A Role of the C-terminal Region of Human Rad9 (hRad9) in Nuclear Transport of the hRad9 Checkpoint Complex*

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Received for publication, March 29, 2002
Published, JBC Papers in Press, May 6, 2002, DOI 10.1074/jbc.M203079200

Rad9, Rad1, and Hus1 are members of the Rad family of checkpoint proteins that are required for both DNA replication and DNA damage checkpoints and are thought to function as sensors in the DNA integrity checkpoint control. These proteins can interact with each other and form a stable proliferating cell nuclear antigen-related Rad9/Rad1/Hus1 heterotrimeric complex that might encircle DNA at or near the damaged sites. In this study, we demonstrate that the human Rad9 (hRad9) protein contains a predicted nuclear localization sequence (NLS) near its C terminus, which plays an essential role in the hRad9-mediated G2 checkpoint. Deletion experiments indicate that the NLS-containing region of hRad9 is critical for the nuclear transport of not only hRad9 but also human Rad1 (hRad1) and human Hus1 (hHus1), although this region is not required for hRad9-hRad1-hHus1 complex formation. In support of the role that hRad9 NLS plays in the nuclear targeting of the hRad9-hRad1-hHus1 complex, overexpression of a deletion mutant of hRad9 lacking the NLS-containing C-terminal region can bypass the G2 checkpoint and result in cell death after ionizing radiation or hydroxyurea treatment. Moreover, knockdown of hRad9 expression by small interfering RNA (siRNA) results in hRad1 accumulation in the cytoplasm and significantly abrogates the G2 checkpoint in the presence of damaged DNA or incomplete DNA replication. Thus, the C-terminal region of human Rad9 protein is important for G2 checkpoint control by operating the transport of the hRad9-hRad1-hHus1 checkpoint complex into the nucleus.

Cell cycle checkpoints are surveillance mechanisms that monitor the structure of the chromosomes and coordinate cell cycle progression with DNA repair to ensure the distribution of accurate copies of the genome to daughter cells (1). If left unrepaired, it can lead to genomic instability, a major contributory factor in the development of cancer and other genetic diseases. To maintain the genomic integrity, multicellular organisms also have the option to eliminate mutated or damaged cells by initiating programmed cell death or apoptosis. Indeed, many of the genes required for cell cycle control and DNA repair are also required for induction of apoptosis in response to DNA damage (2, 3).

In fission yeast Schizosaccharomyces pombe, genetic studies demonstrated that members of the Rad family of proteins including Rad1, Rad3, Rad9, Rad17, Rad26, and Hus1 are required for cell cycle arrest in response to DNA damage or inhibition of DNA synthesis (4–6). The related proteins in human have been identified (i.e. hRad1, ATMS/ATR, hRad9, hRad17, ATTRIP, and hHus1) (7–13), suggesting that the checkpoint signaling cascade controlled by these proteins is evolutionarily conserved.

Rad3 and its human homologues, ATR and ATM, are members of the phosphatidylinositol 3-kinase-related protein family that play critical roles in early signal transduction through cell cycle checkpoints (1). These kinases are likely to be activated in response to checkpoint signals and phosphorylate downstream targets such as Chk1 and Chk2 in order to coordinate cell cycle progression and DNA repair (1, 4). The activated Chk1 or Chk2, in turn, phosphorylates the mitosis-promoting phosphatase Cdc25, which results in the cytoplasmic accumulation and/or catalytic inhibition of Cdc25, thereby preventing activation of the mitotic cyclin B-cdc2 complex and inducing G2 arrest of the cell cycle (1). In fission yeast, the phosphorylation of Chk1 by Rad3 requires Hus1, Rad1, Rad9, Rad17, and Rad26 (14), suggesting that this group of proteins could function as sensors of DNA damage.

Interestingly, molecular modeling analysis has revealed that Rad9, Rad1, and Hus1 as well as their human homologues share regions of sequence similarity to the proliferating cell nuclear antigen (PCNA) (15–18). PCNA is an essential factor that functions in eukaryotic chromosomal DNA replication machinery and plays a critical role in DNA recombination, repair, and several other cellular processes (19–21). The functional complex consists of three PCNA molecules that associate in a head-to-tail manner, thus forming a ring-shaped complex that encircles DNA and functions as a DNA sliding clamp for the replicative DNA polymerases (22, 23). Indeed, evidence has accumulated that Rad9, Rad1, and Hus1 as well as their mammalian homologues can associate with each other and form a stable heterotrimeric complex in intact cells (16, 24, 25). Based on the PCNA model, the Rad9/Rad1/Hus1 complex has been proposed to function as a checkpoint sliding clamp that encircles DNA for the recruitment of the checkpoint signaling machinery to the sites of lesions or stalled replication forks (14). It

* This work was supported by grants CA90315 and CA82197 from the National Institutes of Health. 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.

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1 The abbreviations used are: hRad1, human Rad1; hRad9, human Rad9; hHus1, human Hus1; PCNA, proliferating cell nuclear antigen; HU, hydroxyurea; IR, ionizing radiation; DAPI, 4′,6-diamidino-2-phenylindole; NLS, nuclear localization sequence; PLD, PCNA-like domain; CD, C-terminal domain; GFP, green fluorescent protein; HA, hemagglutinin; siRNA, small interfering RNA.
has recently been shown that Ddc1, the Saccharomyces cerevisiae homologue of Rad9, associates with DNA at the site of double-strands breaks in budding yeast (26, 27). The analogy between PCNA and the Rad9-Rad1-Hus1 complex is extended further by the finding that Rad17 shows structural and functional similarity to the replication factor C subunits (18), a clamp loading complex that controls the loading of the PCNA clamp onto DNA (21). It has been postulated that Rad17 functions as a putative clamp loading complex to recruit the Rad9-Rad1-Hus1 complex and load it onto the damaged DNA (1, 14). In support of this sliding clamp model, Rad17 and its homologues in other organisms have been shown to form a stable complex with four small subunits of replication factor C but do not interact with the large subunit, Rfc1 (14, 28, 29).

Mutagenesis studies indicated that hRad9, hRad1, and hHus1 form a heterotrimeric complex (9-1-1) in a head-to-tail manner similar to those observed in the PCNA homotrimer (30). As predicted by the information from the PCNA alignment, the C-terminal region of hRad9 that does not align with PCNA is not required for interactions with hRad1 and hHus1 (30). In this work, we provide evidence that the C-terminal region of hRad9 plays an essential role in the nuclear localization of the hRad9-hRad1-hHus1 complex and the G2 checkpoint control after DNA damage.

**EXPERIMENTAL PROCEDURES**

**Expression Plasmids**—The pCMV2-FLAG-hRad9 and pEGFP-C2-hHus1 plasmids were described previously (31). The DNA fragments encoding amino acids 1–267 and 261–391 of hRad9 protein, designated as PLD and CD, respectively, were amplified by PCR using hrad9 cDNA as template. The amplified hrad9 DNA fragments were sequenced and subcloned into pCMV2-FLAG, pCDNA3-Myc, or pEGFP-C2 (CLONTECH) vector. To construct pFLAG-hRad9 (ANLS) and pEGFP-hRad9 (ANLS) expression plasmids, hrad9 cDNA (3) was digested with EcoRI and BplI, and the DNA fragment encoding amino acids 1–347 of the hRad9 protein was subcloned into pCMV2-FLAG and pEGFP-C2 vectors. The pcDNA3-HA-hRad1, pcDNA3-Myc-hRad1, and pcDNA3-Myc-hHus1 plasmids were constructed by subcloning hRad1 or hHus1 cDNA (31) into pcDNA3-HA or pcDNA3-Myc vector.

**Cell Cultures**—HeLa and MDA-MB-468 cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 50 units/ml penicillin G, and 50 \( \mu \)g/ml streptomycin. 293 cells and 293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 50 units/ml penicillin G, and 50 \( \mu \)g/ml streptomycin.

**Plasmid DNA Transfections**—293T cells were transfected by a calcium phosphate precipitation method. Ordinarily, a total of 10–15 \( \mu \)g of plasmid DNA was used for transfection of 1–2 \( \times \) 10\(^6\) cells, and cells were collected at 2 days of transfection and applied to experiments. HeLa cells and 293 cells were transfected with LipofectAMINE 2000 reagent (Invitrogen) or TransIT-LT1 reagent (Mirus) according to the respective manufacturer’s recommendations.

**Transfection of Mammalian Cells with siRNA Duplexes**—The 21-nucleotide siRNA duplexes were synthesized and purified by Dharmacon Research (Boulder, CO). The siRNA sequence targeting hRad9 (GenBank accession number U53174) corresponded to the coding region 232–252 (5'-AAGUCUUCUCUGCUUCUCU-3') relative to the first nucleotide of the start codon. The siRNA sequence targeting GFP (GenBank accession number U57606) was from position 322–342 (5'-AAAGACCGCCGCGAAGUGAAG-3') after the start codon. The transfection of siRNA duplexes was performed one to three times over a 3-day period with the manufacturer’s protocol for oligofectamine reagent (Invitrogen). Briefly, 3 \( \mu \)l of oligofectamine reagent was mixed with 12 \( \mu \)l of Opti-MEM (Invitrogen) at room temperature for 7–10 min and then incubated with a mixture of 3 \( \mu \)l of 20 \( \mu \)l siRNA duplex and 50 \( \mu \)l of Opti-MEM for an additional 20–25 min at room temperature. After 32 \( \mu \)l of fresh Opti-MEM was added, 100 \( \mu \)l of siRNA-oligofectamine complexes was applied to each well of cultured cells at ~50% confluence in a 24-well plate.

**Apoptosis Assay and Mitotic Indices**—293 or HeLa cells were exposed to 20 Gy of ionizing radiation (IR) or incubated with medium containing 1 \( \mu \)M hydroxyurea. For determination of mitotic indices, cells were incubated with 400 ng/ml nocodazole for 20 h after 1 h of IR or 6 h of HU treatment. After fixation with 3.7% formaldehyde and staining with DAPI, the mitotic cells were visualized and counted using a fluorescence microscope. Cells with clear condensed chromatin and/or fragmented nuclei were determined to be apoptotic.

**Fluorescence-activated Cell Sorting Analysis**—MCF-7 cells were transfected with siRNA duplexes targeting hRad9 or were mock-transfected for 2 days, followed by irradiation with 40 Gy of IR, or left untreated. After 24 h, cells were fixed with 70% ethanol and incubated for 30 min with 100 \( \mu \)g/ml RNase A and 50 \( \mu \)g/ml propidium iodide at room temperature. DNA content analysis was accomplished by flow cytometry.

**Immunoblot, Immunoprecipitation, and Immunofluorescence Assays**—Immunoblot, immunoprecipitation, and immunofluorescence experiments were basically carried out as described previously (3, 32), with minor modification. For immunoblot and immunoprecipitation analysis, cell lysates were prepared in an appropriate volume of lysis buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM EDTA, 10% glycerol, and 0.5% Triton X-100) with protease inhibitors (32) and normalized for protein content. Briefly, immunoprecipitation was performed by incubating 300 \( \mu \)g of total cell lysate with 15 \( \mu \)l of protein G-agarose preadsorbed with anti-FLAG M2 monoclonal antibody or with protein G-agarose as a negative control for 3 h at 4 °C. After extensive washing in lysis buffer, beads were boiled in 30 \( \mu \)l of Laemmli buffer, and the eluted proteins were subjected to immunoblotting analysis with polyclonal antibodies specific for FLAG, HA, or Myc epitope. For immunofluorescence studies, cells were seeded into 24-well plates containing coverslips pretreated with collagen and cultured overnight, followed by transfection with either expression plasmid DNA or siRNA duplexes, as indicated. Cells were washed twice in phosphate-buffered saline, fixed in 3.7% formaldehyde for 30 min, and permeabilized in 0.5% Triton X-100 for 15 min. After preblocking with 5% bovine serum albumin/phosphate-buffered saline, cells were incubated with primary antibodies at 4 °C overnight, followed by three washes in phosphate-buffered saline and incubation with fluorescein isothiocyanate- or rhodamine-conjugated secondary antibodies (1:30; Dako) for 1 h at 37 °C. Cells were washed twice in phosphate-buffered saline before staining with DAPI for 10 min. After washing free of DAPI, nuclei were analyzed by fluorescence microscopy. The anti-hRad9 polyclonal antiserum was described previously (3). The anti-hRad1 polyclonal antibody was a kind gift from Dr. Larry M. Karnitz (25). The anti-FLAG M2 monoclonal antibody, anti-FLAG polyclonal antibody, anti-HA polyclonal antibody, anti-tubulin monoclonal antibody, and anti-FLAG M2-agarose beads were purchased from Sigma. The anti-FLAG

**FIG. 1.** The nuclear localization of hRad9 is dependent on the presence of its C-terminal domain. A, the structures of the human Rad9 and deletion mutant of Rad9 (PLD, CD, or NLS) proteins are indicated. B, HeLa cells were transiently transfected with 0.1 \( \mu \)g of plasmids encoding GFP-hRad9, GFP-PLD, GFP-CD, GFP-NLS, or control GFP by LipofectAMINE 2000 reagent. After 2 days of transfection, cells were fixed with 3.7% formaldehyde, stained with DAPI, and analyzed by fluorescence microscopy.
Myc polyclonal antibody was obtained from Santa Cruz Biotechnology, and the anti-Myc monoclonal antibody was produced by 9E10 hybridoma (American Type Culture Collection).

RESULTS

Human Rad9 Contains a NLS Near Its C Terminus—The hRad9 protein is 391 amino acids in length and shares a structural similarity to the PCNA (10, 18, 33). In addition, hRad9 also contains a Bcl-2 homology 3-like domain near its N terminus (amino acids 16–30) that is important for its interactions with Bcl-2/Bcl-xL during apoptosis (3, 34). However, the role of the PCNA-unrelated C-terminal region of hRad9, which contains appropriately 120 amino acids, has not been examined. Thus, we constructed deletion mutants encoding the PLD (amino acids 1–267) or the CD (amino acids 261–391) of the hRad9 protein (Fig. 1A). These hRad9 proteins were expressed as GFP fusion proteins in HeLa cells. As shown in Fig. 1B, the wild-type hrad9 protein was mainly localized in the intranuclear space, consistent with the previous studies (3, 24, 35). Surprisingly, nuclear-localized GFP signal was also observed in cells expressing CD fusion protein (Fig. 1B, d and n). In contrast, the C-terminal deletion mutant PLD was dominantly localized in the cytoplasm (Fig. 1B, c and m). These results suggested that the C-terminal region of hRad9 might contain a nuclear localization sequence(s). To explore this possibility, we performed a motif search against the hRad9 protein using the PSORT II computer prediction program (36). This search revealed that a potential NLS lies near the C terminus (amino acids 356–364) of the hRad9 protein (Fig. 1A). To confirm whether this region is required for the nuclear transport of hRad9, we expressed a mutant GFP-hRad9 (∆NLS) protein that lacks the C-terminal 44 amino acids of hRad9 (the region in which the predicted NLS resides) (Fig. 1A) and examined its intracellular localization in HeLa cells. As shown in Fig. 1B, the fluorescence resulting from the GFP-hRad9ΔNLS molecules was also dominantly detected in the cytoplasm, similar to that observed in GFP-PLD-expressing cells, indicating that the NLS-like region plays an essential role in the nuclear localization of the hRad9 protein.

The C-terminal Region of hRad9 Is Not Required for Its Associations with hRad1 and hHus1—Next, we performed co-immunoprecipitation experiments to examine the hRad9/hRad1/hHus1 checkpoint complex formation in cells. 293T cells were transiently co-transfected with plasmids encoding myc-tagged hHus1, HA-tagged hRad1, and FLAG-tagged hRad9 or hRad9 mutants (PLD, CD, or ∆NLS) or parental control vector. Cells were collected and lysed 2 days later, and the protein expression of these transgenes was confirmed by SDS-PAGE/immunoblot analysis with monoclonal antibodies specific for FLAG, HA, or c-Myc epitope (Fig. 2A). A total of 300 μg of cell lysates was subjected to immunoprecipitation using anti-FLAG M2-agarose (Fig. 2B, F) or recombinant protein G-agarose (C) as negative control. The resulting immune complexes were analyzed using polyclonal antibodies specific for FLAG, HA, or c-Myc to detect FLAG-hRad9, HA-hRad1, or myc-hHus1, respectively. As shown in Fig. 2B, hRad9 and hRad9 mutants were immunoprecipitated by anti-FLAG M2-agarose but not control protein G beads. Consistent with the previous reports (8, 24, 25), hRad9 could form a protein complex with hRad1 and hHus1. This association between hRad9, hRad1, and hHus1 was not affected by deletion of the C terminus of hRad9. In addition, the C-terminal region of hRad9 (CD) failed to co-immunoprecipitate with hRad1 and hHus1. These results, together with the previous report (30), indicate that the PCNA-like region of hRad9 is necessary and sufficient for formation of the hRad9/hRad1/hHus1 complex.

The NLS-like Region of hRad9 Is Important for Nuclear Targeting of hRad1 and hHus1—The data presented above revealed that the C-terminal region of hRad9 is not involved in hRad9/hRad1/hHus1 complex formation but is required for the nuclear localization of hRad9. To determine whether this C-terminal region of hRad9 also plays a role in the nuclear targeting of hHus1 and hRad1, HeLa cells were transiently co-transfected with plasmids encoding myc-hHus1 and HA-hRad1 together with GFP-hRad9, GFP-hRad9 mutants (PLD or CD), or control protein GFP expression plasmids. After 2 days of LipofectAMINE 2000 transfection, cells were fixed and stained with anti-Myc 9E10 monoclonal antibody to detect the myc-tagged hHus1 protein. As shown in Fig. 3A, e and m, myc-Hus1 was localized in the cytoplasm of cells co-transfected with myc-hHus1 and control protein GFP in the presence of HA-hrad1 (to stabilize hHus1 expression), consistent with our previous report (31). In contrast, co-expression of GFP-hRad9 resulted in nuclear co-localization of myc-hHus1 and GFP-hRad9 in the presence of HA-hrad1 (Fig. 3A, b, f, j, and n). However, the hRad9 mutant (PLD) that lacks the NLS-containing region of hRad9 (Fig. 1) but is capable of interacting with hHus1 and hRad1 (Fig. 2) sequestered myc-hHus1 in the cytosol (Fig. 3A, c, g, k, and o). Moreover, the C-terminal region of hRad9 was confirmed in the nucleus but failed to target myc-
hHus1 into the nucleus (Fig. 3A, d, h, l, and p), consistent with the failure of this region to form a complex with hHus1 and hRad1 (Fig. 2). Similar results were obtained for hRad1 (Fig. 3B), suggesting that the C-terminal region of hRad9 is required for targeting the hRad9/hHus1 complex into the nucleus.

Dominant Negative Effect of PLD on G2 Checkpoint—Because the hRad9 mutant PLD could retain hHus1 and hRad1 in the cytoplasm, we examined the dominant negative effect of this mutant on the G2 checkpoint of the cell cycle. 293 cells were transiently transfected with 3 µg of either pcDNA3-Myc-PLD or control vector (CV) pcDNA3-Myc for 2 days, followed by treatment with 1 mM HU or 20 Gy of IR or left untreated (UT). After 1 h of IR exposure and 6 h of HU treatment, 400 µg/ml nocodazole was added to the cell cultures, and cultures were incubated for an additional 20 h before fixation with 3.7% formaldehyde and staining with DAPI. Apoptotic cells (A) and mitotic cells (B) were visualized and counted (mean ± S.D.; n = 3) by fluorescence microscopy.

FIG. 3. The C-terminal domain of hRad9 is required for hRad9 to target hRad1 and hHus1 into the nucleus. A, HeLa cells were transfected with 0.45 µg of pcDNA3-Myc-hHus1 and 0.45 µg of pcDNA3-HA-hRad1, together with 0.1 µg of plasmids encoding GFP-hRad9, GFP-PLD, GFP-CD, or control GFP protein. B, HeLa cells were transfected with 0.45 µg of pcDNA3-Myc-hRad1, 0.45 µg of pcDNA3-HA-hHus1, and 0.1 µg of plasmids encoding GFP, GFP-hRad9, GFP-PLD, or GFP-CD fusion protein. Two days after transfection, cells were fixed and stained with anti-Myc 9E10 monoclonal antibody followed by rhodamine-conjugated goat anti-mouse IgG secondary antibody and analyzed by fluorescence microscopy. The nuclear morphology was examined by DAPI staining.

FIG. 4. Overexpression of the PLD protein induces apoptosis and impairs the G2 checkpoint. 293 cells were transiently transfected with 3 µg of either pcDNA3-Myc-PLD or control vector (CV) pcDNA3-Myc for 2 days, followed by treatment with 1 mM HU or 20 Gy of IR or left untreated (UT). After 1 h of IR exposure and 6 h of HU treatment, 400 µg/ml nocodazole was added to the cell cultures, and cultures were incubated for an additional 20 h before fixation with 3.7% formaldehyde and staining with DAPI. Apoptotic cells (A) and mitotic cells (B) were visualized and counted (mean ± S.D.; n = 3) by fluorescence microscopy.

Nuclear Targeting of Rad9 Complex—To further investigate the role of hRad9 in the G2 checkpoint, we treated the human breast cancer cell line MDA-MB-468 with siRNA to inhibit endogenous hRad9 expression. Cells were transfected with siRNAs targeting hRad9 or control GFP two to three times during 3 days of incubation, and the protein levels of hRad9 were evaluated by immunoblot analysis with anti-hRad9 antibody. As shown in Fig. 5A, ~15% of cells died by apoptosis when transfected with the cytosolic hRad9 mutant PLD, compared with <5% of those transfected with control vector. Treating cells with either IR or HU induced apoptosis, which was enhanced by PLD transfection. These results are consistent with the fact that this cytosolic N-terminal fragment of hRad9 contains a Bcl-2 homology 3-like domain, which is critical for its cell death-inducing function and its interactions with the antiapoptotic Bcl-2 family members (3). Indeed, PLD was found to co-immunoprecipitate with the antiapoptotic protein Bcl-xL in 293T cells (data not shown). Interestingly, overexpression of the PLD protein slightly increased (~1.2-fold) the proportion of mitotic cells (Fig. 4B). IR or HU treatment caused a nearly complete block of the G2-M transition of the cell cycle in cells transfected with control vector (Fig. 4B). However, a significant proportion (10–13%) of PLD-transfected cells bypassed the G2 checkpoint after HU or IR treatment (Fig. 4B). These results suggest that the C-terminal deletion mutant of hRad9 could exert a dominant negative effect on the G2 checkpoint in response to DNA damage or inhibition of DNA replication by sequestering the hRad9/hRad1/hHus1 checkpoint complex in the cytosol.

Nuclear Localization of hRad1 Is Dependent on hRad9—To further investigate the role of hRad9 in the G2 checkpoint, we treated the human breast cancer cell line MDA-MB-468 with siRNA to inhibit endogenous hRad9 expression. Cells were transfected with siRNAs targeting hRad9 or control GFP two to three times during 3 days of incubation, and the protein levels of hRad9 were evaluated by immunoblot analysis with anti-hRad9 antibody. As shown in Fig. 5A, the 21-bp RNA duplexes targeting hRad9 dramatically reduced the hRad9 protein in...
MDA-MB-468 cells. This reduction of hRad9 expression was also confirmed by immunofluorescence analysis. Cells transfected with siRNAs were fixed, permeabilized, stained with antibodies specific for either hRad9 or α-tubulin control protein. In B and C, the siRNA-transfected MDA-MB-468 cells were stained with either anti-hRad9 (B) or anti-hRad1 (C) rabbit polyclonal antibodies, which were detected by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG secondary antibodies and analyzed under fluorescence microscopy.

Fig. 5. Reduction of hRad9 causes cytoplasmic distribution of hRad1. A, MDA-MB-468 cells were transfected with siRNAs targeting hRad9 or GFP as control three times over a 3-day period. Cell lysates were prepared and subjected to SDS-PAGE/immunoblot analysis (30 μg total protein/lane) with antibodies specific for hRad9 or α-tubulin control protein. In B and C, the siRNA-transfected MDA-MB-468 cells were stained with either anti-hRad9 (B) or anti-hRad1 (C) rabbit polyclonal antibodies, which were detected by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG secondary antibodies and analyzed under fluorescence microscopy.

Role of hRad9 in G2 Checkpoint

To determine the role of hRad9 in cell cycle checkpoint, MCF-7 cells were transfected with 21-nucleotide siRNA duplexes targeting hRad9 or mock-transfected as control. After 2 days of transfection, cells were exposed to 40 Gy of IR and cultured for an additional 24 h before DNA content analysis by flow cytometry. As shown in Fig. 6A, >40% of the mock-transfected cells accumulated in G2-M phase of the cell cycle after IR treatment. However, treating cells with hRad9 siRNA duplexes apparently reduced IR-induced accumulation of G2-M cells, suggesting that hRad9 is important for DNA damage checkpoint. These results were confirmed in MDA-MB-468 cells transfected with siRNAs targeting hRad9 or control GFP and treated with 20 Gy of IR or 1 mM HU or left untreated (UT). After 1 h of IR or 6 h of HU treatment, cells were incubated with 400 μg/ml nocodazole for an additional 20 h, followed by fixation with 3.7% formaldehyde and staining with DAPI, and mitotic cells were counted (mean ± S.D.; n = 3) by fluorescence microscopy.

Fig. 6. Knockdown of hRad9 promotes the G2-M transition. A, MCF-7 cells were transiently transfected with siRNA duplexes targeting hRad9 or mock-transfected for 2 days. Cells were exposed to 40 Gy of IR or left untreated and cultured for additional 24 h before incubation with 50 μg/ml propidium iodide and DNA content analysis by flow cytometry. B, MDA-MB-468 cells were transfected with siRNAs targeting hRad9 or control GFP and treated with 20 Gy of IR or 1 mM HU or left untreated (UT). After 1 h of IR or 6 h of HU treatment, cells were incubated with 400 μg/ml nocodazole for an additional 20 h, followed by fixation with 3.7% formaldehyde and staining with DAPI, and mitotic cells were counted (mean ± S.D.; n = 3) by fluorescence microscopy.
significant proportion (8–15%) of cells transfected with siRNA against hRad9 bypassed the G2 checkpoint after IR or HU treatment (Fig. 6B), indicating that hRad9 plays an important role in activation of the G2 checkpoint by IR or HU.

**DISCUSSION**

The findings reported here indicate that the hRad9 protein functions to target the hRad9-hRad1-hHus1 checkpoint complex into the nucleus. This is supported by the observations that hRad9 contains a predicted NLS near its C terminus that allows for its transport into the nucleus. This C-terminal region of hRad9 in which the NLS-like domain resides is sufficient to target the GFP protein into the nucleus, whereas deletion of the NLS-containing region from the hRad9 protein results in accumulation of hRad9, hRad1, and hHus1 in the cytoplasm (Figs. 1 and 3). Moreover, the region of hRad9 predicted to share a PCNA-like structure is necessary and sufficient for the hRad9-hRad1-hHus1 complex formation (Fig. 2), consistent with the previous reports in human and yeast (30, 39).

In *S. pombe* and *S. cerevisiae*, molecular and genetic studies have indicated that Rad1 (scRad17), Rad9 (scDdc1), and Hus1 (scMec3) are essential and function in the same pathway for activation of the G2 checkpoint after DNA damage or replication block (4, 14, 40). These proteins interact with each other to form a stable complex in vivo (9, 16), and the stability of the complex depends on the presence of all three proteins (39). Recently, computational modeling studies have predicted that Rad1, Rad9, and Hus1 share structural similarity with PCNA, thus leading to the proposal that these proteins could form a PCNA-like dimeric sliding clamp (15–18). In agreement with this model, all three proteins, Rad1, Rad9, and Hus1, were found in the same complexes (16, 39). Moreover, their human homologues, hRad1, hRad9, and hHus1, have been reported to form a head-to-tail association similar to those seen in the PCNA homotrimer (30). In this study, we also showed that hRad9 could co-immunoprecipitate with hRad1 and hHus1 (Fig. 2). Although the non-PCNA-like region at the C terminus of Rad9 is not required for interactions with Rad1 and Hus1, it is essential for Rad9 to activate the G2 checkpoint signaling cascade (39). Overexpression of a C-terminal deletion mutant of Rad9 in fission yeast sequesters Hus1 and Rad1 in a nonfunctional complex, thus allowing cells to enter mitosis in the presence of HU (39). In agreement with the yeast studies, our results indicate that overexpression of a deletion mutant of hRad9 (PLD) that lacks the C-terminal domain of hRad9 sequesters hHus1 and hRad1 in the cytoplasm (Fig. 3) and hinders activation of the G2 checkpoint after radiation or HU treatment (Fig. 4). This suggests that hRad9 plays an essential role in regulation of the G2 checkpoint by controlling the nuclear localization of hRad1 and hHus1. In fact, reduction of hRad9 expression by siRNA duplexes targeting hRad9 resulted in cytoplasmic accumulation of hRad1 and defects in the G2 checkpoint (Figs. 5 and 6). In *S. pombe*, knockout of Rad9 causes the failure of the G2–M checkpoint after radiation or HU treatment, allowing cells to enter mitosis (10). Moreover, it has been shown that the nuclear localization of Hus1 and Rad9 is dependent on the presence of Rad17 and that Rad9 but not Rad1 is required for Hus1 to be localized to the nucleus (16). Our findings demonstrate that the C-terminal domain of hRad9 is required for targeting the functional hRad9-hRad1-hHus1 complex into the nucleus. Further evidence supporting this conclusion comes from observations that the hRad9 protein contains a predicted NLS at its C terminus and that the NLS-containing region of hRad9 is sufficient to transport the GFP protein into the nucleus (Fig. 1). In contrast, expression of the *S. pombe* Rad9 protein in mammalian cells revealed a cytosolic distribution (data not shown), suggesting that it may use a different mechanism than hRad9 for nuclear transport. Indeed, we could not find any NLS-like region in the yeast Rad9 protein.

Taken together, we conclude that the hRad9 protein contains distinct domains with separate functions. First, the N-terminal Bcl-2 homology 3-like domain is important for the interactions of hRad9 with antiapoptotic Bcl-2 family proteins such as Bcl-2 and Bcl-XL (3, 34). Second, the PCNA-like region is necessary and sufficient for formation of the hRad9-hRad1-hHus1 checkpoint complex. Finally, the non-PCNA-related C-terminal domain located outside of the hRad9-hRad1-hHus1 complex acts to guide this multiprotein complex into the nucleus, where it is involved in activation of the G2 checkpoint in response to DNA damage or inhibition of DNA replication.

**Acknowledgments**—We thank Larry M. Karnitz (Mayo Clinic) for kindly providing anti-Rad1 antibodies. We also thank Hirohito Yamaguchi and Michael W. Lee for helpful discussion and the members of Molecular Biology, Flow Cytometry, and Molecular Imaging core facilities at Moffitt Cancer Center for assistance.

**REFERENCES**

1. Abraham, R. T. (2001) *Genes Dev.* 15, 2177–2196
2. Venclovová, P., and Toczyński, D. (2000) *Cell Stem Cell* 6, 385–396
3. Parker, A. E., Van de Weyer, I., Laus, M. C., Oostveen, I., Yon, J., Verhasselt, P., and Luypen, W. H. (1998) *J. Biol. Chem.* 273, 1832–18339
4. Dell, C. M., Lee, S. K., and Davey, S. (1998) *Nucleic Acids Res.* 26, 3971–3976
5. Cortez, D., Guntuku, S., Qin, J., and Elledge, S. J. (2001) *Science* 294, 1713–1716
6. O’Connell, M. J., Walworth, N. C., and Carr, A. M. (2000) *Trends Cell Biol.* 10, 296–303
7. Aravind, L., Walker, D. R., and Konin, E. V. (1999) *Nucleic Acids Res.* 27, 1223–1242
8. Caspari, T., Dahlen, M., Kantor-Smoler, G., Lindsay, H. D., Hofmann, K., Petropoulos, C., Sumnerpengen, H., and Carr, A. M. (2000) *Cell Biol. Cell Biol.* 124, 1250–1262
9. Kondo, T., Watakeyama, N., Taiki, T., Matsumoto, K., and Sugimoto, K. (2001) *Science* 294, 867–870
10. Melo, J. A., Cohen, J., and Toczyński, D. P. (2001) *Genes Dev.* 15, 2809–2821
11. Rauen, M., Burtelow, M. A., Dufault, V. M., and Karnitz, L. M. (2000) *J. Biol. Chem.* 275, 29677–29772
12. Lindsey-Boltz, L. A., Bermudez, V. P., Hurwitz, J., and Sancar, A. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 11236–11241
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J. Biol. Chem. 2002, 277:25722-25727.
doi: 10.1074/jbc.M203079200 originally published online May 6, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M203079200

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