Human Rad17 Is Phosphorylated upon DNA Damage and also Overexpressed in Primary Non-Small Cell Lung Cancer Tissues

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Abstract

The spRAD17 gene is an essential component of the DNA damage and replication checkpoints in the fission yeast Schizosaccharomyces pombe. Cloning of the human homologue of spRAD17, hRAD17, indicated that it exhibits structural similarity with the replication accessory protein family, which include subunits of the Replication factor C complex. We have analyzed the phosphorylation status of hRad17 in response to DNA damaging agents. Our results showed that phosphorylation of hRad17 occurred immediately after UV and ionizing radiation treatment and reached peak level at ~3 h, suggesting that hRad17 may be a component of the DNA damage checkpoint. When primary tumor samples were analyzed, we observed that the majority (74%) of non-small cell lung carcinoma samples exhibited a significantly higher level of hRad17 expression compared with matched normal tissue controls. In contrast, hRad17 protein levels in a panel of primary colon carcinoma samples did not show an elevated level of expression compared with normal colon tissues. This observation suggests that the function of the hRAD17 gene may be involved in lung cancer development and may serve as a potential tumor marker.

Introduction

The function of DNA damage checkpoints is to maintain genomic stability by controlling cell cycle progression upon the introduction of genotoxic DNA lesions. (1–3). Attenuating cell cycle progression prevents DNA lesions from being converted into heritable mutations and allows time for DNA repair pathways to correct the lesions. Genetic instabilities that arise from checkpoint defects have been firmly linked to cancer development (4, 5).

The spRAD17 gene of S. pombe is one of the key factors involved in the activation of checkpoint signals in response to DNA damage or disruption of DNA synthesis. Loss of spRAD17 function in fission yeast resulted in failure to arrest cell cycle progression and consequently lead to hypersensitivity to genotoxic and DNA synthesis blocking agents (6). The human homologue of spRAD17, hRAD17, has been isolated by a number of groups including ours, and was found to have structural similarity to subunits of RFC3 (7–9). It has been documented (9, 22), and missense substitutions/polymorphisms overexpression of hRad17 in colon cancer and seminoma cells has been identified in tumor samples (23). During an attempt to identify hRAD17 mutations in primary NSCLC samples, we found that hRad17 protein levels increased substantially in the majority of these tumors. In this report, we present data showing that the hRad17 protein was phosphorylated in response to DNA damage and that the protein expression level of hRad17 in a panel of primary NSCLC tumor tissues was elevated.

Materials and Methods

Cell Lines and Tissue Specimens. RKO (human colon epithelial) and HT-1080 (human fibrosarcoma) were maintained in DMEM plus 10% FCS (Life Technologies, Inc.). Protein extracts were prepared by lysing the tissue sample in RIPA buffer [150 mM NaCl, 1.0% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris (pH 8.0)], and protein concentration was determined by standard Bradford assay.
Production of Anti-hRad17 Antibodies and Affinity Purification. A 912-bp XbaI-PstI fragment encoding the COOH-terminal half of hRad17 (Leu131-Ala614) was cloned into the pET28b vector by using an in-frame BamHI-Xbal oligo adapter. The resulting His-tagged protein was overproduced in the BL21-DE3 host and purified through a denaturing nickel column using procedures published by the manufacturer (Novagen, Madison, WI). Rabbit anti-hRad17 serum was generated by Alpha Diagnostic International (San Antonio, TX).

To purify the anti-hRad17 antibody, the same COOH-terminal hRad17 coding sequence (XbaI-PstI) was cloned into pMAL-C2 (New England Biolabs) to produce an MBP-tagged recombinant protein and subsequently purified with an amylose column. The purified MBP-hRad17 was immobilized with the Aminolink Coupling Gel (Fierce, Rockford, IL), and antibody affinity purification was performed by following a standard protocol (24). The anti-phosphorylated hRad17 antibodies (a kind gift from Lee Zou and Steve Elledge, Department of Biochemistry, Baylor College of Medicine, Houston, TX) were raised against peptide epitopes with phosphorylated Ser635 and Ser645 and purified by affinity columns with each peptide.

**DNA Damage Treatment and Immunoblotting.** For ionizing radiation treatment, asynchronous proliferating cells (HT-1080 or RKO) were irradiated with a Nasatron 137Cs source at a dose rate of 4.3 Gy/min in room temperature. UV radiation was conducted in a Hoefer UV (254 nm) cross-linker. Before UV irradiation, asynchronous cells were washed twice with PBS and exposed directly to UV light. For Western analysis, cells were harvested by either direct lysis in 1× SDS loading buffer or in lysis buffer A (50 mM HEPES, 1% Triton X-100, 10 mM NaF, 30 mM Na3PO4, 150 mM NaCl, and 1 mM EDTA) containing freshly added 10 mM glycophosphate, 1 mM Na3VO4, 20 μg/ml pepstatin A, 10 μg/ml aprotinin, 20 μg/ml leupeptin, and 40 μM microcystin-LR). Simultaneous blotting with multiple antibodies and/or different dilutions on the same filter was performed with an incubation manifold (DecaProbe; Hoefer Scientific). Affinity-purified rabbit anti-hRad17 antibody was used in all of the experiments.

**Metabolic Labeling and Phosphatase Treatment.** Exponentially growing RKO cells were placed in phosphate-free DMEM medium 1 h before the addition of 33chloride labeling medium containing 32P phosphate and 10% serum. Cells were immediately exposed to 15 Gy IR and harvested at 0 and 3 h time points. Immunoprecipitation was carried out with purified anti-hRad17 antibody. The level of 32P label of hRad17 against the amount of hRad17 in each immunoprecipitation sample. For phosphatase treatment, total protein (50 μg of extracts prepared in lysis buffer A) was diluted in phosphatase buffer and incubated in the presence or absence of 25 units of CIAP (Roche Biochemicals) for 30 min at 30°C.

**Results**

**DNA Damage-induced Phosphorylation of hRad17.** To produce an antibody against hRad17, we expressed a M, 35,000 COOH-terminal truncation of hRad17 (Leu131-Ala614) in Escherichia coli and purified the polypeptide to near homogeneity through a nickel resin column. The same fragment was also expressed in the form of a fusion protein with the MBP tagged at the NH2 terminus and was used to affinity purify the anti-hRad17 antibody. The purified antibody recognized, with high specificity, a band in HeLa cell extracts that appears to migrate as a M, 76,000 protein (Fig. 1A). To confirm the specificity of the purified antibody, the same COOH-terminal truncation (Leu131-Ala614) was expressed in mammalian cells through transient transfection. Western blot analysis showed that the purified antibody was able to specifically recognize the truncated hRad17 protein along with the endogenous protein (Fig. 1B).

As a putative checkpoint factor, hRad17 may respond to DNA damage by exhibiting post-translational modifications such as phosphorylation and/or altered protein level. We analyzed the migration status of hRad17 in cells exposed to either IR or UV. As shown in Fig. 2A, after treating human HT-1080 fibrosarcoma cells with ionizing radiation, the hRad17 protein became shifted into a more slowly migrating form. The maximum reduction of mobility was observed when cells were harvested 3 h after treatment. However, the mobility shift was diminished 6 h after treatment (data not shown), suggesting a transitory nature of the modification. Likewise, cells irradiated with UV light also produced a slowly migrating form of hRad17 compared with untreated cells (Fig. 2B) with kinetics similar to ionizing radiation-treated cells. To verify whether the damage-induced reduction of hRad17 mobility was caused by phosphorylation, we performed in vivo metabolic labeling of hRad17. We exposed RKO cells cultured in 32P phosphate-containing medium to 15 Gy of ionizing radiation. Protein extracts were prepared 3 h after treatment, and the hRad17 protein was immunoprecipitated by purified anti-hRad17 antibody. As shown (Fig. 2C), hRad17 precipitated from IR-treated cell extracts exhibited an ~4-fold increase in the level of 32P labeling compared with untreated cells. Taken together, these results suggest that hRad17 was a target for phosphorylation in response to DNA damaging agents.

To test whether the damage-inducible reduction of hRad17 mobility was attributable to the phosphorylation of the two SQ motifs located at positions Ser635 and Ser645, we analyzed IR-treated cells with antibodies against phosphorylated Ser635 and Ser645. Indeed, the phosphorylated form of hRad17 was detected in a damage-dependent manner (Fig. 2D). Furthermore, when extracts prepared from IR-treated RKO cells were incubated with CIAP, phosphorylated hRad17 protein could no longer be detected by the phosphor-specific Ser635 and Ser645 antibodies (Fig. 2E). These results suggest that the hRad17 protein was phosphorylated at Ser635 and Ser645 in response to DNA damage. Our data are also consistent with a report by Kim et al. (11) that Ser635 and Ser645 in hRad17 are excellent in vitro substrates for the ATM and ATR kinase activity.

**Expression of hRad17 in Primary NSCLC and Colorectal Carcinoma Tissues.** Using fluorescence in situ hybridization and a monochromosomal hybrid cell mapping panel, we had assigned previously the hRAD17 locus to 5q11 (7). The q arm of chromosome 5 exhibits a high frequency of loss of heterozygosity associated with a variety of thoracic head and neck, and colorectal cancers (25–27). To examine whether hRad17 gene expression and protein levels have been affected in these tumors, we assembled a panel of 28 primary NSCLC samples with pathologically matched normal tissue controls from each patient. PCR analysis revealed no gross deletions at the hRAD17 locus in any of the sample pairs. We then carried out Western blot analysis on these samples with purified anti-hRad17 antibody. As
shown in Fig. 3A, all of the samples contain a single full-length hRad17 protein that migrated as a Mr 76,000 protein, and no aberrant forms of hRad17 were detected in either tissue. Interestingly, in the majority of the tumor samples, the hRad17 protein levels have increased significantly. Of a total of 28 sample pairs, 19 appeared to have substantially elevated hRad17 expression level in tumor samples. Because surgical resections are known for their heterogeneity in cell types and variability in protein yield, we then reexamined a selected group of samples using β-actin as an internal control. Again, high levels of hRad17 expression were detected in most NSCLC tissues when the hRad17 signal in each sample was normalized against β-actin (Fig. 3B).

To test whether hRAD17 overexpression also occurs in other types of tumors, we next examined primary colon carcinoma tissues. Fourteen primary colon carcinoma samples with matched normal tissue controls were examined by Western blot. As shown (Fig. 4), we found no significant difference in hRad17 protein level between normal and colon cancer tissues. Also note that the amount of hRad17 in normal colon tissue appears, in general, to be higher than in normal lung tissues. Taken together, these results suggest that overexpression of the hRad17 protein may be associated with NSCLC development and may perhaps serve as a marker for NSCLC.

As a structural homologue to the RFC family of proteins, hRad17 has been shown to participate in a checkpoint clamp-loading complex (18, 21). Hence, it is plausible that hRad17 may participate in DNA synthesis under certain circumstances. To investigate whether hRad17 overexpression is associated with the high proliferation rate of most tumor tissues, we examined PCNA levels as a proliferating marker in both matched NSCLC and colon cancer samples. In NSCLC samples (Fig. 3C), tumor tissues showed highly elevated levels of PCNA that correlated closely with the level of hRad17. In colon samples (Fig. 4C), a significant number of tumor tissues exhibited higher levels of PCNA, whereas the hRad17 level remained unchanged between tumor and matched controls. However, normal colon tissues appeared to
have an elevated hRad17 protein level, which may be sufficient to support their DNA replication activities. Taken together, these results seem to suggest that hRad17 function might be required in cell proliferation and that it may serve as a potential marker in NSCLC, since normal lung tissues exhibited very low levels of hRad17 protein.

Discussion

The hRAD17 gene was isolated as a structural homologue of the fission yeast checkpoint protein spRAD17 (7, 9, 10) and also by differential display as a highly expressed gene in colon cancer (22). The function of the hRad17 protein in DNA damage checkpoints and its role in tumor development remains to be determined. Using complementary approaches, we obtained clear evidence that hRad17 undergoes DNA damage-induced phosphorylation at the SQ motifs located at Ser635 and Ser645. Our analysis of primary tumor samples indicated that hRad17 was overexpressed in the majority of the NSCLC tissues compared with pathologically matched normal tissues.

Detection of DNA damage is perhaps the least understood step in mammalian checkpoint pathways. In fission yeast, gene products such as rad1, rad9, and rad17 that function upstream of the Rad3 kinase have been considered sensors responsible for the initiation of checkpoint signals upon DNA damage and/or disrupted DNA synthesis. Structural analysis of the hRad17 protein sequence predicted that it might participate in a clamp-clamp loader complex similar to the PCNA-RFC complex (20). This model was supported by the physical interaction between hRad17 and the hHus1/hRad9/hRad1 complex (18, 21). Therefore, it is plausible that hRad17 may interface with DNA repair synthesis and mediate cell cycle regulation.

In a randomized peptide screening of potential ATM kinase consensus substrate sequences, Kim et al. (11) identified two ATM/ATR target sites located at the COOH-terminus of hRad17. When assayed in vitro as an ATM/ATR target, the hRad17 SQ motifs (Ser635Gln636 and Ser645Gln646) were the most potent substrate of ATR and ATM, showing a 10-fold greater activity over the p53 protein using synthetic peptide as the substrates. Consistent with these in vitro results, our data have provided in vivo evidence that hRad17 is subjected to phosphorylation on DNA damage and is likely a substrate of the ATM/ATR kinase activity. While this paper was being prepared, Bao et al. (28) reported DNA damage-stimulated interaction between ATM/ATR and hRad17 as determined by co-IP experiments. This same report also identified Ser635 and Ser645 as the ATM/ATR target sites and showed that damage-induced phosphorylation at these two serine residues were important for the G2 checkpoint. Collectively, these data provide conclusive evidence that hRad17 plays an important role in the DNA damage checkpoint. Moreover, the finding that hRad17 was a target of ATM/ATR kinase consigned the function of hRad17 downstream of the phosphatidylinositol-3 kinase proteins, which suggests that the molecular organization of the damage recognition step in mammalian checkpoint has evolved differently compared with that of fission and budding yeasts.

Our analysis of primary lung cancer tissues showed that hRad17 protein levels were significantly elevated in the majority of NSCLC samples, whereas matched normal tissues exhibited very weak expression. Considering that a surgically resected tumor specimen typically contains a substantial amount of normal tissue, the actual levels of hRad17 overexpression in NSCLC cells are most likely higher than what we have observed. The elevated hRad17 expression level was closely associated with the level of PCNA as a proliferation marker. This correlation seems to suggest that the hRad17 function may be involved in cell proliferation. The exact cause and consequences of such overexpression are not known. Perhaps, rapidly dividing cells may require additional checkpoint activity to assist a high rate of DNA replication, or the hRad17/hHus1/hRad17/hRad9 complex might be an inherent component of the replication machinery, given the structural characteristics of this complex as a clamp-clamp loader. We also analyzed the phosphorylation status of hRad17 in the matched NSCLC sample pairs. Although the amount of hRad17 protein in tumor tissues tested was substantially higher than in control tissues, the levels of phosphorylated hRad17 remained unchanged compared with normal cells (data not shown), suggesting a lower level of basal phosphorylation of hRad17 in NSCLC cells. It is plausible that overexpression of the unphosphorylated form of hRad17 may compromise the normal function of cell cycle checkpoint in NSCLC cells and contribute to oncogenicity. Additional investigation is necessary to verify if overexpression of hRad17 can be characterized as a protein marker for NSCLC.

In summary, our data showed that the hRad17 protein was phosphorylated in response to DNA damage and that the hRad17 protein level was substantially higher in NSCLC tissues compared with matched normal tissue controls. These observations support the notion that hRad17 is a component of the DNA damage checkpoint and provides evidence suggesting a possible role for the hRAD17 gene in NSCLC etiology.

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References


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