Ionizing Radiation Triggers Chromatin-bound kin17 Complex Formation in Human Cells*

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The human DNA-binding HSAkin17 protein cross-reacts with antibodies raised against the stress-activated Escherichia coli RecA protein. We show here that HSAkin17 protein is directly associated with chromosomal DNA as judged by cross-linking experiments on living cells. We detected increased amounts of DNA-bound HSAkin17 protein 24 h after γ irradiation, with 2.6-fold more HSAkin17 molecules after 6 Gy of irradiation (46,000–117,000 molecules). At this time we observed that highly proliferating RKO cells displayed the concentration and co-localization of HSAkin17 and replication protein A in nucleoplasmic foci. Our results suggest that 24 h post-irradiation HSAkin17 protein may localize at the sites of unrepaired DNA damages. RKO clones expressing an HSAKIN17 antisense transcript (RASK.5 and RASK.13 cells) revealed that reduced HSAkin17 protein levels are correlated with a decrease in clonogenic cell growth and cell proliferation, as well as an accumulation of cells in early and mid-S phase. Taken together our observations support the idea that HSAkin17 protein is a DNA maintenance protein involved in the cellular response to the presence of DNA damage and suggest that it helps to overcome the perturbation of DNA replication produced by unrepaired lesions.

Ionizing radiation (IR)1 induces a large range of DNA damage, including DNA double-strand breaks (DSBs), which represent a major threat to the integrity of mammalian genomes through chromosomal breakages and rearrangements (1). In mammalian cells, DSBs are repaired either by the homologous recombinational repair or by nonhomologous end joining (2, 3). DSB repair pathways are usually characterized by the sequestration of many factors into discrete nuclear foci at the sites of DNA lesions and until completion of DSB repair (4). Some of the proteins belonging to these pathways act as a sensor for DNA damage or are involved in cell cycle checkpoints. This is the case for the histone H2AX, 53BP1, RPA, Rad51, BRCA1, or the Mre11-Rad50-Nbs1 nuclease complex (4–8). For instance, the tumor suppressor gene BRCA1, previously involved in the regulation of the replication checkpoint and transcription-coupled repair, forms a multiprotein complex with Mre11-Rad50-Nbs1 and other proteins following irradiation, termed as BASC (BRCA1-associated surveillance complex), which may serve as a sensor of DNA lesions (8, 9).

In this cascade of IR-induced proteins forming nuclear foci, we characterized here the HSAkin17 protein. Murine MMUKin17 protein was identified on the basis of a cross-reactivity with antibodies raised against the Escherichia coli RecA protein, a key enzyme in homologous recombination and recombinational repair of damaged DNA (10, 11). This cross-reactivity stemmed from a sequence homology stretching over 39 amino acids highly conserved during evolution (12). This domain is located in the carboxyl-terminal region of the E. coli RecA protein, a region involved in the regulation of DNA binding (13). Recent data show that kin17 proteins are highly conserved during evolution.2 In particular, a homologous protein has been identified in the yeast Schizosaccharomyces pombe. This conservation from yeast to human points to an essential role of kin17 proteins. For instance, the expression of kin17 proteins is preserved in the phylogeny of the brain of higher vertebrates (14, 15). To date, major features of the kin17 protein are its abilities (i) to bind in vitro to double-stranded DNA and preferentially to DNA with a curved topology (16, 17), (ii) to complement the functions of the H-NS (histone-like nucleoid structuring) protein transcription factor in deficient bacterial strains in controlling gene expression (18), and (iii) to be a stress-activated protein recruited during the cellular response to ionizing radiation or UVC (19, 20). In particular, UVC irradiation induced a stabilization of MMUKIN17 mRNA from 80 min to more than 8 h in mouse fibroblasts (21). Interestingly, ΔXPA mouse cells, which are unable to repair UVC-induced DNA damage, accumulated MMUKIN17 mRNA at lower doses of UVC (5–10 J/m²) than repair-proficient mouse fibroblasts (20–30 J/m²), suggesting that DNA damage per se is required for the stabilization of MMUKIN17 mRNA (21).

In human cells, the HSAKIN17 gene is localized on chromosome 10 at position p15-p14. HSAKIN17 transcripts were ubiquitously found at low levels in all human organs tested, displaying an expression profile akin to that of housekeeping genes. Heart, skeletal muscle, and testis displayed the highest HSAKIN17 mRNA levels compared with the other tissues analyzed.

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¶ The abbreviations used are: IR, ionizing radiation; DSB, double-strand break; RPA, replication protein A; EBV, Epstein-Barr virus; Gy, gray; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; PBS, phosphate-buffered saline; DMS, dimethylsulfate; BrdUrd, bromodeoxyuridine; PCNA, proliferating cell nuclear antigen.

2 J. F. Angulo, unpublished result.
lyzed (12). HSAkin17 protein expression is associated with the differentiation program of human keratinocytes cultivated in the in vitro reconstructed skin model (22). Mouse and human kin17 proteins are able to arrest cell proliferation of human tumor cells when their expression is transiently increased after transfection (23). Only the immortalized but not tumorigenic HEK 293 cell line tolerates ectopic expression of either MMUkin17 or HSAkin17 proteins and can be propagated in mass culture for several weeks. However, the constitutive overexpression of MMUkin17 protein in HEK 293 cells entailed major growth defects and nuclear abnormalities (23).

We show here for the first time that the HSAkin17 protein is mainly present in the nuclear compartment associated with nuclear structures in human cells. We demonstrate that a fraction of nuclear HSAkin17 protein is directly associated with DNA. HSAkin17 protein is localized in discrete nuclear foci spread throughout the nucleoplasm. Strikingly, γ irradiation induces an increase in the DNA-bound fraction of HSAkin17 protein that correlates with the appearance of foci containing both HSAkin17 protein and replication protein A (RPA) 24 h after IR. To ascertain the requirement of HSAkin17 in the immortalized phenotype, we have expressed HSAKIN17 antisense transcript in RKO carcinoma cells. These RKO cells expressing the antisense HSAkin17 (called RASK cells) displayed markedly reduced proliferation rates associated with a defect in S phase progression. All of our observations support the idea that HSAkin17 protein is involved in the cellular re-

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D. S. F. Biard, unpublished data.
sponse to the presence of DNA damage and suggest that it may help to overcome the DNA replication arrest produced by unrepaired lesions.

MATERIALS AND METHODS

Cell Cultures—Human colorectal carcinoma RKO cells were obtained from M. F. Poupon. Human cervical carcinoma HeLa cells were obtained from E. May. The cell lines were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin, under 5% CO2.

Cloning of EBV vectors carrying HSAkin17 cDNA in an antisense orientation has been performed as described elsewhere (23). Transfected RKO cells carrying EBV plasmids were propagated in culture in the presence of 500 μg/ml hygromycin B (Invitrogen). We used the following vectors: pEBVCMV (pB482) and pEBVCMVasHSAkin17 (pB399as). Transfection experiments were carried out using LipofectAMINE 2000 (Invitrogen). RKO clones transfected with the pB399as EBV vector carrying the HSAkin17 cDNA-SV40 polyadenylation signal cartridge in an antisense orientation were termed RASK, for RKO antisense HSAkin17 cDNA. Control clones carrying the pB482 plasmid were named R482.

The cells were irradiated using a 137Cs source (IBL 637 CisBio International) with a dose rate of 1.97 Gy/min. For clonogenic cell growth, the RKO cells were seeded as indicated in the table and cultivated for 2 weeks in the presence of hygromycin B. Growing clones were fixed with 4% paraformaldehyde and stained with methylene blue, and the clones containing more than 50 cells were counted. Each experiment was done three times.

Monoclonal Antibodies against HSAkin17 Protein and ELISA—Monoclonal antibodies (mAb K3, mAb K31, mAb K36, and mAb K58) were obtained after inoculation of recombinant His-tagged human HSAkin17 protein (His6-HSAkin17) purified by metal chelation and heparin column chromatography from baculovirus-infected Sf9 Spodoptera frugiperda cell extracts and injected in mice as described previously. We used either hybridoma supernatants (mAb K36) or IgG anti-HSAkin17 protein (Ig K36; Ig K58) purified from ascites fluid. Purified immunoglobulin from rabbit polyclonal antibody anti-His6-HSAkin17 (IgG 77P) was obtained as described elsewhere.

A conventional two-site immunometric assay (sandwich immunoassay) based on two specific monoclonal antibodies recognizing nonoverlapping epitopes was developed essentially as described by Grassi et al. (24). We chose the mAb K3 and mAb K31 monoclonal antibodies, the
23 weeks after transfection, the cells were seeded as indicated in the presence of 500 μg/ml hygromycin B. The clones were fixed, stained, and counted 16 days after seeding. Control clone, R482.1 cells.

### Table I

| Cell line | Number of cells seeded | Plating efficiency ± S.D. |
|-----------|------------------------|---------------------------|
| RKO       | 10                     | 53.2 ± 5.3                |
| Control clone | 10               | 70.8 ± 4.5               |
| RAS5      | 33                     | 11.6 ± 1.9                |
| RAS13     | 100                    | 5.3 ± 4.9                 |

mAb K3 being conjugated to acetylcholinesterase as reporter enzyme (25).

**Indirect Immunofluorescence Staining**—The cells were plated at 5,000 cells/cm² on glass coverslips and treated. At the indicated times after treatment, the cells were fixed for 5 min in 70% acetone, 30% methanol at −20 °C. The primary antibodies were diluted in the incubation buffer B (0.5% Tween 20, 12% bovine serum albumin, 0.036% NaN₃ in PBS) and incubated for 45 min. The following antibodies were used: hybridoma supernatant mAb K36 anti-HSAkin17 (diluted by half), purified Ig K36 anti-HSAkin17 (450 ng/ml), purified rabbit immunoglobulin IgG 77P against HSAkin17 (5 μg/ml), and anti-RPA70 (directed against the 70-kDa subunit; mouse monoclonal NA13 antibody, 500 ng/ml; Oncogene Research Products). Primary antibodies were revealed with either Cy2-conjugated affinity-purified goat anti-mouse IgG or Cy3-conjugated affinity-purified goat anti-rabbit IgG (Jackson Laboratories, 25 ng/ml). The cells were counterstained with 4′,6-diamino-2-phenylindole (4 μg/ml). Immunofluorescence staining was viewed using a Zeiss Axioscop 2 epifluorescence microscope coupled to a cooled SenSys 1400 camera from Photometrics monitored by the Zeiss KS300 3.0 program. The use of a CCD camera-based imaging system allows high resolution and a wide dynamic range for acquiring and analyzing fluorescent staining. Representative fields for each cell line are presented. Irradiation experiments were reproduced more than 10 times under different culture conditions.

**Protein Extraction and Western Blot**—The cells were seeded at 5,000 cells/cm² 3 days before trypsinization and treated as indicated. The cells were lysed using RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Igepal, 0.1% SDS, 0.1% sodium deoxycholate, protease inhibitor mixture from Roche Molecular Biochemicals) or buffer N (50 mM Tris-HCl, pH 7.9, 150 mM NaCl, 1% Igepal, 1 mM EDTA, protease inhibitor mixture from Roche Molecular Biochemicals), as indicated in the legends of the figures. The lysates were kept on ice for 30 min with the buffer N, and soluble proteins recovered after centrifugation (20,000 × g for 15 min) were quantified by Bradford assay (Bio-Rad) and analyzed by SDS-PAGE.

**RESULTS**

**Association of Human HSAkin17 Protein with Chromosomal DNA in Vivo—**Mazin et al. (16, 17) demonstrated that in vitro mouse mHSAkin17 protein recognizes DNA, particularly double-stranded DNA with a curved topology. In agreement with these results, we have observed that most HSAkin17 protein was localized in nuclei of different cultured human cell lines (data not shown). We then tested whether in vivo endogenous human HSAkin17 protein could be associated with chromosomal DNA. We used a method based on a limited cross-linking of living HeLa cells with formaldehyde to stabilize DNA-protein interactions prior to the extraction (25). This method minimized the formation of nonspecific cross-links and further excluded noncross-linked material by two consecutive cesium chloride density gradient centrifugations. Equilibrium density gradient centrifugation separates cellular components according to their density. Under these experimental conditions, DNA-protein complexes exhibited a density near to that of native chromatin (1.4 g/ml). Covalent bonds introduced by formaldehyde are reversible by boiling in SDS-containing buffers, thus allowing the analysis of proteins by SDS-PAGE. After a first centrifugation, we observed a co-migration of HSAkin17 and PCNA proteins as components of a high molecular weight complex displaying similar densities (Fig. 1A). Fractions 6–8 containing DNA were pooled and purified by a second isopycnic centrifugation. Most HSAkin17 protein was detected in fractions corresponding to DNA-protein complexes, suggesting that HSAkin17 protein was bound in vivo to DNA in HeLa cells (Fig. 1B).

We asked whether HSAkin17 protein is associated with the chromatin in a cell cycle-dependent manner. HeLa cells were treated with a microtubule poison (nocodazole) to trigger an anaphase arrest and to trap cells into mitosis (27). HeLa cells arrested in G₂-M retained HSAkin17 tightly associated with the
chromatin structure, as did mock treated cells (Fig. 1C). Therefore, HSAkin17 remains associated with the chromatin structure in both proliferating or G2-M-arrested cells. Hence, \textit{in vivo} association of HSAkin17 protein with DNA structures was independent of the cell cycle.

We next examined whether HSAkin17 protein was directly bound to DNA. We used DMS to convert heat-reversible methylene bonds induced by formaldehyde to stable DNA-protein covalent bonds that are resistant to boiling in SDS-containing buffers. Under these conditions, if HSAkin17 protein is directly associated with the chromatin, the DNA-protein complex is stabilized and cannot be separated by SDS-PAGE. Conversely, if HSAkin17 protein is linked to proteins of the chromatin, covalent methylene bonds are reversed by boiling, and the protein can migrate in SDS-PAGE. After DMS treatment the HSAkin17 band disappeared, showing that \textit{in vivo} most HSAkin17 protein is directly linked to DNA (Fig. 1D, lane 2).

\textbf{FIG. 5.} Flow cytometric analysis of BrdUrd incorporation in RASK.5 cells versus R482.1 cells. The cells were seeded 3 days before irradiation (6 Gy) and analyzed 24 h later. BrdUrd (30 \mu M) was added to the culture medium for 15 min before fixation and labeling with fluorocyanine isothiocyanate-conjugated BrdUrd antibody and counterstaining with propidium iodide as described under "Materials and Methods." A, C, E, and G, BrdUrd incorporation is shown as log fluorescence using the FL1-H channel and relative DNA content (propidium iodide) is measured by FL3-A. B, D, F, and H, cell cycle using propidium iodide (FL3-A channel). The experiments were done twice with the different clones isolated.
by serum stimulation. Under these conditions, about 25% of the cells were in S phase as determined by BrdUrd pulse incorporation and flow cytometry analysis (see Fig. 5A and data not shown). Total proteins were recovered, and the HSAkin17 protein level was assessed at different times following irradiation. Although a tremendous induction of p53 was always observed 6 and 24 h after irradiation, we usually observed an increased HSAkin17 protein level only 24 and 48 h after irradiation (Fig. 2A). At these times, most of the RKO cells were arrested in the G2 phase, as evidenced by flow cytometry (see Fig. 5E and data not shown). Under these conditions, the PCNA protein level remained unchanged (Fig. 2A).

Second, we assessed the level of HSAkin17 protein anchored to the DNA structure 24 h after IR. In this approach, we used a lysis buffer containing 1% Igepal™ (buffer N) to discriminate between cytoplasmic and soluble nuclear proteins (detergent-soluble fraction) and nuclear proteins highly anchored to DNA (DNA-bound fraction). We observed a dose-dependent increase in the DNA-bound HSAkin17 protein levels 24 h after γ irradiation starting with a dose of 0.5 Gy (Fig. 2B). No significant induction was noted in the detergent-soluble fraction. Therefore, γ irradiation mainly induced DNA-bound HSAkin17 protein. The p53 protein level increased in both fractions, suggesting that γ irradiation induced both DNA-bound p53 as well as detergent-soluble p53 (Fig. 2B). We also noted a slight increase in the PCNA content in the soluble fraction as well as an increase in the insoluble fraction only at 10 Gy. A similar increase in DNA-bound PCNA protein has already been reported at doses higher than 10 Gy (28).

To confirm that IR increased the level of HSAkin17 protein
At higher ionic strength, the number of HSAkin17 molecules associated with DNA were observed after damage to DNA (Fig. 2). Considering that 100% of HSAkin17 molecules were recovered with 1 M NaCl after irradiation at 6 Gy, we obtained only 73% at 0.5 M NaCl and 35% at 0.15 M NaCl. At 1 M NaCl we reached roughly the same percentage recovery as that obtained with an RIPA buffer. This indicated that increased amounts of HSAkin17 tightly associated with DNA were observed after damage to DNA (Fig. 2C). Considering that 100% of HSAkin17 molecules were recovered with 1 M NaCl after irradiation at 6 Gy, we obtained only 73% at 0.5 M NaCl and 35% at 0.15 M NaCl. At 1 M NaCl we reached roughly the same percentage recovery as that obtained with an RIPA buffer. This indicated that increased amounts of HSAkin17 tightly associated with DNA were observed after damage to DNA (Fig. 2C). At higher ionic strength, the number of HSAkin17 molecules increased 2.6-fold 24 h after irradiation at 6 Gy (46,000–117,000 molecules). A similar result was obtained with the parental RKO cell lines, implying that the expression of the viral EBNA-1 protein did not interfere with the HSAkin17 response (data not shown).

Localization of HSAkin17 Protein in Large Nucleoplasmic Foci Following γ Irradiation—Because several nuclear proteins involved in DNA repair/DNA damage recognition pathways concentrate into nuclear foci after irradiation, we performed immunocytochemical staining using the mAb K36 antibody to determine HSAkin17 sublocalization in RKO cells. The cells were seeded 3 days before irradiation to avoid serum stimulation of proliferation-associated proteins such as HSAkin17. In nonirradiated RKO cells, HSAkin17 showed a weak and diffuse staining pattern throughout the nucleoplasm (Fig. 3). Twenty-four hours after irradiation, enhanced HSAkin17 protein levels were clearly detected at 2 and 6 Gy. At these times, HSAkin17 protein coalesces into large foci that might correspond to the HSAkin17 fraction tightly associated with DNA.

Isolation of RASK Cells Expressing an Antisense HSAKIN17 Transcript—To evaluate the importance of foci-forming HSAkin17 protein during cell proliferation and the cellular response to ionizing radiation, we generated several clones displaying low levels of HSAkin17 protein.

In a first step, we analyzed endogenous HSAkin17 protein levels in different human cells by ELISA and Western blot. We conclude that endogenous HSAkin17 protein is tightly associated with DNA, whatever the cell line used (data not shown). We also observed that human carcinoma cells, such as RKO cells, exhibited the greatest number of HSAkin17 molecules/cell as compared with either normal human fibroblasts or other tumoral cells. For this reason, we decided to reduce the HSAkin17 protein level in RKO cells. RKO clones were isolated after transfection of the pEBVCMVAsHSAKIN17 vector (pB399as) carrying a HSAKIN17 cDNA-SV40 polyadenylation signal cartridge in an antisense orientation, followed by subsequent hygromycin B selection. These clones were termed RASK (RKO antisense HSAKIN17). From the 60 clones isolated, half died rapidly, and the others grew very slowly. After several weeks of cultivation, three RASK clones were selected and characterized in more detail (RASK.1, RASK.5, and RASK.13). Three clones carrying the pEBVCMV vector (pB482) were selected at random and used as controls (R482.1, R482.2, and R482.3).

Because RASK cells expressing the antisense HSAKIN17 cDNA were usually unstable, we systematically assessed HSAkin17 protein levels by either immunocytochemical staining or Western blot. As judged by indirect immunofluorescence, more than 95% of HSAkin17 protein was essentially localized in nucleoplasmic speckles of diameters ranging from 0.1 to 2 μm in proliferating R482 cells (Fig. 4A). In highly proliferating cells, the greater number of HSAkin17 foci inside nuclei of R482 cells led to an intense nuclear staining. We further noted that

### Table II

Flow cytometry analysis of cells pulse-labeled with BrdUrd

| Cell line   | Treatment | G0-G1 | S  | G2-M |
|-------------|-----------|-------|----|------|
| Control     | 0 Gy, 24 h| 64.5 ± 2.1 | 23.5 ± 2.1 | 11.5 ± 0.7 |
| Control     | 6 Gy      | 29.0 ± 4.2 | 6.5 ± 4.9 | 64.5 ± 9.2 |
| RASK.5      | 0 Gy      | 44.5 ± 2.1 | 34.0 ± 1.4 | 19.5 ± 0.7 |
| RASK.5      | 6 Gy      | 30.5 ± 0.7 | 8.0 ± 2.8 | 60.5 ± 3.5 |

![Fig. 6. Co-localization of HSAkin17 and RPA proteins in response to γ rays.](http://www.jbc.org/)

RKO cells were plated 1 day before treatment. Exponentially growing RKO cells untreated (A) or irradiated at 6 Gy for 24 h (B and C). Two representative figures are shown for irradiated cells. Magnification is ×500.
a few R482 cells presented the staining of an extranuclear structure close to the nuclei (indicated by arrows in Fig. 4A). In the parental RKO cells, we currently detected an extranuclear structure close to the nuclei and probably in the vicinity of the nucleosome, which indicated the HSAkin17-specific fluorescence signal. This concentrated the expression of a viral nuclear protein (EBNA-1) coded by EBV vectors could not account for the observed distribution of the HSAkin17 protein. Seven weeks after transfection and hygromycin B selection, the production of antisense HSAkin17 mRNA in RASK cells appeared to decrease strikingly the number of HSAkin17 nucleoplasmic foci, leading to a weak diffuse nuclear staining (Fig. 4A). Curiously, RASK cells maintained one or two extranuclear stained structures/cell, suggesting that HSAkin17 protein presented here was certainly very stable (arrows in Fig. 4A).

Western blot analysis of HSAkin17 protein also revealed a dramatic decrease in the HSAkin17 basal level (70–80% less) in the three antisense clones selected, as compared with controls (Fig. 4B). This reduction was specific for HSAkin17 protein because the expression of PCNA remained unchanged. Interestingly, RASK cells failed to induce HSAkin17 protein 24 h after irradiation, as did control clones. Therefore, we used RASK cells to study the biological consequences of a reduced HSAkin17 protein content in a human tumor cell.

Early and Mid-S Phase Accumulation of RASK Cells—We asked whether reduced HSAkin17 protein levels affect cell proliferation. Plating efficiencies of the different clones were assessed after seeding the same number of control and RASK cells/cm². Under these culture conditions, RASK cells exhibited a markedly decreased proliferation rate, with plating efficiencies 15-fold lower than those observed for control clones (data not shown). When cells were plated at different densities to account for their specific plating efficiencies, we also observed a dramatic decrease of growing clones in both RASK clones (Table I). Therefore, decreased levels of HSAkin17 protein strongly affected cell growth.

This decreased cell proliferation observed in RASK cells that express the antisense HSAkin17 mRNA could stem from alteration of their cell cycle. Therefore, we analyzed the cell cycle of BrdUrd pulse-labeled RASK clones by flow cytometry analysis. The cells were seeded 3 days before irradiation (6 Gy) and analyzed 24 h later. The cells were pulse-labeled for 15 min with 30 μM BrdUrd. Incorporation of BrdUrd into cellular DNA was measured by fluorescence-activated cell sorter analysis, and the percentage of BrdUrd-positive cells corresponded to S phase cells actively synthesizing DNA (Fig. 5).

Comparison of the cell cycle of RASK cells versus control cells revealed a low number of RASK.5 cells in the G1 phase (45 ± 2%) as compared with control (65 ± 2%) and an enhanced proportion of BrdUrd-positive cells with a DNA content between 2 and 4 N (34 ± 1% versus 24 ± 2%) (Fig. 5 and Table II). RASK.5 cells accumulated in early and mid-S phase, but only a few cells were detected in late S phase (Fig. 5C). This suggested that low HSAkin17 protein levels resulted in better entry into the S phase, but replicating cells were hampered in their progress to the S phase. We also detected an elevated percentage of cells in the G2 phase (20 ± 1%) as compared with control cells (12 ± 1%). We obtained similar results using RASK.13 cells (data not shown). These cell cycle modifications could explain the reduced proliferation rates observed in the RASK cells. R482.1 cells and RASK.5 cells displayed a G2 arrest 24 h after 6 Gy, with 65 ± 9% and 61 ± 4% of cells in G2 phase, respectively, indicating that HSAkin17 protein is not essential for the γ-ray-induced G2 arrest (Fig. 5, F versus H).

Endogenous HSAkin17 Co-localized with the RPA Protein 24 h after Irradiation—Because HSAkin17 is (i) associated with DNA and (ii) induced after IR at later times post-irradiation (24 h), we hypothesized that after irradiation HSAkin17 protein could be associated with remaining DNA lesions. This prompted us to compare the intracellular localization of HSAkin17 with a protein known to participate in the DNA repair processes.

In mock-irradiated proliferating RKO cells, RPA was uniformly distributed throughout the nucleoplasm as a dispersed and punctate pattern that corresponds to replication foci (Fig. 6A). After γ irradiation, RPA concentrated in nuclear foci of very strong intensity and almost all the bright RPA foci co-localized with HSAkin17 (Fig. 6, B and C). These results suggested that HSAkin17 and RPA could cooperate in response to IR-induced DNA lesions. However, RPA and HSAkin17 foci of strong intensity were never detected soon after irradiation at 6 Gy (3 h and 6 h) in proliferating RKO cells nor at later times post-irradiation in slowly proliferating RKO cells (data not shown). Co-immunoprecipitation experiments were unsuccessful in demonstrating a strong physical interaction between endogenous HSAkin17 and RPA-70 proteins in RKO cells. We assumed that (i) HSAkin17 and RPA belong to a same high molecular weight complex without direct interaction between them, (ii) only a small fraction of both proteins participate in the same nuclear foci, and (iii) constitutive amounts of both HSAkin17 and RPA proteins were too low to be detected under our experimental conditions.

DISCUSSION

The well conserved kin17 proteins are DNA-binding proteins activated in response to ionizing and UVC irradiations (19, 20). Prior studies were mainly performed at the mRNA level. Recently, the production of large amounts of human recombinant HSAkin17 protein made it possible to obtain a panel of new monoclonal antibodies and to develop biochemical approaches. This also affords us the opportunity to quantify endogenous HSAkin17 protein levels in different human cells using ELISA. Normal, immortalized, or tumoral cells present a wide range of HSAkin17 protein levels. Although proliferating normal human fibroblasts elicited a low level, non-small cell lung carcinoma cells (H1299) and colorectal carcinoma cells (RKO) display the highest level. Therefore, high HSAkin17 protein levels are observed during carcinogenesis that may be a consequence of uncontrolled proliferation or genomic instability. Alternatively, elevated levels could be required during the process of cancer development. Immunochemical staining performed with the mAb K36 antibody identified HSAkin17 as a protein mainly localized in nuclei with a staining pattern resembling those observed for other proteins involved in DNA replication. This observation is consistent with the previously reported localization of kin17 protein in HeLa cells using antibodies directed against the mouse MMUkin17 protein and raised in rabbits (12). Interestingly, certain tumor-derived cells concentrate a part of the HSAkin17 protein in a dense fluorescence focus neighboring to the nucleus, particularly in resting cells. At present, we have no explanation for this observation.

We identify here HSAkin17 as a protein tightly associated in vivo with the chromosomal DNA. We show that HSAkin17 protein exists in the cells as both a soluble fraction and a DNA-bound fraction. Other nuclear proteins involved in the DNA metabolism, such as PCNA, exhibited a similar distribution. IR triggers a redistribution of HSAkin17 protein from a soluble form to DNA-bound complexes comparable with that observed...
for PCNA (28). However, whereas an increased level of the PCNA insoluble fraction was observed at higher doses of irradiation (e.g., 10 Gy in our experiment), the DNA-bound RASK fraction increases at a lower dose (0.5 Gy). This enhanced amount of RASK bound to DNA observed after irradiation coincides with the appearance of large intranuclear focal sites of RASK scattered throughout the nucleus. We show that RASK and RPA proteins co-localized in large foci 24 h after irradiation at 6 Gy. IR-induced RPA-RASK foci were observed only in highly proliferating cells, suggesting that DNA replication is required.

The heterotrimeric RPA protein is a single-stranded DNA-binding protein required for DNA replication, recombination, nucleotide excision repair, DSB repair, and transcriptional regulation (29). RPA is a crucial component of the early stage of nucleotide excision repair, because it binds synergistically with XPA to damaged single-strand DNA, allowing the subsequent recruitment of the other repair factors at the site of DNA damage (30, 31). RPA is also involved in the gap-filling step of nucleotide excision repair, which requires PCNA, RF-C, and DNA polymerase δε (32). RPA forms discrete foci after irradiation in many cell lines. The rate of dispersion of RPA-forming proteins is usually compared with the rate of DSB repair. Kinetic experiments of DSB repair have previously shown a biphasic response with a fast component for repair of most breaks (half-time ranging from 20 to 30 min) followed by a slow component for repair of the remaining breaks (90–300 min) (33). MacPhail and Olive (34) have demonstrated that RPA co-localized with the DNA recombination protein Rad51 through its 70-kDa subunit. The highest number of Rad51-RPA co-localizations was observed 1 day following irradiation (5 Gy) of mouse fibroblasts. Furthermore, 30 h after irradiation RPA co-localizes with Rad51 in micronuclei (36). Taken together these data indicate that RPA foci are associated with unrepaired DNA damage and/or with DNA sites that are unable to replicate. The idea that a fraction of RASK protein co-localized with RPA foci at these sites of damaged DNA is further supported by the observation that RASK protein co-purifies with proteins of the replication complex of human cells.5

If RASK protein acts at the site of DNA replication, in particular of damaged DNA, a lowered RASK protein level might impede cell proliferation and decrease resistance to IR. The antisense strategy used here to constitutively decrease the RASK protein level confirmed this idea. The overexpression of an antisense RASK17 transcript led to a 75% decrease in the RASK protein level, which correlates with a reduced cell growth and increased radiosensitivity. Flow cytometry analysis of BrdUrd incorporation showed an accumulation of RASK17 antisense cells in early and mid-S phase and a subsequent increase in the number of cells in the G2 phase. The increased number of cells in the G2 phase may correspond to RASK cells undergoing DNA repair. We hypothesized that a premature entry of RASK cells into the S phase, as evidenced by the low number of cells in the G1 phase, may lead to an accumulation of DNA damage and a subsequent arrest in the G2 phase. The accumulation of RASK cells in early and mid-S phase indicates that RASK protein participates in DNA repair. Indeed, ongoing experiments revealed that RASK protein co-purifies with RPA and PCNA proteins in elution fractions corresponding to DNA replication complexes.5

Our results show that human PCNA and RASK proteins are components of the same set of replication proteins activated by ionizing radiation. The decrease in the intracellular concentration of RASK protein affects the cell cycle, apparently by interfering with proteins localized at damaged DNA sites unable to replicate. The RASK cells described here compared with other human cells unable to repair double-strand breaks will be used to test this idea further and to shed some light on the molecular role played by the human RASK protein.

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