRad1 (Fig. 1) did not reveal such complexes. Thus, we propose that hRad9, hHus1, and hRad1 form a heterotrimeric 9-1-1 complex that does not include self-multimers of any individual component.

Examination of the interactions within the 9-1-1 complex revealed that each member interacted in a pair-wise manner. We also mapped the pair-wise interactions using amino- and carboxy-terminal expression constructs. These results showed that: 1) the amino terminus of hRad9 interacted selectively with hRad1, 2) the amino terminus of hRad1 interacted with hHus1, and 3) the amino terminus of hHus1 interacted with hRad9. Further mapping, using the carboxy-terminal fragment of hRad9 predicted PCNA fold, demonstrated that this region associated with the amino terminus of hHus1. Thus, the PCNA-like portion of hRad9 is flanked by hRad1 on its amino terminus and by hHus1 on its carboxy terminus. Although we were unable to fully map the interactions among the subunits, our studies support a PCNA-like clamp model (Fig. 8) and define the orientation of the three proteins in the complex. It is

---

2 M. A. Burtelow and L. M. Karnitz, unpublished data.
Reconstitution of the hRad9-hHus1-hRad1 Complex

important to bear in mind, however, that confirmation of the structure awaits biophysical analysis of purified 9-1-1 complex. Studies in *S. pombe* demonstrated that Myc-tagged spHus1 eluted as a complex with an apparent molecular mass of 450 kDa (27). Moreover, only very small fractions of the total spRad9 and spRad1 pools were associated with the tagged spHus1. These results suggest that either the complex is very unstable or that the proteins do not form a clamp-like structure. In contrast, we found that all the hHus1 and hRad9, and about one-half of the hRad1 were assembled into a stable, discrete complex in human cell lysates, indicating that the complex was the favored form in cell lysates and presumably in intact cells. Such results suggest that the complexes may vary significantly between yeast and mammals. Alternatively, the addition of 13 Myc tags to spHus1, even though the tagged protein complemented a *sphus1* null mutant, may dramatically affect the stability of protein-protein interactions with spHus1.

The present studies also differ from the studies in yeast with respect to interactions between individual complex members. Two studies have shown that *S. pombe* spHus1 and spRad1 do not co-immunoprecipitate in the absence of spRad9 (6, 10), suggesting that spRad9 links spRad1 and spHus1. This result differs from our data and is difficult to reconcile with the clamp model. We found that each protein interacted efficiently in pair-wise reconstitution experiments, which is consistent with a clamp model. The reasons for the discrepancies between the yeast and human studies are not known. The two organisms may differentially regulate complex assembly. Alternatively, the discrepancy may stem from dissimilar technical approaches used to study yeast and mammalian cells. For example, the harsher conditions required to disrupt yeast cells may also destabilize the interactions among the 9-1-1 complex members, especially if one complex member is missing as is the case in the studies with the spRad9 mutant.

The results presented here may also provide insight into a disparity between the clamp model and genetic studies in *S. pombe*. Although the PCNA-like clamp model for the 9-1-1 complex is compelling, it does not account for all the available data. A previous analysis of telomere maintenance in *S. pombe* revealed that *spraak1* null mutants had telomere-shortening defects, whereas *sphus1* null and *sprad9*-192 (null for all tested phenotypes) mutants had normal telomeric lengths (38). These observations are not compatible with a clamp composed of three different subunits; if any one subunit were missing, the clamp would no longer be able to encircle DNA. Thus, null mutants of *sprad1* and *sphus1* should have identical phenotypes. A possible explanation for this discordance is that the functions of these proteins may have diverged. Evidence for this possibility includes the observation that cells derived from *mHus1* knockout mice are sensitive to ultraviolet but not ionizing radiation (40). In contrast, *sphus1* null *S. pombe* are sensitive to both forms of radiation, suggesting that mammals may have evolved additional mechanisms to respond to DNA damage. An alternative explanation is that the monomeric hRad1 that we observed in human cell lysates might have functions independent of a 9-1-1 clamp. For instance, spRad1 and the related *Ustilago maydis* Rec1 checkpoint protein possess 3'-5' exonuclease activity (11, 41). Thus, hRad1 may have dual biochemical functions; in a complex with hRad9 and hHus1 it might be part of a clamp, and as a monomer it may operate as an exonuclease.

On the basis of the genetic, biochemical, and structural prediction data available for Rad9, Hus1, Rad1, and Rad17, the following model has arisen. Rad17, in complex with the small RFC subunits, may recognize directly or indirectly DNA damage. The Rad17-RFC clamp loader will then open and load the 9-1-1 clamp onto the DNA. Once loaded, the 9-1-1 clamp becomes resistant to extraction, and by analogy with PCNA, tethers proteins to sites of DNA damage. Once clamped onto the DNA, the function of the 9-1-1 clamp may be to “mark” DNA lesions, thereby recruiting repair machinery and downstream signaling components that activate the checkpoint signal. The studies presented here provide compelling evidence that hRad9, hHus1, and hRad1 form a clamp-like structure, providing further impetus to test the predicted clamp model.

Acknowledgments—We thank Scott Kaufmann, Junjie Chen, and Jann Sarkaria for thoughtful discussions and critical evaluations of the manuscript.

REFERENCES

1. Dasika, G. K., Lin, S.-C. J., Zhao, S., Sung, P., Tomkinson, A., and Lee, E.-Y.-H. P. (1999) Oncogene 18, 7883–7899

2. Paulovich, A. G., Toczyski, D. P., and Hartwell, L. H. (1997) Cell 88, 315–321

3. Weinert, T. (1998) Cell 94, 555–558

4. Longhese, M. P., Foa, M., Mazi-Falconi, M., Lucchini, G., and Plevani, P. (1998) EMBO J. 17, 5552–5562

5. Lowndes, N. F., and Murgia, R. J. (2000) Curr. Opin. Genet. Dev. 10, 17–25

6. Casparsi, T., and Carr, A. M. (1999) Biochimie (Paris) 81, 173–181

7. Bluyssen, H., Raay, van Os, J., Naz, H., Racis, I., Hoeijmakers, J. H. J., and de Klein, A. (1998) Genes Dev. 12, 2560–2573

8. Udelli, C. M., Lee, S. K., and Davey, S. (1998) Nucleic Acids Res. 26, 3971–3976

9. Lieberman, B. H., Hopkins, K. M., Nuss, M., Demetrick, D., and Davey, S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 13890–13895

10. Kostub, C. F., Knudsen, K., Subramani, S., and Enoch, T. (1998) EMBO J. 17, 2055–2066

11. Parker, A. E., Van de Weyer, I., Labs, M. C., Oostveen, I., Yon, J., Verhasselt, D. A., Smith, S., Uziel, T., and Sfez, S., et al. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1233–1243

12. Savitsky, K., Bar-Shira, A., Gilad, S., Rotman, G., Ziv, Y., Vanagaite, L., Tagle, D. A., Smith, S., Uziel, T., and Sfez, S., et al. (1995) Science 268, 1749–1753

13. Bao, S., Chang, M.-S., Auclair, D., Sun, Y., Wang, Y., Kong, X. P., Gary, S., Burgers, P. M., and Kuriyan, J. (1994) Science 269, 4047–4054

14. Dean, F. B., Lian, L., and O'Donnell, M. (1998) Genes Dev. 12, 2560–2573

15. Lieberman, B. H., Hopkins, K. M., Nuss, M., Demetrick, D., and Davey, S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 13890–13895

16. Thelen, M. P., Onel, K., and Holloman, W. K. (1994) J. Biol. Chem. 269, 747–754

17. Thelen, M. P., Onel, K., and Holloman, W. K. (1994) J. Biol. Chem. 269, 747–754
Reconstitution and Molecular Analysis of the hRad9-hHus1-hRad1 (9-1-1) DNA Damage Responsive Checkpoint Complex
Matthew A. Burtelow, Pia M. K. Roos-Mattjus, Matthew Rauen, Jeremy R. Babendure and Larry M. Karnitz

J. Biol. Chem. 2001, 276:25903-25909.
doi: 10.1074/jbc.M102946200 originally published online May 4, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M102946200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 41 references, 21 of which can be accessed free at http://www.jbc.org/content/276/28/25903.full.html#ref-list-1