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Abstract

In response to replication block or DNA damage in S phase the DNA replication and DNA damage checkpoints are activated. The current model in human predicts, that a Rad17/Replication factor C (RF-C) complex might serve as a recruitment complex for the Rad9/Hus1/Rad1 complex to sites of replication block or DNA damage. In this study we have investigated the fate of the Rad17/RF-C complex after treatment of synchronized Hela cells with the replication inhibitor hydroxyurea. In hydroxyurea treated cells the RF-C p37 subunit became more resistant to extraction. Moreover, co-immunoprecipitation studies with extracts of hydroxyurea treated cells showed an interaction of RF-C p37 with Rad17 and of PCNA with Rad9 and RF-C p37. An enhanced colocalization of Rad17 and PCNA in late S phase after hydroxyurea treatment was observed. Our data suggested, that upon replication block a Rad17/RF-C complex is recruited to sites of DNA lesions in late S phase, binds the Rad9/Hus1/Rad1 complex and enables it to interact with PCNA. An interaction of Rad17/RF-C with PCNA appears to be mediated by the small RF-C p37 subunit, suggesting that PCNA might provide communication between replication checkpoint control and DNA replication and repair.
Colocalization of human Rad17 and PCNA in late S phase of the cell cycle upon replication block

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In response to replication block or DNA damage in S phase the DNA replication and DNA damage checkpoints are activated. The current model in human predicts, that a Rad17/Replication factor C (RF-C) complex might serve as a recruitment complex for the Rad9/Hus1/Rad1 complex to sites of replication block or DNA damage. In this study we have investigated the fate of the Rad17/RF-C complex after treatment of synchronized Hela cells with the replication inhibitor hydroxyurea. In hydroxyurea treated cells the RF-C p37 subunit became more resistant to extraction. Moreover, co-immunoprecipitation studies with extracts of hydroxyurea treated cells showed an interaction of RF-C p37 with Rad17 and of PCNA with Rad9 and RF-C p37. An enhanced colocalization of Rad17 and PCNA in late S phase after hydroxyurea treatment was observed. Our data suggested, that upon replication block a Rad17/RF-C complex is recruited to sites of DNA lesions in late S phase, binds the Rad9/Hus1/Rad1 complex and enables it to interact with PCNA. An interaction of Rad17/RF-C with PCNA appears to be mediated by the small RF-C p37 subunit, suggesting that PCNA might provide communication between replication checkpoint control and DNA replication and repair.

Keywords: DNA replication checkpoint; hRad17; PCNA; hRad9; confocal microscopy

Introduction

In response to replication block or DNA damage in the DNA synthesis phase (S phase) of the cell cycle eukaryotic cells slow down progression through S phase and delay the onset of mitosis (M). These responses are summarized as intra S and S/M replication checkpoint and DNA damage checkpoint. Studies in budding yeast showed, that the slowing down of S phase progression is due to suppression of replication from late origins (Santocanale and Diffley, 1998, Shirahige et al., 1998) and allows the stabilization of existing replication forks (Tercero and Diffley, 2001, Lopes et al., 2001). The replication checkpoint also directly targets components of the replication machinery. It is assumed, that the DNA replication checkpoint serves to maintain the replisome and fork in an open and competent structure (Donaldson and Blow, 2001). Studies in fission yeast have shown a role for members of the so-called Rad protein family, including Rad17, Rad1, Rad9 and Hus1, in the activation of DNA replication and DNA damage checkpoints (Mitchell, 2001). Replication factor C (RF-C), a pentameric protein complex, serves as a clamp loader for proliferating cell nuclear antigen (PCNA) in DNA replication and repair (Mossi and Hübscher, 1998). In yeast, RF-C subunits also function in the DNA replication and DNA damage checkpoint control pathways. Fission and budding yeast cells carrying a deletion or mutation in genes encoding small RF-C subunits are defective in the DNA replication and DNA damage checkpoint functions (Reynolds et al., 1999; Shimada et al., 1999; Noskov et al., 1998; Gary Schmidt et al., 2001; Sugimoto et al., 1996, 1997). An alternative RF-C complex consisting of the four small RF-C subunits and cell cycle checkpoint protein Rad24/Rad17 was isolated from uncompromised budding yeast (Green et al., 2000) and human cells (Lindsey-Boltz et al., 2001). Using in vitro transcribed/translated human (h) RF-C subunits and hRad17 it was shown, that complex formation is dependent on interaction of hRad17 with the p38 subunit of hRF-C. Purified hRad17/hRF-C complex has similar properties like replicative hRF-C: it exhibits ATPase activity, which can be strongly stimulated by ssDNA and binds preferentially to primed DNA. In contrast to replicative hRF-C, however, the ATPase activity of hRad17/ hRF-C complex is not stimulated by PCNA (Lindsey-Boltz et al., 2001).

By yeast two hybrid analysis it was shown that hRad17 interacts with hRad1 (Parker et al., 1998). The checkpoint proteins hRad9, hHus1, and hRad1 can be co-immunoprecipitated with hRad17 from human cell extracts (Rauen et al., 2000). Structure and sequence based alignments predict that hRad9, hHus1, and hRad1 form a PCNA like clamp (Venclovos and Thelen, 2000). hRad9, hHus1, and hRad1 expressed in baculovirus infected insect cells indeed form a trimeric complex like PCNA (Burtelow et al., 2001). Electron microscopical analyses showed that Rad17/RF-C...
complexes form U-shaped particles resembling replicative RF-C and that hRad9/hHus1/hRad1 (9-1-1) complexes form flat rings with a distinct hole very similar to PCNA (Griffith et al., 2002). The hRad17/hRF-C complex is thought to load the 9-1-1 complex onto the DNA at the site of a DNA lesion during an early stage of checkpoint activation (Mitchell, 2001). Recombinant hRad1 and hRad9 purified from E. coli exhibit 3'-5' exonuclease activity (Parker et al., 1998; Bessho and Sancar, 2000), suggesting that these proteins might play a role in the processing of DNA lesions.

Upon treatment with hydroxyurea (HU) or ionizing radiation the interaction of hHus1 with PCNA is enhanced, suggesting that PCNA recruits the 9-1-1 complexes to sites of replication block or DNA damage (Komatsu et al., 2000). PCNA is a protein with multiple interaction partners in DNA metabolism: it serves as a sliding clamp for polymerase δ in DNA replication and interacts with proteins involved in Okazaki fragment processing and joining, DNA repair, DNA methylation, chromatin assembly and cell cycle checkpoint control (Tsurimoto, 1999). This suggests that PCNA has a recruiting or coordinating function for proteins playing a role in the manipulation and the preservation of the genetic material. PCNA could provide communication between DNA replication, DNA repair and cell cycle checkpoint control (Loor et al., 1997).

In this study we investigated the fate of the hRad17/hRF-C complex upon treatment of Hela cells with HU or the DNA damaging agent 4-nitroquinoline (4-NQO) in S phase. By immunocytochemical analysis we showed that hRad17 is a constitutively nuclear protein throughout the cell cycle. hRad17 could be co-immunoprecipitated with hRF-C p37 from extracts of HU treated cells, indicating that the hRad17/hRF-C complex is present in Hela cells under conditions of replication block. Upon checkpoint activation hRF-C p37 was recruited to the DNA. Further immunoprecipitation experiments revealed that PCNA interacted with hRad9 and with hRF-C p37 in HU treated cells. In untreated Hela cells hRad17 localized with sites of BrdU incorporation and PCNA in early S phase, to a low extent in mid S phase and only marginally in late S phase. Upon treatment with HU or 4-NQO hRad17 and PCNA showed enhanced colocalization in the late S phase of the cell cycle. Our data suggest that a hRad17/hRF-C complex is recruited to the DNA upon replication block or DNA damage in late S, binds the 9-1-1 complex and enables it to interact with PCNA. Our immunocytochemical data point to a surveyor/sensor role of hRad17 in early S phase and to a sensor/signal transmitter role in late S phase.

Results

hRad17 is a constitutively nuclear protein during the cell cycle

In order to investigate the subcellular localization of hRad17 during the cell cycle we performed immuno-

cytotoxicity with Hela cells synchronized in the G0/G1, S or G2/M phases of the cell cycle using anti-hRad17 antibody sc-5613. The antibody was monospecific in immunoblot analysis with Hela total extracts for a protein of approximately 76 kD, which corresponds very well to the theoretical molecular weight of hRad17 of 75.8 kD. The antibody was also specific for native GSThRad17 in dot blot analysis (Figure 1b). In all synchronized cell populations hRad17 is located in the nucleus showing that it is a constitutively nuclear protein in Hela cells during the cell cycle (Figure 1a). We detected a punctuate hRad17 pattern in the nucleoplasm and in some cells a partial overlap with nucleoli. In contrast to Chang et al. (1999), however, we did not detect a constitutive and strong localization of hRad17 in nucleoli in uncompromised cells. The same result was obtained, irrespective whether we used synchronized or unsynchronized cells, paraformaldehyde fixation alone or paraformaldehyde fixation in combination with a stepwise dehydration and rehydration with different methanol dilutions. The differences between our data and the report by Chang et al. (1999) are likely due to the usage of different antibodies with different specificities for immunocytochemical analysis and of different fixation procedures.

Recruitment of hRF-C p37 to the DNA upon checkpoint activation

Yeast mutants carrying a deletion of or a mutation in the gene encoding the hRF-C p37 homologue RF-C2 are deficient in the DNA replication checkpoint function (Reynolds et al., 1999; Noskov et al., 1998) or are defective in the S phase DNA damage checkpoint (Gary Schmidt et al., 2001). By immunoblot analysis of total extracts from equivalent Hela cell numbers we detected increased extraction resistance of hRF-C p37 at physiological salt conditions in the presence of ethidium bromide and 0.1% Triton X-100 after treatment with HU or with the DNA damaging agent 4-NQO (Figure 2a). Extraction resistant hRF-C p37 could be released by treatment of cells with S7 nuclease, showing that hRF-C p37 was less extractable due to recruitment to the DNA (Figure 2b). In these cells we also detected hyperphosphorylated hRad9 reflecting checkpoint activation (Figure 2a, Chen et al., 2001b). The increased DNA binding of hRF-C p37 in Hela cells treated with HU or 4-NQO is therefore caused by the activation of the DNA replication and DNA damage checkpoint responses. It could reflect prolonged progression through S phase, in the case of 4-NQO treatment DNA repair or in the case of HU treatment other checkpoint mediated responses like stabilization of replication forks. We additionally observed an increased amount of hRF-C p140 and hRad9 in S phase cells in comparison to unsynchronized cells, which consist predominantly of cells in G0/G1. In contrast to hRF-C p140, hRad17 is already quantitatively extracted at physiological salt conditions in the presence of ethidium bromide and 0.1% Triton
X-100 and showed a similar extraction pattern as the replication and repair protein PCNA (Figure 2a). The abundance of hRad17 or hRF-C p37 did not change following a block to DNA synthesis or DNA damage (Figure 2c,d).

Interactions of replication proteins and checkpoint Rad proteins in HU treated cells

In budding yeast the interaction of the hRad17 homologue Rad24 with the small RF-C subunits is essential for DNA damage checkpoint control throughout the cell cycle (Naiki et al., 2000). Furthermore, Rad24 interacts functionally with the small RF-C subunit RF-C5 in the DNA replication and in the DNA damage checkpoint pathways. Rad24 can be co-immunoprecipitated with the small RF-C subunits from uncompromised yeast (Shimomura et al., 1998; Green et al., 2000). We could co-immunoprecipitate hRad17 with the hRF-C subunit p37 from extracts of Hela cells treated with HU (Figure 3a), showing that both proteins interacted under conditions of replication block in human cells. Additional co-immunoprecipitation experiments revealed, that PCNA interacted with checkpoint protein hRad9 (Figure 3b), suggesting that PCNA recruits the 9-1-1 complex to sites of replication block. The hRad17/hRF-C complex is thought to load the 9-1-1 complex onto the DNA at the site of a DNA lesion during an early stage of checkpoint activation (Mitchell, 2001). After immunoprecipitation of PCNA from extracts of HU treated cells we could detect co-immunoprecipitated hRF-C p37 (Figure 3c), but not hRad17 (Figure 3c). This indicates, that PCNA and hRad17 do not interact in HU treated cells. Alternatively, the interaction might be transient or mediated by other proteins, e.g. the small hRF-C subunits and was therefore not detected under our immunoprecipitation conditions.
Colocalization of hRad17 with sites of DNA replication in S phase

Genetic studies in yeast positioned Rad17/Rad24 and RF-C subunits at the basis of the cell cycle responses to DNA replication block and DNA damage (Lindsay et al., 1998; Walworth and Bernards, 1996; de la Torre-Ruiz et al., 1998; Sugimoto et al., 1996; Shimada et al., 1999). Genetic and biochemical analyses in yeast also proved a functional and physical interaction between Rad17/Rad24 and RF-C subunits (Shimada et al., 1999; Shimomura et al., 1998; Green et al., 2000; Naiki et al., 2000). This suggests a sensor or signal transmitter role for the Rad17/RF-C or Rad24/RF-C complexes in the DNA replication and/or DNA damage checkpoint pathways. A sensor of replication block or DNA damage is expected to accompany the replisome to survey the smooth progression of DNA replication and to be recruited to stalled replication forks or to sites of DNA damage. The distribution of replication sites shows distinct patterns in different stages of S phase and can be monitored by immunocytochemical detection of bromodeoxyuridine (BrdU) incorporation or of PCNA. In early S phase replication sites are distributed throughout the nucleus with the exception of condensed heterochromatin and nucleoli. Replication sites are very small, discrete and number several hundred. As cells proceed from early to mid S phase, nuclei show no unlabelled areas and a fairly uniform staining of the nucleus. In the late S phase the perinucleolar region replicates first, often leading to ring structures. Replication of the bulk of heterochromatin follows appearing as a pattern of large discrete foci. At this stage heterochromatin at the nuclear periphery is also replicated. At the end of S phase replication is confined to the nuclear periphery (Bravo and MacDonald-Bravo, 1987; Fox et al., 1991).

We investigated the subnuclear localization of BrdU incorporation and of hRad17 in S phase Hela cells. hRad17 and sites of DNA replication colocalized in the early S phase of the cell cycle and to a low extent in mid S phase. Only a very petty colocalization was found in late S phase (Figure 4). Thus, hRad17 might
directly monitor the accuracy of DNA replication by associating with the replication machinery in early S phase. We could confirm these data by investigating the subnuclear localization of hRad17 and the replication protein PCNA (Figure 5a).

Enhanced colocalization of hRad17 with PCNA in late S phase upon treatment with HU or 4-NQO

Our immunocytochemical studies combined with epifluorescence microscopy showed, that hRad17 colocalized with PCNA in the early S phase and to a low extent in the mid S phase of the cell cycle in untreated Hela cells (Figure 5a) and in cells treated with HU (Figure 5b) or 4-NQO (Figure 5c). By immunocytochemistry, epifluorescence and confocal microscopy we observed enhanced colocalization of hRad17 with PCNA in the late S phase of the cell cycle upon replication block or DNA damage. In untreated cells hRad17 only weakly colocalized with perinucleolar PCNA assemblies characteristic for an early stadium in late S (Figure 5a,d, upper panel). It only marginally colocalized with large PCNA speckles observed in later stages of the late S phase (Figure 5a,d, middle and lower panel). After treatment of Hela cells with HU hRad17 showed enhanced colocalization with PCNA arranged around nucleoli (Figure 5b,e, upper panel), with large PCNA speckles and with PCNA distributed in small foci throughout the nucleoplasm (Figure 5b,e, middle and lower panel).

In cells treated with 4-NQO hRad17 colocalized stronger with perinucleolar PCNA assemblies than in untreated cells (Figure 5c,f, upper panel). In later stages of late S we observed only marginal colocalization of hRad17 with PCNA (Figure 5c,f middle and lower panel). In untreated and treated cells in S phase hRad17 was distributed in foci throughout the nucleoplasm, which form regions of higher and lower density and often are arranged in horseshoe-like assemblies (Figure 5a–f). Upon treatment of Hela cells with HU or 4-NQO the nucleoplasmic PCNA immunofluorescence was slightly or strongly enhanced due to recruitment of PCNA to the chromatin (Sasaki et al., 1993; Miura, 1999).

Discussion

Recently Chen et al. (2001a) reported the discovery of alternative hRad17 splicing products encoding different hRad17 polypeptides. The hRad17 version we investigated in our study corresponds to the usually described and by far most abundant 670 amino acid version, which was detected in all cell cycle phases in total extracts of unirradiated and γ-irradiated human fibroblasts. We could additionally show that in Hela cells hRad17 is a constitutively nuclear protein during the cell cycle. Therefore, its function is not regulated by nuclear import or export. In contrast, hHus1 is translocated into the nucleus upon DNA damage or upon replication inhibition (Komatsu et al., 2000). Our discovery, that endogenous hRad17 and hRF-C p37 interact under conditions of replication block is consistent with data from studies in fission yeast.
These show that the hRF-C p37 homologue Rfc2 associates with overexpressed Rad17 in untreated as well as HU treated yeast (Kai et al., 2001). Like in fission yeast, hRF-C p140 did not co-immunoprecipitate hRad17 in extracts of HU treated Hela cells (our unpublished data), indicating that the two proteins participate in two distinct hRF-C complexes.

Zou et al. (2002) demonstrated that in Hela cells UV or HU treatment enhances the phosphorylation of hRad17 on the chromatin. Furthermore, hRad17 or hHus1 are required to load hRad9 onto the chromatin upon UV treatment or HU treatment. Phosphorylation of hRad17 by checkpoint kinases regulates its interaction with hRad1 (Bao et al., 2001). Our co-immunoprecipitation experiments revealed an interaction of PCNA with hRad9 and with hRF-Cp37 in HU treated S phase Hela cells. Previous studies by Komatsu et al. (2000) reported an association of hHus1 with PCNA upon treatment of cells with HU or ionizing radiation. Our immunocytochemical studies additionally showed that hRad17 colocalized with sites of BrdU incorporation or PCNA in the early S phase and to a low extent in mid S phase, suggesting that in these stages of S phase hRad17 accompanies the replisome. The hRad17/RF-C complex could function as a surveyor or sensor monitoring the accuracy of DNA replication and the integrity of the DNA. In the late S phase of the cell cycle the colocalization of hRad17 with PCNA was enhanced after treatment with HU or 4-NQO. After HU treatment hRad17 was found to colocalize with perinucleolar PCNA assemblies, with large PCNA speckles and with small PCNA foci in the nucleoplasm. Bravo (1986) and Bravo and MacDonald-Bravo (1985) reported, that the staining of PCNA does not change significantly upon treatment of cells with HU or aphidicolin at any stage during S phase and that the staining pattern is maintained as long as DNA synthesis is inhibited. This suggests, that hRad17 is recruited to sites of replication block. In the light of the known data our failure to co-immunoprecipitate PCNA and hRad17 most probably reflects the indirect nature of this interaction. hRF-Cp37 could serve as the connecting link between hRad17 and PCNA, because it interacted in HU treated cells with both proteins. Since it has been shown that hRad17 interacts with the small hRF-C subunits (Lindsey-Boltz et al., 2001) and that small hRF-C subunits interact with PCNA, an interaction of hRad17 with PCNA could also be mediated by one of the other small RF-C subunits, e.g. hRF-C p36 (Mossi et al., 1997). In the presence of UV like damage hRad17 showed enhanced colocalization with perinucleolar foci in late S phase, but not in later stages of late S. This suggests a recruitment of hRad17 to sites of DNA damage in an early stage of late S.

We propose the following model for the role of hRad17 in the DNA replication checkpoint pathway (Figure 6): hRad17 is recruited to sites of replication block in late S phase and functions as a sensor or as a signal transducer. hRad17 loads the hRad9/hHus1/hRad1 complex onto the chromatin and enables it to interact with PCNA arrested at sites of replication block. hHus1 mediates the phosphorylation of hRad17 by the checkpoint kinase ATR, which could represent an additional sensor for replication block (Zou et al., 2002). Phosphorylation of hRad17 regulates its interaction with hRad1. PCNA then might serve as a coordinating platform for DNA replication checkpoint activation, DNA repair and the resumption of DNA replication. The role of the hRad9/hHus1/hRad1 complex in the recovery process from replication block is currently a subject of intensive studies.

Materials and methods

Synchronization of Hela cells

Synchronization of Hela cells in G1 was achieved by serum starvation for 48 h with DMEM/0.5% (v/v) FCS and treatment with aphidicolin (3 µg/ml medium) for 26 h. Hela cells were synchronized in S phase by serum starvation with DMEM/0.5% (v/v) FCS for 48 h, treatment with 3 µg/ml medium aphidicolin for 24 h and subsequent growth in DMEM/10% (v/v) FCS for 5 h 30 min. Synchronization in G2/M was achieved by addition of 150 ng/ml nocodazole to the medium for 15 h and 1 h release from cell cycle arrest at G2/M. The cell cycle status of synchronized cells was analysed by propidium iodide staining of the DNA and FACS scan analysis.

Treatment of cells to cause replication block or DNA damage

Hela cells synchronised in S phase were incubated for 1 h with medium containing 10 mM HU or 2 µg/ml 4-NQO.

Dot blot analysis

500 ng of GST-hRad17 (103 kD), aldolase (40 kD) or GST-GEF2 (43 kD) were spotted onto a nitrocellulose membrane and subjected to immunoblot analysis using anti hRad17 antibody sc-5613.

Extraction test

Hela cells were synchronized in S phase, left untreated or treated with HU or 4-NQO as described above. Cells were harvested by trypsinization. Total extracts were prepared by adding 170 µl HEPES/Triton lysis buffer per 1.5 × 10⁶ cells and incubation for 22 min on ice. Lysis buffer contained 140 or 400 mM KCl in 40 mM HEPES pH 7.4, 10 mM MgCl₂, 1 mM EDTA, 2 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml bestatin, 1 mM PMSF, 50 mM NaF, 1 mM Na₃VO₄, 20 ng/ml microcystin LR, 10 mM glycerophosphate, 50 µg/ml ethidiumbromide, 1 mM DTT, 0.1% (v/v) Triton X-100. S7 nuclease digestion of cellular DNA was performed by lysing Hela cells in the same way as described above in HEPES/Triton lysis buffer containing 140 mM KCl without EDTA and ethidiumbromide, but supplemented with 2 mM CaCl₂ and 100 µg S7 nuclease/170 µl lysis buffer. After lysis extracts were centrifuged 5 min at 20800 g at 4°C. To the supernatants 20 µl 10 × SDS–PAGE sample buffer were added, samples were boiled for 5 min at 100°C and subjected to immunoblot analysis.
Immunoprecipitations

Hela cells were synchronized in S phase, treated with HU and harvested by trypsinization. For immunoprecipitation of hRF-C p37 total extracts were prepared by incubating cells 22 min on ice in 170 µl Tris/NP40 lysis buffer (50 mM Tris pH 7.5, 140 mM KCl, 10 mM MgCl₂, 1 mM EDTA, 2 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml bestatin, 1 mM PMSF, 50 mM NaF, 1 mM Na₃VO₄, 20 ng/ml microcystin LR, 10 mM glycerophosphate, 50 µg/ml ethidiumbromide, 1 mM DTT, 0.1% (v/v) NP40) per 1.5 x 10⁶ cells. Extracts were centrifuged 5 min at 20,800 g at 4°C. Extracts were diluted 1:1 to adjust the KCl concentration to 100 mM and the NP40 concentration to 0.5% (v/v). To 240 µl diluted extract 5 µl anti hRF-C p37 antiserum or control serum from unimmunized rabbits were added and incubated for 2 h 30 min on a rocking platform at 4°C. 20 µl equilibrated protein A sepharose were added to each sample and the samples were incubated on a rocking platform for further 60 min at 4°C. After washing four times with Tris/NP40...
wash buffer (50 mM Tris pH 7.5, 100 mM KCl, 10 mM MgCl₂, 1 mM EDTA, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml bestatin, 1 mM PMSF, 50 mM NaF, 1 mM Na₃VO₄, 20 ng/ml microcystin LR, 10 mM glycerophosphate, 50 μg/ml ethidium bromide, 1 mM DTT, 0.5% (v/v) NP-40) bound proteins were eluted from protein A sepharose by addition of 70 μl 2× SDS–PAGE sample buffer. For immunoprecipitation of PCNA total extracts were prepared by lysing HeLa cells in HEPES/Triton lysis buffer containing 140 mM KCl as described in the paragraph ‘Extraction test’. To 120 μl total extract 1 μg PC10 or control antibody HA.11 were added and incubated for 2 h 30 min on a rocking platform at 4°C. After addition of 20 μl equilibrated protein G sepharose the samples were incubated for further 90 min on a rocking platform at 4°C. After washing four times with HEPES/Triton lysis buffer containing 200 mM KCl bound proteins were eluted from protein G sepharose by addition of 70 μl 2× SDS–PAGE sample buffer. Samples were subjected to immunoblot analysis.

Immunocytochemistry

Hela cells were seeded on Lab-Tek chamber slides (Nalge Nunc International), synchronized in G₀/G₁, in S phase or in the G₂/M phase of the cell cycle. For subcellular localization of hRad17 and simultaneous DAPI (4,6-diamidino-2-phenylindole) staining cells were fixed 20 min at room temperature with 4% (w/v) PFA/PBS, then washed three times with PBS at room temperature and incubated for 5 min with PBS/0.2% (v/v) Triton X-100. Unspecific antibody binding sites were blocked by incubation with blocking solution (PBS/1% (w/v) BSA/0.03% (v/v) Tween-20) for 20 min. Cells were incubated for 1–2 h with anti hRad17 antibody sc-5613 (1:300 in blocking solution), then washed twice 5 min with PBS and once with blocking solution at room temperature. Cells were incubated for 45 min at room temperature with Cy.3 conjugated goat anti rabbit antibody (1:300 in blocking solution), washed three times 5 min with PBS at room temperature, incubated 5 min at room temperature with

Figure 5 Enhanced colocalization of hRad 17 with PCNA in late S phase after HU or 4-NQO treatment. Hela cells synchronized in S phase were left untreated or treated with HU or 4-NQO. (a–f) The subnuclear localizations of hRad17 (red fluorescence, left panels) and PCNA (green fluorescence, middle panels) were investigated by immunocytochemistry using antibody sc-5613 in combination with Cy.3 coupled secondary antibody and antibody PC10 in combination with DTAF coupled secondary antibody. Panels on the right show the merged hRad17 and PCNA immunofluorescences. (a–e) Representative cells in early, mid and late S phase without treatment (a), after HU treatment (b) and after 4-NQO treatment (c) are shown. Captures were made using an Olympus BX51 fluorescence microscope coupled to a DP50 digital camera system. Panels (d–f) show pictures of representative cells in the late S phase taken using a Leica TCS SP2 confocal laser scanning microscope: (d) without treatment, (e) after HU treatment and (f) after 4-HQO treatment. (g) FACS scan analysis of the cell cycle distribution at the timepoint of treatment (S phase control 1), of untreated cells (S phase control 2), of HU or of 4-NQO treatment cells (S phase + HU, S phase + 4-NQO). The top panel shows the cell cycle distribution of unsynchronized cells (unsynch.).
For colocalization of hRad17 with sites of BrdU incorporation unsynchronized Hela cells were incubated 30 min with medium containing 10 \( \mu \text{M} \) BrdU. Cells were washed with PBS and fixed for 2 min with 3% (w/v) paraformaldehyde/PBS at room temperature, washed with PBS, then fixed with 70\% (v/v) MetOH/PBS, 60\% (v/v) MetOH/PBS, 100\% MetOH, 60\% (v/v) MetOH/PBS, 30\% (v/v) MetOH/PBS for 2 min each. Cells were washed twice with PBS at room temperature and incubated for 5 min with PBS/0.2\% (v/v) Triton X-100. Unspecific antibody binding sites were blocked by incubation with blocking solution (PBS/1\% (w/v) BSA/0.03\% (v/v) Tween-20) for 20 min. Cells were incubated with sc-5613 (1:300 in blocking solution) for 1–2 h at room temperature, then washed twice 5 min with PBS and once with blocking solution at room temperature. Cells were then incubated for 45 min at room temperature with Cy.3 conjugated goat anti rabbit antibody (1:300 in blocking solution) and then incubated with anti BrdU antibody 3D4 (1:100 in blocking solution) for 1 h 30 min. After washing twice with PBS for 5 min and once with blocking solution for 5 min cells were incubated 45 min with DTAF conjugated goat anti mouse antibody (1:500 in blocking solution). After washing the cells three times 5 min with PBS at room temperature, cells were covered with vectashield mounting medium (Vector Laboratories), and coverslips were fixed with nail polish.

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References


Figure 6 Model showing possible interactions of DNA replication and replication checkpoint proteins. Our and previously published data suggest the following model for the role of hRad17 and PCNA in the DNA replication checkpoint pathway: the heteropentameric hRad17/hRF-C complex is recruited to sites of replication block in late S phase and functions as a sensor or as a signal transducer. It binds the hRad9/hHus1/hRad1 (9-1-1) complex and enables it to interact with PCNA arrested at sites of replication block. An interaction of hRad1-1/hRF-C with PCNA could be mediated by small hRF-C subunits, e.g. hRF-C p37. PCNA could provide communication between replication checkpoint control, DNA repair and the resumption of DNA replication. RPA: Replication Protein A