Participation of kin17 Protein in Replication Factories and in Other DNA Transactions Mediated by High Molecular Weight Nuclear Complexes

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Abstract

The Homo sapiens kin17 (HSAkin17) protein is a chromatin-associated protein conserved during evolution and overproduced in certain human tumor cell lines. For the first time, immunoelectron microscopy analysis of endogenous HSAkin17 protein revealed an ultrastructural co-localization of HSAkin17 and bromodeoxyuridine (BrdUrd) at sites of DNA replication after either short (15 min) or long (120 min) pulses of BrdUrd labeling. After hydroxyurea (HU) or l-mimosine (Mimo) block and withdrawal, we observed that HSAkin17 was recruited onto the chromatin during the re-entry and the progression in the S phase. These results are consistent with a major role of HSAkin17 protein in DNA replication factories. Other treatments hampering replication fork progression and/or inducing double-strand breaks also triggered an accumulation and a concentration of the chromatin-bound HSAkin17 protein into large intranuclear foci 24 h post-treatment. Moreover, HU- and Mimo-induced HSAkin17 foci were retained in the nucleus after detergent extraction, suggesting a strong association with nuclear structures. Gel filtration analyses of cellular extracts showed that endogenous HSAkin17 protein co-eluted with both replication proteins RPA32 and RPA70 in a fraction containing complexes of Mr, 600,000. Interestingly, HU-induced G1-S arrest triggered an increase in the molecular weight of complexes containing HSAkin17 protein. Hence, treatments interfering with either initiation and/or elongation of DNA replication also recruited chromatin-bound HSAkin17 protein. We hypothesize that in the presence of unrepaired DNA damage, HSAkin17 protein concentrated into high molecular weight complexes probably to create a bridge that contributes to the harmonization of DNA replication and repair.

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mouse _MMUKIN17_ pathway in ΔXPA mouse cells. Furthermore, the integrity of the human _global genome repair_ is crucial for the up-regulation of _HSAKIN17_ gene. In particular, the presence of functional XPA and XPC proteins is a prerequisite for the up-regulation of _HSAKIN17_ gene expression after UVC (13). Interestingly, XPA, XPC, and replication protein A (RPA) have been implicated in DNA damage recognition (16). Together, our results indicate that _HSAKIN17_ gene may help to overcome damage provoked by several types of genotoxic stress.

Recently, we have detected a wide diversity of _HSAkin17_ protein expression among 16 cell lines in culture. In non-small cell lung cancer H1299 cells, chronic myelogenous leukemia K562 cells, and immortalized embryonic kidney HEK 293 cells, _HSAkin17_ is a relatively abundant protein (10^5 molecules per cell nucleus). Strikingly, melanoma MeWo cells present low levels of _HSAkin17_ protein (4.5 × 10^5 molecules per cell nucleus) (12). In most of the cells, these contents are near to that observed for other nuclear proteins involved in DNA metabolism such as RPA (3 × 10^5 to 2 × 10^6), ligase 1 (10^3), DNA pol β (5 × 10^5), or Ogg 1 (1.23 × 10^3) (17, 18). The endogenous _HSAkin17_ protein exists in two distinct pools in cultured human cells and the balance between them depends on the physiological state of the cells. The major pool is extracted from cells under isotonic conditions and a non-ionic detergent (detergent-soluble fraction). The minor pool corresponds to the chromatin-bound fraction (or detergent-insoluble fraction) (4). Similar results have been reported for other essential proteins involved in DNA replication such as RPA70 and PCNA (18, 19). The chromatin-bound _HSAkin17_ fraction is enriched in a dose-dependent manner 24 h after IR or etoposide treatment, both of them creating double-strand breaks (DSBs) (4, 12). RPA70 binds to chromatin at the onset of S phase forming discrete foci that co-localize with sites of early-, but not late-replicating chromatin. Interestingly, detergent-insoluble forms of RPA70 and PCNA co-localized with active S-phase DNA replication forks (20). A co-localization between _HSAkin17_ and RPA70 proteins has been observed after lasting DNA damage, reinforcing the idea that _HSAkin17_ accumulated at sites of unrepaired DNA damage (4).

Herein, we demonstrate for the first time, that the _HSAkin17_ protein strictly localizes at sites of DNA replication by immunoelectron microscopy after BrdUrd labeling. We also show that chromatin-bound fraction of _HSAkin17_ protein was recruited during the re-initiation of DNA replication and the progression throughout the S phase after either hydroxyurea (HU) or 1-mimosine (Mimo) blocks followed by drug withdrawal. Both Mimo- and HU-induced _HSAkin17_ foci were preserved in attached cells after detergent extraction in cells, indicating a strong association of _HSAkin17_ with nuclear structures during late G1 and early S phases. Gel filtration analysis revealed that endogenous _HSAkin17_ protein was distributed into three protein complexes of _M_r 400,000, 600,000, and 1,800,000. _HSAkin17_ and RPA co-eluted in a _M_r 600,000 elution fraction, which certainly contained replication complexes. These observations support that _HSAkin17_ plays a role in DNA replication during normal cell division. We extended our study by demonstrating that drugs known to interfere with elongation of DNA replication and generating DSBs in the DNA also triggered increased chromatin-bound _HSAkin17_ protein levels. Gel filtration analysis demonstrated a shift of the _HSAkin17_ protein to complexes of higher molecular weight upon HU treatment. After HU treatment, _HSAkin17_ was mainly concentrated into two complexes of _M_r 1,000,000 and 2,000,000.

Collectively, our results highlighted that _HSAkin17_ may be a component of DNA replication factories. In the presence of unrepaired DNA damage, _HSAkin17_ concentrated into high molecular weight complexes that may create a bridge between DNA replication and DNA repair.

**Results**

**Ultrastructural Localization of _HSAkin17_ Protein During DNA Replication**

_HSAkin17_ protein is directly bound to chromatin _in vivo_ in cultured human cells. Furthermore, the immunocytochemical localization of _HSAkin17_ is similar to those of different proteins involved in DNA replication such as RPA (4, 5). Therefore, we analyzed the nuclear distribution of _HSAkin17_ protein in rapport to the replication sites. We localized _HSAkin17_ protein inside nuclei of non-synchronous proliferating cells by immunoelectron microscopy after pulse BrdUrd labeling. Cells were pulse-labeled with BrdUrd for 15 or 120 min and specimens were processed for immunoelectron microscopy analysis and labeled with anti-BrdUrd and anti-_HSAkin17_ antibodies. In this approach, we used a mix of two monoclonal antibodies (purified immunoglobulins Ig K36 and Ig K58) known to recognize different parts of the recombinant _HSAkin17_ protein. BrdUrd was revealed with 10-nm-diameter gold particles and _HSAkin17_ with 5 nm particles as mentioned in the legend of Fig. 1. We show two representative images obtained with proliferating RKO cells.

_HSAkin17_ protein was detected in almost all of the BrdUrd foci scattered throughout the nucleoplasm, particularly in the peripheral chromatin 15 min after BrdUrd labeling (Fig. 1A, large arrows). Slowly proliferating cells presented 90% of the _HSAkin17_ grains in the vicinity of BrdUrd incorporation sites. Because the ultrastructural co-localization of BrdUrd with either PCNA or cyclin A has been previously demonstrated (21), it is likely that these _HSAkin17_/BrdUrd foci correspond to DNA replication domains as described elsewhere (22). Note that RKO cells displayed a chromatin structure essentially in a dispersed state as several rapidly proliferating tumor cells. After 120 min of BrdUrd incorporation, _HSAkin17_ protein foci overlapped almost all the BrdUrd foci corresponding to newly synthesized DNA (Fig. 1B). These _HSAkin17_/BrdUrd foci were larger and propagated either in the chromatin surrounding the nucleolus or in huge domains close to the lamina, certainly at sites of perinuclear and intranuclear dense chromatin. Note that few _HSAkin17_ foci did not overlap BrdUrd dots (Fig. 1, A and B, small arrows). Similar results were obtained with MRC5-V2 human fibroblasts immortalized with SV40 virus (data not shown).

To conclude, these results indicate the presence of _HSAkin17_ protein in nearly all DNA replication sites in the absence of genotoxic injuries. These sites might correspond to the so-called replication factories (22). We also observed that the _HSAkin17_ protein remains on newly replicated DNA.
Stalled Replication Forks Induced by DNA Synthesis Inhibitors Triggered Accumulation of Chromatin-Bound HisAkin17 Protein

Because HisAkin17 protein localized at sites of DNA replication and appeared to participate in this process, we sought to determine whether HisAkin17 remained bound to the sites of stalled replication forks. We first assessed the efficiency of the cell cycle arrest induced by replication inhibitors, by means of flow cytometry analysis. The percentages of S-phase cells actively synthesizing DNA were assessed after short incorporation of BrdUrd into cellular DNA (Fig. 2A). In RKO cells, the HisAkin17 immunocytochemical pattern depended on...
FIGURE 2. Recruitment of $\text{HSA} \text{kin17}$ tightly associated with a DNA structure 24 h after HU and Mimo treatment. RKO cells were seeded 4 days before treatment, treated with HU (2 mM) or Mimo (400 $\mu$M), and analyzed 24 h later. A, Flow cytometry analysis of BrdUrd-labeled cells. BrdUrd (30 $\mu$M) was added to the culture medium for 15 min before fixation and cells were labeled with FITC-conjugated BrdUrd antibody and counterstained with propidium iodide. BrdUrd incorporation is shown as log fluorescence using the FL1-H channel and relative DNA content (propidium iodide) is measured by FL3-A. Green, G0-G1 phase; red, S phase; blue, G2-M phase. B, Immunocytochemical staining of $\text{HSA} \text{kin17}$ 24 h after HU and Mimo treatment. RKO cells were fixed with PFA (4%, 20 min and permeabilized with Triton X-100 for 5 min) or C. Proteins were extracted with Triton X-100 for 5 min before PFA fixation.
their proliferation rate. While highly proliferating cells exhibited an intense dot-like nuclear staining, slowly proliferating cells and resting cells elicited a dramatic decrease in this staining. Therefore, cells were plated 4 days before recovery and displayed low endogenous levels. Hence, asynchronously and slowly proliferating RKO cells were treated with either HU (2 mM) or Mimo (400 μM) for 24 h. Cells were pulse-labeled with 30 μM of BrdUrd for 15 min before fixation.

We used HU and Mimo to perturb DNA replication. HU is known to reduce the deoxynucleotide triphosphate pool and to block the elongation of DNA replication. HU treatment resulted in a population of RKO cells predominantly stalled at the G1-S border and in very early S phase (Fig. 2A). Mimo induces a cell cycle arrest of human cells in late G1 phase close to the G1-S border, before the establishment of active DNA replication forks (21). Under our conditions, an overnight treatment of RKO cells with 400 μM Mimo resulted in a population synchronized in late G1 phase before entry into S phase (Fig. 2A). We detected a clear increase in the intranuclear amount of HSAkin17 protein [without Triton X-100 extraction (Fig. 2A)]. We detected a clear increase in the intranuclear amount of HSAkin17 protein [without Triton X-100 extraction before parafomaldehyde (PFA) fixation] after either HU or Mimo treatment in RKO cells (Fig. 2B). Note that more intense stainings were obtained after PFA fixation as compared to acetone/methanol fixation (Fig. 2B versus Fig. 4C). Under these conditions, the immunocytochemical detection of HSAkin17 protein was similar to that of RPA32. The in situ detection of the chromatin-bound HSAkin17 protein in attached cells was performed after detergent extraction. Cells were permeabilized with 0.5% Triton X-100 in modified CSK buffer (for 5 min) to remove soluble cytoplasmic and nuclear proteins and then fixed. Under this condition, remaining HSAkin17 and RPA32 proteins were detected in nuclear aggregates (Fig. 2C). In mock-treated cells, HSAkin17 protein was barely visible after detergent extraction.

Our data indicate that HU or Mimo treatment provokes the concentration of a fraction of the nuclear HSAkin17 protein onto particular nuclear structures (chromatin and nuclear matrix) at sites of stalled replication forks.

**The DNA Replication Re-Start Observed After Either HU or Mimo Treatment Correlates With Increased Levels of HSAkin17 Protein**

Because HSAkin17 protein seems associated to sites of DNA replication as evidenced by immunoelectron microscopy, we sought to determine a relationship with the progression (or alternatively the arrest) of replication forks. Cells were seeded 4 days before treatment and confluent cells were treated with either HU (2 mM) or Mimo (400 μM) for 24 h. At different times following drug removal, cells were analyzed for either DNA content (flow cytometry) or HSAkin17 protein content (Western blot). Cytoplasmic and soluble nuclear proteins (soluble fraction) and chromatin-bound proteins (insoluble fraction) were recovered separately and analyzed as reported (4).

The determination of the DNA content by means of propidium iodide and flow cytometry clearly revealed that HU-treated cells re-entered into the S phase more rapidly than Mimo-treated cells (Fig. 3A). Cells arrested in late G1-early S phase after the HU treatment progressed in the cell cycle 1 h after drug withdrawal and crossed over the S phase 3 and 6 h later. Cells got out the S phase 9 h after drug removal. In opposition, Mimo-treated cells blocked in late G1, re-entered into the S phase only 6 h after drug withdrawal, and passed through the S phase at 9 h (Fig. 3B). During this period, different kinetics of HSAkin17 protein recruitment were observed. After 24-h HU treatment, HSAkin17 protein content increased in both soluble and DNA-bound protein fractions (Fig. 3C). When cells traversed the S phase, a regular increase of DNA-bound HSAkin17 was observed accompanied by a decrease in the soluble fraction. In confluent RKO cells, HU withdrawal induced a similar protein expression profile for PCNA. On the contrary, cyclin A protein recruitment was mainly detected in the fraction of proteins weakly associated with DNA structures at early times following HU withdrawal, suggesting a rapid re-entry of cells into the S phase. After Mimo treatment, cells encountered major hindrances to enter into the S phase and remained blocked at the G1-S border for a longer period of time. As a consequence, we observed elevated DNA-bound HSAkin17 protein levels during the whole period of time, as well as an increased cyclin A (soluble fraction) protein content (Fig. 3D).

Our data revealed that the amount of HSAkin17 protein increases during the re-initiation of DNA replication as well as during the progression through the S phase, suggesting its recruitment in the so-called replication factories.

**HSAkin17 Protein Is Recruited During Initiation and/or Elongation of DNA Replication**

To further investigate the relationship between replication and the intranuclear concentration of kin17 protein, we treated RKO cells for 24 h with agents able to inhibit the elongation of DNA replication. Slowly proliferating cells were treated with Mimo (400 μM), aphidicolin (APH, 15 μM), HU (2 mM), camptothecin (CPT, 0.1 μM), VP16 (20 μM), or γ irradiation (6 Gy). We determined the efficiency of the cell cycle arrest induced by these replication inhibitors (Figs. 2A and 4A, and 12). APH, an inhibitor of DNA polymerase α and δ, blocks the elongation of DNA replication and APH-treated cells accumulated in early S phase (Fig. 4A). We also tested drugs which interfere with either DNA topoisomerase I or II (topo I and topo II) creating single- and DSBs in DNA. CPT interferes with the resealing activity of topo I. This stabilizes a topo-I-DNA intermediate which hampers the relaxation of topo-I linker single-strand breaks creating DNA damage (e.g., DSBs). VP16 is a specific inhibitor of topo II. Interestingly, DNA replication can initiate in the absence of both enzymes, but elongation stops after a couple of thousand base pairs. While CPT exerts its effect predominantly during the mid and late S phase of the cell cycle, VP16 leads to an arrest of cells in the late S phase (Fig. 4A and 12). Treatment with one of these drugs resulted in a prominent G2 arrest, which may correspond to the accumulation of DNA-damaged cells.

HU-treatment increased the level of chromatin-bound HSAkin17 protein followed by the relocalization of HSAkin17 protein in dot-like structures with a pattern characteristic of replication loci (Figs. 4, B and C; Fig. 2B). This result suggests that stalled replication forks due to nucleotide deprivation in early S phase recruited DNA-bound HSAkin17 protein. We cannot rule out the possibility that HSAkin17 protein was also required after HU-induced DSBs. Furthermore, HSAkin17...
FIGURE 3. Chromatin-bound HSAkin17 protein levels increased during the re-initiation of DNA synthesis and the progression into the S phase. RKO cells were seeded 4 days before treatment with either HU (2 mM) or Mimo (400 μM). Twenty-four hours later, fresh medium was added and cells were recovered at the indicated times. A, B. Cell cycle analysis of RKO cells after either HU or Mimo blocks. C, D. HSAkin17 protein content analysis after either HU or Mimo blocks. At the indicated times, cells were trypsinized, counted, and lysed in the buffer N to recover separately chromatin-bound proteins and detergent-soluble proteins. Proteins were analyzed by Western blot.
FIGURE 4. Stalled replication forks and DSBs triggered the accumulation of the chromatin-bound HSAkin17 protein in RKO cells. RKO cells were seeded 4 days before treatment and analyzed 24 h later. A, Flow cytometry analysis of BrdUrd-labeled cells after APH or CPT treatment. B, RKO cells were trypsinized, counted, and lysed to recover separately chromatin-bound proteins and detergent-soluble proteins. Proteins were analyzed by Western blot. C, RKO cells were fixed with ethanol/acetone and HSAkin17 was identified with purified Ig K36 mAb.
protein accumulates after Mimo and APH in a manner similar to that observed after HU. These data suggest that stalled replication forks at the G1-S border or in early S phase induce the accumulation of HSAkin17 protein (Fig. 4B).

We asked further if the formation of DSBs after IR, CPT, and VP16 was able to enhance the level of HSAkin17 protein. We showed that irradiation, CPT, or VP16 treatment led to the accumulation of DNA-bound HSAkin17 protein into discrete nuclear foci (Fig. 4, B and C). It was noteworthy that an increase of chromatin-bound RPA70 protein was detected after these treatments in parallel with a decrease of the nucleoplasm-associated RPA70, suggesting a shift in the equilibrium (Fig. 4B). In opposition to Fig. 3, where RKO cells were at confluence before treatment, cells in Fig. 4 were in a slowly proliferating state contributing to an elevated PCNA basal level. In these conditions, we failed to detect significant variation in PCNA in both protein fractions.

We conclude that the inhibition of DNA replication by several types of chemical and physical agents producing DSBs is correlated with increased intranuclear levels of HSAkin17 protein but not of other nuclear proteins like PCNA.

G1-S Arrest Leads to the Association of HSAkin17 Protein to High Molecular Weight Complexes

We tested whether the dot-like nuclear structures formed by HSAkin17 protein and detected by immunocytochemical staining corresponded to the presence of HSAkin17 protein in discrete high molecular weight complexes that may be detected by biochemical methods. Firstly, we analyzed the molecular mass of purified HSAkin17 protein. The (His)$_6$HSAkin17 protein was overexpressed and purified to homogeneity from extracts of baculoviral infected Sf9 cells using metal-affinity and heparin-based chromatography (5). Purified (His)$_6$HSAkin17 protein was mainly eluted as a monomer with an apparent molecular mass of 49,000 Da.

FIGURE 5. Chromatographic separation of endogenous HSAkin17 protein into high molecular weight complexes after HU treatment. A. Gel filtration analysis of (His)$_6$HSAkin17 protein. Five hundred nanograms of (His)$_6$HSAkin17 protein were separated by size exclusion chromatography through a Superdex 200 HR 10/30 column and fractions were analyzed by SDS-PAGE using anti-His monoclonal antibody. The column was calibrated using apoferritin ($M_r$ 440,000), $\alpha$-amylase ($M_r$ 200,000), aldolase ($M_r$ 158,000), and BSA ($M_r$ 67,000) (data not shown). B. H1299 cells were seeded 1 day before treatment and buffer N-soluble proteins were recovered and fractionated through a Superose 6 HR 10/30 column as described in “Materials and Methods.” Proteins were analyzed by 12% SDS-PAGE. The numbers refer to the analyzed column fractions. The arrows above the gel indicate the fraction numbers at which the standards eluted: apoferritin ($M_r$ 440,000); thyroglobulin ($M_r$ 660,000); and dextran blue ($M_r$ 2,000,000).
FIGURE 6. Co-elution of HSAkin17 and RPA into high molecular weight complexes after HU treatment. Fractionation of both buffer N-soluble and chromatin-bound proteins from (A) mock-treated H1299 cells or (B) H1299 cells treated with 2 mM HU for 24 h. Legend is the same as in Fig. 5.
HSAkin17 Is a Component of Active Replication Factories

molecular weight lower than $M_r$ 67,000 corresponding to BSA (Fig. 5A), as evidenced by gel filtration using a Superdex 200 HR 10/30 column. These data indicate that the purified (His)$_{6}$-HSAkin17 has a monomeric structure and behaves as a globular protein.

Secondly, we determined the molecular weight of the endogenous HSAkin17 protein in H1299 cells. Detergent-soluble proteins and chromatin-bound proteins were recovered from either mock-treated or HU-treated H1299 cells and analyzed separately by gel filtration. Under gentle conditions of lysis in buffer N, most of the HSAkin17 and RPA70 protein content was detected as detergent-soluble proteins in the absence of genotoxic injuries (as shown for RKO cells in Fig. 4B). Gel filtration revealed the absence of monomeric HSAkin17 protein in the extracts of H1299 cells with or without treatment (Fig. 5). This result suggested that endogenous HSAkin17 protein was mainly present in huge nuclear complexes. Indeed, HSAkin17 protein was distributed into three protein fractions with molecular masses corresponding to about $M_r$ 400,000 and 600,000 and 1,800,000 named here peaks I, II, and III (Figs. 5B and 6A). Note that HSAkin17 and RPA proteins (both the $M_r$ 70,000 and 32,000 subunits) co-eluted in fraction 19 (Fig. 6A). HSAkin17 and RPA proteins were also co-eluted into very high molecular weight structures of about $M_r$ 1,800,000 (peak III, Fig. 6A).

The three HSAkin17-containing peaks displayed a significant shift toward higher molecular weights in cells presenting a cell cycle arrest at the G1-S transition after a 24-h HU treatment (Figs. 5B and 6B, fraction 13). RPA conserved the same expression profile after HU treatment and was eluted in fractions 19–21. Nevertheless, HU treatment led to the concentration of both HSAkin17 and RPA proteins in peak III at roughly $M_r$ 2,000,000 (Fig. 6B, fractions 3–5). It was noteworthy to observe a population of slower migrating RPA32 after HU treatment corresponding to hyperphosphorylated forms. Interestingly, the hyperphosphorylation of RPA32 is a cellular marker for activation of the DNA damage response pathway.

Peak II was clearly detected in fractions of chromatin-bound proteins soluble in RIPA buffer, a relatively stringent buffer that sustains only high-affinity interactions. This peak clearly shifted to higher molecular weights after HU treatment (Fig. 6, A versus B). Under our experimental conditions of protein extraction using RIPA buffer to recover chromatin-bound proteins, we failed to detect RPA. However, each fraction analyzed corresponded to a very low number of cell equivalents (3,000 cells) opening the possibility to be below the detectable threshold. The fact that these peaks were preserved in RIPA buffer suggests strong protein interactions between the different building blocks.

These data indicate that the re-localization of HSAkin17 protein into intranuclear foci after an HU treatment corresponds to a re-distribution of HSAkin17 protein in stable complexes of high molecular weight and suggest that HSAkin17 and RPA are part of a multiprotein complex of $M_r$ 600,000 eluted in the peak II of detergent soluble proteins.

Discussion

The notion that HSAkin17 protein is involved in DNA replication stemmed from the following observations: (a) the distribution of endogenous HSAkin17 protein in intranuclear dots in proliferating cells resembled those of proteins, such as RPA70, involved in DNA replication (4); (b) endogenous kin17 protein physically interacts with the SV40 large T antigen when this protein is bound to the SV40 DNA origin of replication; (c) both T antigen and human HSAkin17 protein are part of the same high molecular weight multiprotein complex in infected human cells; (d) the overexpression of human HSAkin17 protein in vitro or the introduction of increased amounts of HSAkin17 protein in an in vitro assay reduced T antigen-dependent DNA replication (5); and (e) RKO cells expressing HSA KIN17 antisense transcripts displayed a premature entry into S phase and an accumulation of cells in early and mid S phase (4). All these data are reinforced by the fact that DSB-generating agents, such as IR, induce the accumulation of chromatin-bound HSAkin17 protein and its re-localization into larger foci in different human cell lines probably at sites of unrepaired DNA damage that may inhibit or slow down the replication process (4, 12).

Replication foci are commonly detected as numerous granules distributed throughout the nucleus as shown by light microscopic analysis (24). The ultrastructural localization of DNA replication sites by means of immunoelectron microscopy reveals replication factories at the periphery of condensed chromatin areas and that the neosynthesized DNA moves rapidly from replication sites toward the interior of condensed chromatin areas (25, 26). Nascent DNA, DNA polymerase α, cyclin A, or PCNA are located at the border of condensed chromatin (21, see 26). For the first time, we identified HSAkin17 protein near or at the sites of DNA replication. Almost all of the HSAkin17 foci co-localized with BrdUrd incorporation sites. It is difficult to affirm that HSAkin17 protein is required preferentially during the initiation of DNA replication because newly synthesized DNA moves rapidly toward the interior of condensed chromatin, after 5 min of BrdUrd incorporation. After a period of 1 h, DNA replication in one replication cluster present in a “replication factory” is nearly complete (24). Under these conditions, the co-localization of HSAkin17 with BrdUrd after a pulse of 120 min indicated that either (a) HSAkin17 protein remains associated with sites of newly duplicated DNA, or (b) HSAkin17 re-associated with new replication sites because waves of DNA synthesis consist of different groups of foci activated in turn (26). These data strengthened the notion that endogenous HSAkin17 protein is involved in DNA replication.

While HSAkin17 protein is expressed during the whole cell cycle of asynchronous proliferating cells (unpublished data), the chromatin-bound fraction of HSAkin17 protein increased after treatment that completely abolished DNA replication like Mimo or HU. Although Mimo alters nucleotide pool levels in vivo inhibiting the elongation step of DNA synthesis, it also inhibits the initiation of DNA replication as well (27). As a consequence, cells were blocked since the late G1, before the entry into the S phase. The increase in the chromatin-bound HSAkin17 fraction after Mimo treatment and 1 h after drug withdrawal suggests its participation during the re-initiation of DNA replication. This observation was confirmed by HU treatment which mainly blocked cells in early S phase. We observed a time-dependent increase of the chromatin-bound HSAkin17 protein content after drug withdrawal when cells
traversed the S phase. If HSAkin17 protein is involved in DNA replication and especially during the early phases of this process, this progressive increase could be required either (a) to prepare the second wave of DNA replication after HU withdrawal, or (b) to avoid a re-entry in S phase before the completion of DNA replication. This expression profile is reminiscent of those observed for other proteins associated with the initiation of DNA replication (6).

Certain inhibitors of DNA replication may trigger the redistribution of DNA ligase I and PCNA from replisome patterns leading to the disassembly of replication factories, such as the topoisomerase II inhibitor VP16 (28). Other inhibitors, such as APH (inhibitor of DNA polymerase α and δ), were unable to disperse the enzymes belonging to replication factories. In the particular case of HSAkin17 protein, all the tested drugs interfering with either initiation and/or elongation of DNA replication induced its recruitment to chromatin together with RPA70. This suggests that HSAkin17 and RPA70 proteins remained strongly anchored to chromosomal DNA after genotoxic injuries. In the absence of genotoxic injury, most of the nuclear HSAkin17 and RPA70 proteins are found free in the nucleosol or slightly associated with nuclear structures (buffer N-soluble fraction), whereas only a minor fraction of both proteins is associated with chromatin (buffer N-insoluble fraction). This observation confirms data previously reported for RPA70 (18).

It was noteworthy that since endogenous HSAkin17 protein level has been reported to increase in S-phase cells, APH, VP16, or CPT treatments could indirectly increase the HSAkin17 protein level by increasing the number of cells arrested in S phase. However, HSAkin17 protein was also recruited by other treatments (HU, MIMO, or γ rays), triggering a dramatic decrease of the number of cells in S phase. This observation outlined that the HSAkin17 protein could be required for both DNA replication and repair.

HSAkin17 protein forms nuclear foci in normal proliferating cells as well as in response to aberrant DNA structures including DSBs and stalled replication forks. These dot-like structures are tightly anchored to DNA structures as shown by detergent extraction before cell fixation. This type of distribution is characteristic of proteins involved in DNA metabolism such as PCNA (19, 29). On the other hand, the co-localization of HSAkin17 with BrdUrd might correspond to high molecular weight complexes devoted to either DNA replication or DNA repair. This view is consistent with the biochemical detection of HSAkin17 protein in three peaks of high molecular weight in H1299 cells before and after HU treatment (Fig. 6). Interestingly, HSAkin17 protein co-eluted with RPA in the peak II of Mr 600,000 corresponding to the replication complex as mentioned elsewhere (30). HSAkin17 could also participate in the formation of other complexes displaying different functionalities. The increase in the molecular weight of the three peaks containing HSAkin17 protein after HU-induced G1-S arrest is not surprising. Indeed Chiba and Parvin (31) have shown the appearance of a specific complex induced by HU and termed HUIC. Our results suggest that a HU-induced G1-S arrest might lead to a molecular remodeling of nuclear complexes containing HSAkin17 protein and other proteins involved in DNA replication and repair. This idea is also supported by the recent finding that RKO cells overproducing HSAkin17 protein present higher DNA content as judged by flow cytometry analysis (12). Now, the biochemical characterization of peaks I, II, and III will help us to further determine the role of HSAkin17 protein in DNA replication.

Materials and Methods

Cell Cultures

RKO (colorectal carcinoma) and H1299 (human non-small cell lung cancer) were maintained in a DMEM (Invitrogen Life Technologies, Inc., Carlsbad, CA) supplemented with 10% FCS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin, under 5% CO₂.

Cells were seeded 3 or 4 days before treatment. Irradiation was performed using a 137Cs source (IBL 637, CisBio International, Gif sur Yvette, France) with a dose rate of 1.9 Gy/min and chemical treatments were done with either APH, etoposide (VP16), CPT, HU, or MIMO (all from Sigma Chemical Co., St. Louis, MO) at the doses indicated below. Samples were analyzed 24 h later. Experiments were repeated more than five times under different culture conditions.

Indirect Immunofluorescence Staining

Cells were plated at 5,000 cells/cm² on glass coverslips 4 days before treatment. Twenty-four hours later, cells were fixed for 5 min in 70% acetone/30% methanol at −20 °C. Alternatively, cells were washed in PBS and extracted with 0.5% Triton X-100 in modified cold cytoskeleton buffer [CSK: 100 mM NaCl/300 mM sucrose/1 mM MgCl₂/1 mM EGTA in 20 mM potassium phosphate buffer (pH 6.8)] for 5 min at room temperature. After two washes in PBS, cells were fixed with 4% PFA for 20 min at room temperature, washed with PBS, and treated for 10 min with 0.5% Triton X-100 + 0.5 mM CuSO₄ in PBS min. Coverslips were stored in PBS at 4 °C before staining. Primary antibodies were diluted in buffer B (0.5% Tween 20, 12% BSA, 0.036% NaN₃ in PBS) and incubated for 45 min. Cells were stained with the purified Ig K36 anti-HSAkin17 (400 ng/ml). Primary antibodies were revealed with Cy3-conjugated affinity-purified goat anti-mouse IgG (2 μg/ml; The Jackson Laboratory, Inc., West Grove, PA). Cells were counterstained with 4',6-diamino-2-phenylindole (DAPI; 4 μg/ml). Immunochemistry staining was viewed using a Zeiss Axioshot 2 epifluorescence microscope coupled to a cooled Sensys 1400 camera from Photometrics monitored by the Zeiss KS500 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.

Protein Extraction and Western Blot

RKO cells were seeded at 5,000 cells/cm² 4 days before treatment. Cells were treated at about 50% of confluence and 24 h later, cells were trypsinized, counted, and washed in PBS. To discriminate between chromatin-bound proteins versus detergent-soluble proteins, cells were lysed with 100 μl per 10⁶ cells of buffer N [50 mM Tris-HCl (pH 7.9)/150 mM NaCl/1% Igepal/1 mM EDTA/protease inhibitor cocktail (complete from Roche, Indianapolis, IN)]. Lysates were maintained on ice.
for 30 min. Soluble proteins were recovered after centrifugation (20,000 × g for 15 min). Remaining pellets (insoluble proteins) were directly denatured with 100 µl per 10^6 cells of 2 × Laemmli buffer. Both fractions were analyzed by Western blot. Purified IgG IgK36 and IgK58 were used at the concentration of 40 ng/ml. Other antibodies used were rabbit polyclonal anti-RPA32 protein (diluted to 1/300; kindly provided by R. Knippers), anti-RPA70 (mAb NA13, 50 µg/ml; Oncogene Research Products, Calbiochem, Darmstadt, Germany), and anti-PCNA (mAb PC10 diluted to 50 µg/ml; Novo Castra, Newcastle, United Kingdom).

**Gel Filtration Analysis of Endogenous HSAkin17 Protein**

Cells were seeded 1 day before treatment and collected 24 h later. After trypsinization and counting, chromatin-bound proteins and detergent-soluble proteins were recovered separately as described above except for chromatin-bound proteins which were resuspended in a RIPA buffer [50 mM Tris-HCl (pH 7.4)/150 mM NaCl/1% Igepal/0.1% SDS/0.1% sodium deoxycholate/protease inhibitor cocktail (complete from Roche)]. The equivalent of 5 × 10^6 cells of both fractions was fractionated by gel filtration through a Superose 20 HR 10/30 column (Smart System, Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) equilibrated in 50 mM Tris (pH 8)/150 mM NaCl. The columns were run at 40 µl/min at 4°C. We used protein standards analyzed under the same conditions to calibrate the column. Protein fractions (40 µl) were collected and proteins were denatured in Laemmli sample buffer, boiled for 10 min at 100°C, and analyzed by Western blot.

Purified recombinant (His)_6-HSAkin17 protein was analyzed by gel filtration through a Superdex 200 HR 10/30 column as described (5). Fractions containing (His)_6-kin17 protein were detected by Western blotting with anti-His antibody (Clontech, Palo Alto, CA) and revealed by chemiluminescence.

**Flow Cytometry Analysis of BrdUrd Incorporation**

Flow cytometry analysis of BrdUrd incorporation was performed as already described (4).

**Immunelectron Microscopy Detection of HSAkin17 and BrdUrd**

**Fixation and Embedding.** Specimens were processed as described elsewhere (21). After pulse-BrdUrd labeling, cells were fixed for 1 h at 4°C with either 4% formaldehyde (Merck, Darmstadt, Germany) in 0.1 M phosphate buffer (pH 7.3), or 1.6% glutaraldehyde (Taab Lab. Equip. Ltd., Reading, United Kingdom) in 0.1 M phosphate buffer (pH 7.3), or 1.6% glutaraldehyde (Taab Lab. Equip. Ltd., Reading, United Kingdom) equilibrated in 50 mM Tris (pH 8)/150 mM NaCl. The columns were run at 40 µl/min at 4°C. We used protein standards analyzed under the same conditions to calibrate the column. Protein fractions (40 µl) were collected and proteins were denatured in Laemmli sample buffer, boiled for 10 min at 100°C, and analyzed by Western blot.

Purified recombinant (His)_6-HSAkin17 protein was analyzed by gel filtration through a Superdex 200 HR 10/30 column as described (5). Fractions containing (His)_6-kin17 protein were detected by Western blotting with anti-His antibody (Clontech, Palo Alto, CA) and revealed by chemiluminescence.

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Participation of kin17 Protein in Replication Factories and in Other DNA Transactions Mediated by High Molecular Weight Nuclear Complexes \(^1\) EDF contract no. 8702.

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