Human Homologs of Schizosaccharomyces pombe Rad1, Hus1, and Rad9 Form a DNA Damage-responsive Protein Complex*

(Received for publication, October 15, 1998)

Elias Volkmer and Larry M. Karnitz‡
From the Division of Radiation Oncology and Department of Immunology, Mayo Foundation, Rochester, Minnesota 55905

DNA damage activates cell cycle checkpoints in yeast and human cells. In the yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe checkpoint-deficient mutants have been characterized, and the corresponding genes have been cloned. Searches for human homologs of S. pombe rad1, rad9, and hus1 genes identified the potential human homologs hRad1, hRad9, and hHus1; however, little is known about the roles of these proteins in human cells. The present studies demonstrate that hRad1 and hHus1 associate in a complex that interacts with a highly modified form of hRad9, but hHus1 and hRad1 do not associate with hRad17. In addition to being a key participant in complex formation, hRad9 is phosphorylated in response to DNA damage. Together, these results suggest that hRad9, hRad1, and hHus1 are central components of a DNA damage-responsive protein complex in human cells.

DNA damage triggers a variety of cellular responses in eukaryotic cells, including the induction of a regulatory signaling network that activates checkpoint controls (1–3). Checkpoint activation transiently blocks cell cycle progression by arresting cells in G2 and G2/M and slowing progression through S phase. Genetic studies in the yeast Saccharomyces cerevisiae and Schizosaccharomyces pombe have identified many of the relevant players in DNA damage-induced checkpoint activation (4, 5). A common theme that emerges from these studies is that checkpoint-deficient yeast are dramatically more sensitive to genotoxins than their wild-type counterparts (1–3), suggesting that the checkpoint proteins play critical roles in cellular responses to DNA damage.

Recent studies suggest that key checkpoint regulators may be conserved between yeast and humans. Cloning of the human gene mutated in AT (ATM) revealed that the ATM gene exhibited significant homology with the S. pombe rad3 (sprad3) and S. cerevisiae MECl (scMECl) checkpoint genes (6). The corresponding proteins scMECl, spRAD3, and ATM are protein kinas

---

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Mayo Foundation, 200 First Street Southwest, Oncology Research, 13 Guggenheim, Rochester, MN 55905. Tel.: 507-284-3124; E-mail: karantz.larry@mayo.edu.
expression vector was prepared by amplifying hHus1 using primers that added a tandem HA epitope tag to the carboxyl terminus of hHus1. The PCR-derived DNA fragment was cloned into pEF-BOS-ARI (19) to yield pEF-BOS-ARI-hHus1-HA."  

Antibodies—Bacterial expression vectors for hexahistidine-tagged hRad1, hHus1, and hRad17 were generated by cloning PCR-amplified DNA fragments into pET24a+ (Novagen, Madison, WI). Histidine-tagged proteins were induced and purified according to manufacturer’s instructions. The hRad9 DNA was cloned into pGEX-KG (20) to generate an in-frame fusion with glutathione S-transferase. Bacterially produced GST-hRad9 was purified by affinity chromatography on glutathione agarose. The purified proteins were used to immunize rabbits with standard procedures. The anti-HA and anti-AU1 mAbs were from Babco (Berkeley, CA), and the anti-FLAG mAb was from Eastman Kodak Co.

Coimmunoprecipitation Experiments—Exponentially growing K562 cells (1 × 10⁷ per sample) were either transfected as indicated above or used directly for immunoprecipitation studies. Cells were washed in phosphate-buffered saline, and lysed in lysis buffer (50 mM HEPES, 1% Triton X-100, 10 mM NaF, 30 mM Na₃P₂O₇, 150 mM NaCl, 1 mM EDTA, 0.5 mM microcystin-LR). The cell lysates were immunoprecipitated with the indicated mouse monoclonal antibodies and protein G-Sepharose (Sigma) or rabbit antisera and protein A-Sepharose (Sigma) for 1 h. The immunoprecipitates were washed three times with lysis buffer and fractionated by SDS-PAGE (10% gel). The gels were transferred to Immobilon-P (Millipore, Bedford, MA) and immunoblotted. All membranes were developed with SuperSignal chemiluminescent substrate (Pierce). Membranes that were sequentially blotted were stripped with two 30-min washes of 8 M guanidine hydrochloride prior to blocking and immunoblotting.

**RESULTS**

**hRad1, hRad9, and hHus1 Interact.** Cells were transfected with 40 µg of pCDNA3 empty vector only (lanes 1, 2, and 4), with 5 µg of pEF-BOS-ΔRI-hHus1-HA, or 5 µg of pCDNA3-FLAG-hRad1 expression vectors (lane 3), with 20 µg AU1-hRad9 alone (lane 5 ) or with these amounts of all three checkpoint expression vectors (lane 6). The following day, cell lysates were immunoprecipitated with either preimmune serum (PI, lane 1), anti-hRad9 (lanes 2 and 3), or anti-AU1 mAb (lanes 4–6). Immunoprecipitations (IP) were immunoblotted with anti-hRad9. The arrows indicate apparent molecular masses of AU1-hRad9 calculated using commercially available protein standards.

**RESULTS**

**hRad1, hRad9, and hHus1 Associate in a Checkpoint Complex—Comparisons of the predicted human and yeast protein sequences indicate that the human proteins are 25–30% identical and 53–57% similar to their respective homologs, with homologies extending over extensive portions of each protein (12–18). In S. pombe, spRad1 and spHus1 associate in wild-type, but not rad9, mutant yeast. One interpretation of this result is that spRad9 may physically link spRad1 to spHus1, although this hypothesis has not been validated experimentally (18). To address whether conservation extends to a functional level, we examined the ability of the human homologs to form biochemical complexes similar to those reported in yeast.

hHus1 immunoprecipitates (Fig. 1A, upper left panel) contained a 34-kDa protein, which is similar in size to hHus1’s form biochemical complexes similar to those reported in yeast.
predicted mass of 32 kDa. The hHus1 immunoprecipitates also contained an anti-hRad1-reactive 33-kDa band (Fig. 1A, middle left panel), which comigrated with immunoprecipitated hRad1 (Fig. 1A, middle right panel), demonstrating that hRad1 associated with hHus1. Immunoblotting of the hHus1 immunoprecipitate with an anti-hRad9 antiserum revealed a 70-kDa band (Fig. 1A). Samples were processed as in A. C, K562 cells were treated with nothing or 50 Gy IR and cultured for 5 h. hRad9 immunoprecipitates were washed with lysis buffer, followed by phosphatase buffer, with nothing or 50 Gy IR and cultured for 5 h. hRad9 immunoprecipitates were washed with lysis buffer, followed by phosphatase buffer, and treated with nothing or with 0.25 unit of calf intestinal alkaline phosphatase (CIAP), in the absence and presence of 50 mM β-glycerophosphate (β-GP). The immunoprecipitates were washed once with lysis buffer and immunoblotted with anti-hRad9.

To further verify that these proteins associate, we immunoprecipitated hRad9. In these precipitates we readily observed a hHus1-reactive band (Fig. 1A, upper center panel) that comigrated with immunoprecipitated hHus1 (Fig. 1A, upper left panel). We also immunoprecipitated hRad1 and demonstrated that hHus1 (Fig. 1A, upper right panel) and hRad9 (Fig. 1A, lower right panel) were present in anti-hRad1 immunoprecipitates. However, we did not find hRad1 in anti-hRad9 immunoprecipitates (Fig. 1A, middle center panel), even though we did observe hRad9 in anti-hRad1 immunoprecipitates (Fig. 1A, lower right panel). One explanation for these discrepant results is that hRad1 and the immunodominant anti-hRad9 antibodies share an overlapping binding site, which precludes simultaneous interaction. In support of this, we demonstrated that anti-epitope immunoprecipitates of AU1-tagged hRad9 contain hRad1. These results suggest that this group of human checkpoint proteins assembles into a multimolecular complex even in the absence of genotoxic stimuli.

hRad1 and hHus1 Do Not Interact with hRad17—Epistasis studies identify genetic interactions and are frequently indicative of biochemical interactions as well. Studies in S. cerevisiae have shown genetic and biochemical interactions among the members of the scRAD24 epistasis group (21, 22), which includes S. cerevisiae homologs for HRAD1 (scRAD17), HRAD17 (scRAD24), and HRAD9 (partially homologous to scDDC1). Additionally, previous work demonstrated that hRad17 and hRad1 interacted in a two-hybrid system (16). Therefore, we addressed the possibility that hRad1 and hHus1 might interact with the checkpoint protein hRad17. We immunoprecipitated hHus1 and hRad17 (Fig. 1B, left panel) and hRad1 and hRad17 (Fig. 1B, right panel). The immunoprecipitates were immunoblotted first with anti-hHus1 (Fig. 1B, lower left panel) or anti-hRad1 (Fig. 1B, lower right panel), followed by anti-hRad17 (Fig. 1B, upper panels). We found no interactions between hRad17 and hRad1 or hHus1. Thus, these results suggest that the human checkpoint complexes may be differentially assembled in undamaged cells. Alternatively, the interactions may be transient and not detectable under our experimental conditions.

hRad9 Undergoes Complex Post-Translational Modifications—To generate a system amenable to further biochemical analysis, we prepared epitope-tagged expression vectors for hRad1, hHus1, and hRad9. When analyzed by SDS-PAGE (Fig. 1), hRad9 migrated with an apparent molecular mass (70 kDa) that was much larger than predicted (45 kDa), suggesting that the cellular pool of hRad9 may undergo extensive post-translational modifications (Fig. 2, lanes 2 and 3). Consistent with this observation, overexpression of AU1-tagged hRad9 revealed multiple species when resolved by SDS-PAGE (Fig. 2, lane 5). The major detectable band had an apparent molecular mass of 55 kDa. This is significantly smaller than the 70-kDa endogenous hRad9 (Fig. 2, lanes 2 and 3), but still larger than the predicted molecular mass (45 kDa), even when the 2-kDa epitope tag is taken into account. Thus, the major 55-kDa band may be an unmodified form that migrates anomalously or a partially modified version. In addition to the 55-kDa form, multiple slower migrating bands were present above this band, suggesting several steps of post-translational modification (Fig. 2, lane 5). Remarkably, coexpression of hRad9 with hHus1 and hRad1 increased the amount of a highly modified, 72-kDa form of hRad9 (Fig. 2, lane 6). This slow-migrating form of hRad9 had an apparent molecular mass slightly greater than endogenous hRad9, which is due to the addition of the tandem AU1 tag on hRad9.

Epitope-tagged Checkpoint Proteins Associate in a Modification-dependent Manner—To test whether the epitope-tagged checkpoint proteins recapitulate complex formation, K562 cells were transfected with empty vector, a combination of hHus1 and hRad1, hRad9 alone, or expression vectors for all three proteins. The anti-hRad9 immunoblots of cell lysates (Fig. 3, lanes 1–4) revealed that epitope-tagged hRad9 was highly overexpressed compared with the 70-kDa endogenous hRad9 (Fig. 3C, lanes 3 and 4), which was not visible on this exposure. Consistent with the results presented in Fig. 2, there were multiple forms of AU1-tagged hRad9, and coexpression of hRad1 and hHus1 enhanced the accumulation of the highly modified form (Fig. 3C, lane 15 versus lane 16).

To assess associations among the transfected proteins, we immunoprecipitated hRad1 (Fig. 3, lanes 5–8) and found epitope-tagged hRad1 (Fig. 3A), hHus1 (Fig. 3B), and both endogenous hRad9 (Fig. 3C, lane 6) and epitope-tagged hRad9 (Fig. 3C, lane 8) in these immunoprecipitates. We also immunoprecipitated with anti-HA (Fig. 3, lanes 9–12) and looked for associated hRad1 (Fig. 3A), hHus1 (Fig. 3B), and hRad9 (Fig. 3C). Again, as in the hRad1 (anti-FLAG) immunoprecipitates, endogenous and AU1-tagged hRad9 were present in the complex. Strikingly, in both the hRad1 and hHus1 precipitations, only the most highly modified form of transfected hRad9 asso-
cated with these proteins, suggesting that the modification is essential for interaction.

In the reciprocal experiment (Fig. 3, lanes 13–16) we observed that hRad1 (Fig. 3A) and hHus1 (Fig. 3B) coprecipitated with hRad9. Unlike our observations with the endogenous proteins, hRad1 (Fig. 3A) was detected readily in the anti-AU1-hRad9 immunoprecipitations, thus suggesting strongly that the rabbit anti-hRad9 antiserum indeed masks or disrupts the hRad1 interaction (see Fig. 1). Taken together, these results revealed that the human checkpoint proteins hRad1 and hHus1 associated selectively with modified hRad9 in a protein assembly that mimics the endogenous complex.

hRad9 Is Phosphorylated in Response to DNA Damage—
sCdC1 is a putative S. cerevisiae homolog of spRad9 and hRad9. Because scDcd1 is phosphorylated in response to DNA damage (22), we explored the possibility that hRad9 may also be phosphorylated in response to genotoxins. It is important to note that endogenous hRad9 (although highly modified) migrates as a single band when isolated from undamaged cells. However, we noticed that the single endogenous hRad9 band (70-kDa form) exhibited a progressively greater reduction in electrophoretic mobility when isolated from cells irradiated with increasing doses of IR (Fig. 4A). We also explored the time course of the mobility shift, which showed that hRad9 was modified within 30 min, was maximal at 2 h, and persisted for at least 6 h (Fig. 4B).

To determine whether the mobility shift reflected DNA damage-induced phosphorylation, we treated hRad9 immunoprecipitates isolated from irradiated cells with calf intestinal phosphatase (Fig. 4C). As expected for phosphorylation, the DNA damage-induced mobility shift was readily reversed by treatment with the phosphatase. Moreover, the phosphatase inhibitor β-glycerophosphate blocked the effects of the phosphatase, suggesting that effects of the phosphatase preparation are not the result of contaminating activities.

DISCUSSION

The present results demonstrate that hRad9 undergoes extensive and quantitative modification even in the absence of exogenous genotoxic stimuli. This modification is significant, because it is crucial for hRad9 to interact with the checkpoint proteins hRad1 and hHus1. However, there is an intricate interplay between hRad9 modification and complex formation. Overexpression of hRad1 and hHus1 promote the appearance of fully modified hRad9. One possible explanation for this result is that hRad1 and hHus1 are required for hRad9 modification. Another, perhaps more plausible explanation is that hRad9 is not stable when overexpressed singly, but once modified it associates with hRad1 and hHus1 and forms a stable multimolecular complex.

In addition to the modification required for hHus1 and hHus1 interactions, hRad9 is phosphorylated in response to DNA damage, like its S. cerevisiae homolog scDcd1 (22). Phosphorylation of scDcd1 requires the Atm homolog scMec1 (23). However, in SV40-transformed AT fibroblasts, we observed DNA damage-induced hRad9 phosphorylation (data not shown), suggesting that other PIKKs, possibly Atm, may be key mediators of this signaling pathway. Although we do not yet know the significance of hRad9 phosphorylation, it has no effect on association with hRad1 or hHus1 (data not shown), suggesting that phosphorylation regulates interactions with other members of the checkpoint signaling cascade.

There is precedent for other checkpoint proteins undergoing extensive modifications that dramatically alter their apparent molecular mass and regulate their interactions with other proteins. The unrelated scRad9 is phosphorylated in response to DNA damage, and this modification is essential for scRad9's interaction with the checkpoint protein kinase scRad53 (24). There are several potential molecular modifications that may contribute to hRad9's mobility shift, including phosphorylation that is resistant to phosphatase treatment, possibly due to protection by hHus1 and hRad1 interaction. Although the nature of the hRad9 modification is currently unknown, we are intensively investigating these and other possible molecular alterations of the protein.

Previous studies in yeast demonstrated that hRad1 and hHus1 genetically and biochemically interact and are required for activation of checkpoints in response to DNA damage and replication inhibitors (18). Although much genetic evidence attests to their importance in cell cycle arrest and survival, even in the well studied yeast models little is known about their functions. To add further complexity, spRad9, hRad9, hHus1, and hHus1 have no signature sequences or homologies with other proteins that yield clues to their functions. However, spRad1 and hRad1 have significant homology with Ustilago maydis Rec1 exonuclease (12–14, 25). Additionally, hRad1 may possess 3′ → 5′ exonuclease activity (13), although other groups could not confirm this hypothesis (12). The presence of a putative DNA-metabolizing protein in the multimolecular checkpoint complex, coupled with genetic data that place spRad1, spHus1, and spRad9, and their S. cerevisiae counterparts, early in the response pathway (2, 3, 21), suggests that the complex may function as a sensor that scans the genome for damaged DNA. Once damaged DNA is detected, this complex may initiate endonucleolytic processing of the lesions and trigger interactions with downstream signaling elements. Alternatively, the checkpoint complex may link unknown damage recognition components to downstream signal-transducing pathways that include ATM and hChk1, both of which are implicated in actively enforcing cell cycle arrest after DNA damage. Thus, the present data provide the first identification of a DNA damage-responsive human checkpoint complex that is fundamentally conserved between yeast and humans.

REFERENCES

1. Elledge, S. J. (1996) Science 274, 1664–1672
2. Weinert, T. (1998) Cell 94, 555–558
3. Longhese, M. P., Paciotti, V., Fraschini, R., Zaccarini, R., Plevani, P., and Luyten, W. H. M. L. (1998) EMBO J. 17, 5555–5560
4. Carr, A. M. (1994) Int. J. Radiat. Biol. 66, S133–S139
5. Paulovich, A. G., Toczycki, D. P., and Hartwell, L. H. (1997) Cell 88, 315–321
6. Savitsky, K., Bar-Shira, A., Gilad, S., Rotman, G., Ziv, Y., Yanai, A., Tagle, D. A., Smith, S., Uziel, T., Sefz, S., et al. (1995) Science 268, 1749–1753
7. Zakian, V. A. (1995) Cell 82, 685–687
8. Weinert, T. (1997) Science 277, 1450–1451
9. Benham, S., Moral, L., Shieh, S. Y., Taya, Y., Anderson, C. W., Chessa, L., Smorodinsky, N. I., Prives, C., Beizas, Y., Shiloh, Y., and Ziv, Y. (1998) Science 281, 1674–1677
10. Cann, C. E., Lin, D.-S., Cimprich, K. A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M. B., and Siliciano, J. D. (1998) Science 281, 1677–1679
11. Walworth, N. C., and Bernards, R. (1996) Science 271, 353–356
12. Freire, R., Murguia, J. R., Tarsonas, M., Lowndes, N. F., Moens, P. B., and Appella, E., Kastan, M. B., and Siliciano, J. D. (1998) Science 281, 1677–1679
13. Parker, A. E., Van de Weyer, I., Laus, M. C., Oostveen, I., Yon, J., Verhasselt, P., and Luyten, W. H. M. L. (1998) J. Biol. Chem. 273, 18332–18339
14. Uziel, T., Sefz, S., Uziel, T., Sefz, S., et al. (1995) Science 268, 1749–1753
15. Lieberman, B. H., Hopkins, K. M., Nuss, M., Demetrich, D., and Davey, S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 13890–13895
16. Parker, A. E., Van de Weyer, I., Laus, M. C., Verhasselt, P., and Luyten, W. H. M. L. (1998) J. Biol. Chem. 273, 18340–18346
17. Sanchez, Y., Wong, C., Thoma, R. S., Richman, R., Wu, Z., Piwnica-Worms, H., and Elledge, S. J. (1997) Science 277, 1497–1501
18. Kostrub, C. F., Krudn, K., Subramani, S., and Enoch, T. (1998) EMBO J. 17, 2055–2066
19. Mizushima, S., and Nagata, S. (1990) Nucleic Acids Res. 18, 5322
20. Guan, K. L., and Dixon, J. E. (1994) Annu. Rev. Biochem. 63, 262–267
21. Lydall, D., and Weinert, T. (1995) Science 270, 1488–1491
22. Longhese, M. P., Paciotti, V., Fraschini, R., Zaccarini, R., Plevani, P., and Luyten, W. H. M. L. (1998) EMBO J. 17, 4199–4209
23. Sun, Z., Hsiao, J., Fay, D. S., and Stern, D. F. (1998) Science 281, 272–274
24. Long, K. E., Suennerhagen, P., and Subramani, S. (1994) Gene (Amst.) 148, 155–159