Rad51 Accumulation at Sites of DNA Damage and in Postreplicative Chromatin

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Abstract. Rad51, a eukaryotic RecA homologue, plays a central role in homologous recombinational repair of DNA double-strand breaks (DSBs) in yeast and is conserved from yeast to human. Rad51 shows punctuate nuclear localization in human cells, called Rad51 foci, typically during the S phase (Tashiro, S., N. Kotomura, A. Shinohara, K. Tanaka, K. Ueda, and N. Kamada. 1996. *Oncogene.* 12:2165–2170). However, the topological relationships that exist in human S phase nuclei between Rad51 foci and damaged chromatin have not been studied thus far. Here, we report on ultraviolet microirradiation experiments of small nuclear areas and on whole cell ultraviolet C (UVC) irradiation experiments performed with a human fibroblast cell line. Before UV irradiation, nuclear DNA was sensitized by the incorporation of halogenated thymidine analogues. These experiments demonstrate the redistribution of Rad51 to the selectively damaged, labeled chromatin. Rad51 recruitment takes place from Rad51 foci scattered throughout the nucleus of nonirradiated cells in S phase. We also demonstrate the preferential association of Rad51 foci with postreplicative chromatin in contrast to replicating chromatin using a double labeling procedure with halogenated thymidine analogues. This finding supports a role of Rad51 in recombinational repair processes of DNA damage present in postreplicative chromatin.

Key words: Rad51 • DNA damage • microirradiation • postreplicative DNA repair • indirect immunofluorescence

Introduction

DNA double strand breaks (DSBs)[1] are major threats to the genomic integrity of cells. Eukaryotic cells have multiple pathways to repair DSBs, such as recombinational repair and end-joining. In *Saccharomyces cerevisiae*, genes of the Rad52 epistasis group are involved in homologous recombination based DSBs repair (Baumann and West, 1998; Kanaar et al., 1998). Rad51 plays a central role in this process and also in normal meiotic recombination (Shinohara et al., 1992). Rad51 forms helical filaments on both single stranded DNA (ssDNA) and double stranded DNA, promoting homologous pairing and strand exchange (Shinohara et al., 1992, 1993; Baumann et al., 1996). Human Rad51 protein interacts with Rad52, replication protein A, and the tumor suppressors p53, Brca1 and Brca2 (Shinohara et al., 1992; Buchhop et al., 1997; Scully et al., 1997b; Sharan et al., 1997; Golub et al., 1998).

In yeast, lilies, mice, and humans, Rad51 forms nuclear protein complexes on meiotic chromosomes (Bishop, 1994; Haaf et al., 1995; Terasawa et al., 1995; Scully et al., 1997b). Human Rad51 protein shows discrete foci in nuclei of somatic cells, called Rad51 nuclear foci, typically during S phase (Tashiro et al., 1996). Interestingly, S phase Rad51 foci colocalize with foci containing Brca1, replication protein A, and proliferating cell nuclear antigen (PCNA; Scully et al., 1997a; Haaf et al., 1999). Several lines of evidence suggest that Rad51 foci correspond with nuclear protein complexes for recombinational DNA repair (Scully et al., 1997a; Raderschall et al., 1999). However, the colocalization of Rad51 with sites of DNA damage has not been conclusively demonstrated thus far. A previous study based on partial exposure of nuclei to soft x-rays showed that another DNA repair complex, including Mre11 and Rad50, is assembled directly at sites of radiation-induced DNA damage, but failed to show that the same is true for Rad51 (Nelms et al., 1998). Therefore, the

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[1]*Abbreviations used in this paper:* BrdU, bromodeoxyuridine; CldU, chlorodeoxyuridine; DSBs, DNA double strand breaks; IdU, iododeoxyuridine; SSBs, DNA single strand breaks; ssDNA, single stranded DNA.

relationship of Rad51 foci with DNA repair and DNA replication in human S phase nuclei is still not clear.

In this study we describe the results of two types of UV irradiation experiments performed with a human fibroblast cell line: (i) laser microirradiation of small nuclear areas (using a laser line, $\lambda = 337$ nm, in the UVA range) and (ii) UVC exposure of whole cells. Before irradiation, nuclear DNA was sensitized by the incorporation of halogenated thymidine analogues bromodeoxyuridine (BrdU) or iododeoxyuridine (IdU). Previously it had been established that UV light exposure of sensitized chromatin induces single and double strand breaks (SSBs and DSBs) (Krasin and Hutchinson, 1978; Limoli and Ward, 1993). Our experiments demonstrate the redistribution of Rad51 to damaged, BrdU- or IdU-labeled chromatin. Rad51 recruitment to damaged sites takes place from Rad51 foci, which are regularly observed in S phase nuclei and apparently are scattered throughout the nucleus. In addition to these irradiation experiments, we used a double labeling procedure with IdU and chlorodeoxyuridine (CldU) to visualize postreplicative and actively replicating chromatin in nuclei of nonirradiated cells. In these nuclei we observed the preferential association of Rad51 foci with postreplicative chromatin but not with actively replicating chromatin. This result supports a role of Rad51 in recombinational repair complexes associated with postreplicative chromatin.

Materials and Methods

Cell Culture and DNA Labeling with Halogenated Thymidine Analogues

GM02063, an SV-40 transformed fibroblast cell line showing normal DNA repair, was established from a patient with Lesch-Nyhan syndrome and cultured in DME with 10% FCS (Xia et al., 1996). For DNA labeling with halogenated thymidine analogues, BrdU (Boehringer), CldU (Sigma-Aldrich) or IdU (Sigma-Aldrich) were added to the culture medium at final concentrations of 10 μM. To visualize postreplicative and replicating sites simultaneously, a double labeling scheme with CldU and IdU was applied as described in Results.

Detection of Replication Label and Rad51 Protein

Cells were fixed with 4% paraformaldehyde in 1× PBS. Next, nuclei were permeabilized with 1% SDS/0.5% Triton X-100/1× PBS for 10 min. For the detection of BrdU, CldU, or IdU in denatured DNA, cells were incubated for 30 min at 37°C with mouse anti-BrdU (Boehringer), rat anti-BrdU (Serolab), which recognizes BrdU and CldU (Aten et al., 1992), or mouse anti-BrdU antibodies (Becton Dickinson), which recognize BrdU and IdU (Aten et al., 1992), diluted in 0.5% BSA/0.5× PBS/30 mM Tris/0.3 mM $MgCl_2$/0.5 mM 2-mercaptoethanol/10 μg/ml DNase I (Boehringer), respectively. For the detection of in vivo–generated ssDNA, fixed nuclei were incubated with mouse anti-BrdU antibody diluted in 1% BSA/1× PBS without DNA denaturation and DNase treatment. Rabbit anti-Rad51 antibody (Tashiro et al., 1996) was mixed to these primary antibodies for the simultaneous detection of Rad51. FITC- or Cy3-conjugated sheep anti–mouse (Dianova), Cy3-conjugated goat anti–rat (Amersham Pharmacia Biotech), and FITC- or biotin-conjugated goat anti–rabbit (Tago) were used as secondary antibodies. Avidin-Cy5 (Dianova) was used for the detection of biotin-conjugated goat anti–rabbit antibody.

Microirradiation Using a UVA Pulse Laser

For microirradiation, cells were seeded on round coverslips (Schubert & Weiss) and incubated with medium containing BrdU for 20 h. Before microirradiation the coverslips were mounted in a living cell chamber model FCS2 (Bioptechs). During microirradiation, the cells were kept in RPMI medium containing 25 mM Hepes and 10% FCS (Biochrom). The cells were kept at 37°C with an objective heater (Bioptechs). Microirradiation was carried out with a laser microdissection system (P.A.L.M.) coupled into a ZEISS Axiovert 100.

Whole Cell UVC Irradiation

Whole cell UVC irradiation was performed for 10 s with a UV lamp Typ 600 352 (Waldmann) at 10 J/m^2.

Image Acquisition

Confocal sections were taken with a ZEISS LSM410. For the colocalization analysis, a macro written for the operating software of the ZEISS LSM410 was developed in our laboratory. Adobe Photoshop was used for presentation of images.

Quantitative Analysis for Colocalization of Rad51 Foci with Postreplicative or Replicating Chromatin

The percentage of Rad51 foci showing overlap with IdU-labeled (postreplicative) or CldU-labeled (replicating) chromatin was measured in 20 nuclei with an average of 10 Rad51 foci. Analyses were performed using two threshold levels for the segmentation of IdU and CldU signals. A high level was chosen to detect only intense IdU and CldU pixels clearly distinct from background noise, and a low level was applied to distinguish virtually all IdU- and CldU-positive pixels but possibly including a fraction of background pixels as well. At each level thresholds were adjusted to yield the same numbers of IdU- and CldU-positive pixels roughly corresponding to similar amounts of differentially labeled postreplicative and replicating chromatin. As a criterion for the colocalization of a segmented Rad51 focus with postreplicative or replicating chromatin we required that >50% of the pixels reflecting a Rad51 focus should show colocalization with a coherent cluster of either IdU- or CldU-positive pixels.

Results

Recruitment of Rad51 to Sites of DNA Damage Produced by Laser UV Microirradiation of Small Nuclear Areas in BrdU-labeled Human Fibroblasts

All experiments described below were carried out with the human fibroblast cell line GM02063. A fraction of nuclei in growing cell cultures showed scattered Rad51 foci. To test whether cells showing these foci were in S phase, cultures were pulse-labeled with BrdU for 30 min and double stained for Rad51 and BrdU. 42% of the cells ($n = 200$) showed focally concentrated signals of Rad51 scattered throughout the nuclei. Since 93% of these Rad51 foci–positive cells also showed incorporation of BrdU, we conclude that most cells with Rad51 foci were indeed in S phase as reported previously for other human cells (Tashiro et al., 1996; Scully et al., 1997b). The few nonlabeled nuclei exhibiting scattered Rad51 foci should be mostly in G2, since it has been shown that Rad51 is not present in cells before late G1 phase (Tashiro et al., 1996; Yamamoto et al., 1996). The significance of these scattered Rad51 foci is not clear at present. Foci may indicate nuclear repair sites of endogenous DNA damage or they may represent Rad51 storage sites.

Before microirradiation experiments, fibroblast cultures were labeled with BrdU for 20 h, i.e., the time roughly required for one cell cycle (data not shown). Labeling of DNA with BrdU enhances the efficiency of UVA to induce SSBs or DSBs (Limoli and Ward, 1993). Cells from labeled and unlabeled cultures were microirradiated at a single nuclear site with a laser microbeam ($\lambda = 337$ nm; 10 MJ/m^2). Approximately 30 min later microirradiated and

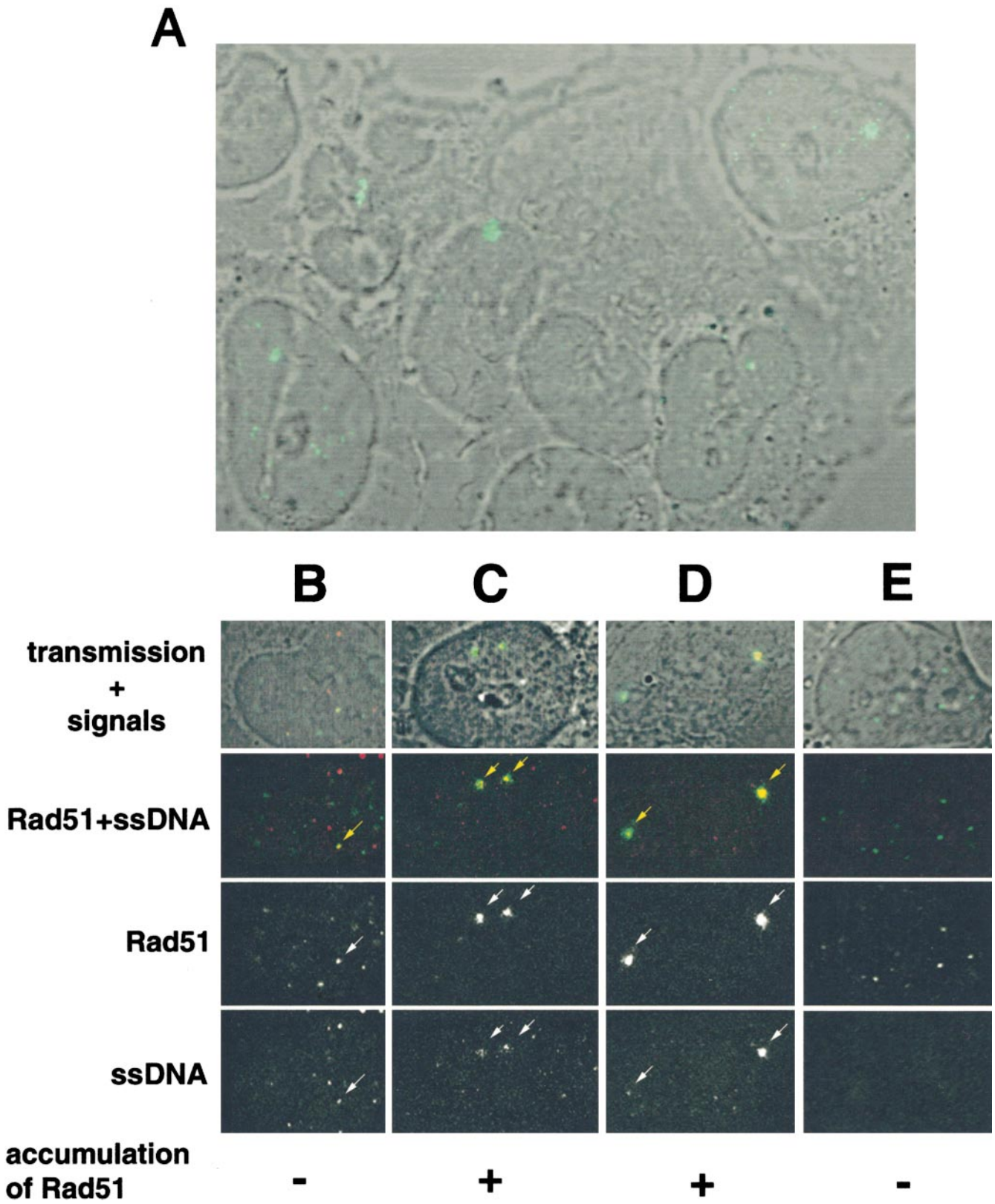

Figure 1. Accumulation of Rad51 in microirradiated nuclear areas. GM02063 cells were irradiated with a pulse laser beam (λ = 337 nm). Rad51 was visualized in green, and ssDNA (BrdU signal detected without DNA denaturation) in red. (A) A merged transmission and Rad51 image 30 min after microirradiation of one nuclear site. (B–E) Transmission images are merged with Rad51 (green) and ssDNA (red) in the top row. The second row shows merged images of Rad51 and ssDNA. The third and fourth rows show raw images for Rad51 and ssDNA, respectively. Arrows indicate Rad51 foci that show colocalization with ssDNA regions. (B) BrdU-labeled nucleus without microirradiation. (C) BrdU-labeled nucleus 30 min after microirradiation of closely adjacent sites. (D) BrdU-labeled nucleus 30 min after microirradiation of two distant sites. (E) Nucleus of a cell without BrdU labeling 30 min after microirradiation.

nonirradiated cells on the same slide were fixed and subjected to immunostaining for Rad51. Light optical nuclear sections were obtained with a confocal laser scanning microscope. We noted a single intense accumulation of Rad51 in 38% of the microirradiated nuclei from BrdU-labeled cultures (see below for evidence that this site truly reflects the site of microirradiation). In addition, these nuclei showed a scattered distribution of Rad51 foci (Fig. 1 A), indicating that they were in S or G2 phase. These intense accumulations were never observed in nonirradiated cells on the same slides (Fig. 1 B) or in microirradiated cells from nonlabeled cultures (Fig. 1 E). We conclude that the formation of intense Rad51 accumulations required both BrdU labeling and microirradion in S or G2 phase. 62% of microirradiated cells did not show any Rad51 accumulation at the microirradiated nuclear site. Some of these cells did not show any Rad51 foci, indicating that they were probably microirradiated in G1. However, in other cells the presence of scattered Rad51 foci suggested microirradiation in S or G2. The lack of Rad51 accumulation at the microirradiated nuclear site suggested that these cells probably had not passed through S phase during the labeling period with BrdU and thus were not sensitive to UVA microirradiation.

To demonstrate unequivocally that sites of intense Rad51 accumulation are identical with microirradiated nuclear sites, we microirradiated BrdU-labeled nuclei both at two closely adjacent sites and at two distant sites. As expected, nuclei with two closely adjacent microirradiation sites also showed two closely adjacent accumulations of Rad51 (Fig. 1 C), whereas two distant microirradiation sites resulted in two distant sites of Rad51 accumulations (Fig. 1 D).

In a time series, where BrdU-labeled cells were microirradiated at a single site and fixed 10–60 min later, accumulation of Rad51 was detected in nuclei as early as 10–20 min after microirradiation (Table I). This recruitment to the microirradiated nuclear site was more rapid than the increase of the percentage of cells with Rad51 foci noted after whole cell UVC or γ irradiation (Haaf et al., 1995; Raderschall et al., 1999), which was first observed 60 min after the induction of DNA damage.

As described above, we noted that nuclei microirradiated at one site showed scattered Rad51 foci in addition to the Rad51 accumulation induced at the site of microirradiation. In contrast, when the total irradiation dose was doubled in nuclei microirradiated at two sites, we noted a strong decrease in the number of scattered Rad51 foci, and in some cases these foci even disappeared entirely (Fig. 1, B–D). This result indicates that most if not all Rad51 was redistributed to the two strong Rad51 accumulations seen at the two microirradiated nuclear areas. Thus far, we do not know whether entire Rad51 foci are able to move towards microirradiated chromatin. Alternatively, it seems possible that the positions of Rad51 foci are largely fixed, but that each Rad51 focus is able to bind and release individual Rad51 molecules in a highly dynamic fashion. Released Rad51 may diffuse rapidly throughout the nuclear space (possibly channeled within the interchromatin domain space; see Discussion) and be captured by binding sites newly created in microirradiated chromatin. According to the latter scenario, a major part (even the majority) of Rad51 contained in the nucleus at any given time could be present in a free form, whereas only a minor part is present in scattered Rad51 foci.

Table I. Recruitment of Rad51 into the Microirradiated Area

	Intense Rad51 accumulation	
Min	BrdU(−)	BrdU(+)
0–10	−	−
10–20	−	+
20–30	−	+
30–40	−	+
40–50	−	+
50–60	−	+

In addition to immunostaining of Rad51, BrdU was detected at microirradiated nuclear sites without a DNA denaturing step. This protocol allowed us to detect ssDNA in BrdU-labeled cells, since anti-BrdU antibodies recognize incorporated BrdU only in ssDNA (Raderschall et al., 1999). 72% of the intense Rad51 accumulations seen in BrdU-labeled cells ($n = 25$) after microirradiation showed colocalization with ssDNA regions, as shown in Fig. 1, C and D. Thus, we conclude that not only Rad51 but also ssDNA regions accumulate at sites of microirradiation, probably as a requirement of DNA repair processes.

Recruitment of Rad51 to Sites of DNA Damage Produced by Whole Cell UVC Irradiation in IdU-labeled Chromatin Foci

The experiments described above demonstrated the recruitment of Rad51 from scattered Rad51 foci to microirradiated chromatin containing SSBs and DSBs. The following whole cell UVC irradiation experiment was designed to confirm this result. UVC, i.e., ultraviolet light in the range between 200 and 280 nm, is well known to produce thymidine dimers, but at the dose range applied in our experiments rarely produces DSBs. As the incorporation of IdU into DNA-like BrdU enhances the effect of UVC irradiation to induce DSBs (Hutchinson, 1973), UVC irradiation produces many more DSBs in IdU-labeled replication foci than in unlabeled chromatin. For this reason, we labeled cells in S phase before UVC irradiation by the incorporation of IdU for 1 h. After UVC irradiation we expected the redistribution of Rad51 to IdU-labeled chromatin. Immediately before UVC irradiation IdU-containing medium was replaced by normal medium. After a variable recovery time (10–45 min) to provide cells with sufficient time for Rad51 recruitment to damaged chromatin sites, cells were labeled with CldU for 15 min before they were fixed. This second label step yielded very little CldU incorporation in UVC-irradiated cells, indicating a strong UVC-induced inhibition of DNA replication (Fig. 2). As expected UVC-irradiated cells showed colocalization of numerous Rad51 foci with IdU-labeled chromatin (Fig. 2, left), in contrast to unirradiated control cells (Fig. 2, right). The percentage of Rad51 foci that showed colocalization with IdU-labeled chromatin was 2.5–4.9 times higher after UVC irradiation as compared with nonirradiated control cells (Table II). As the average numbers of Rad51 foci in UVC-irradiated nuclei did not change sig-

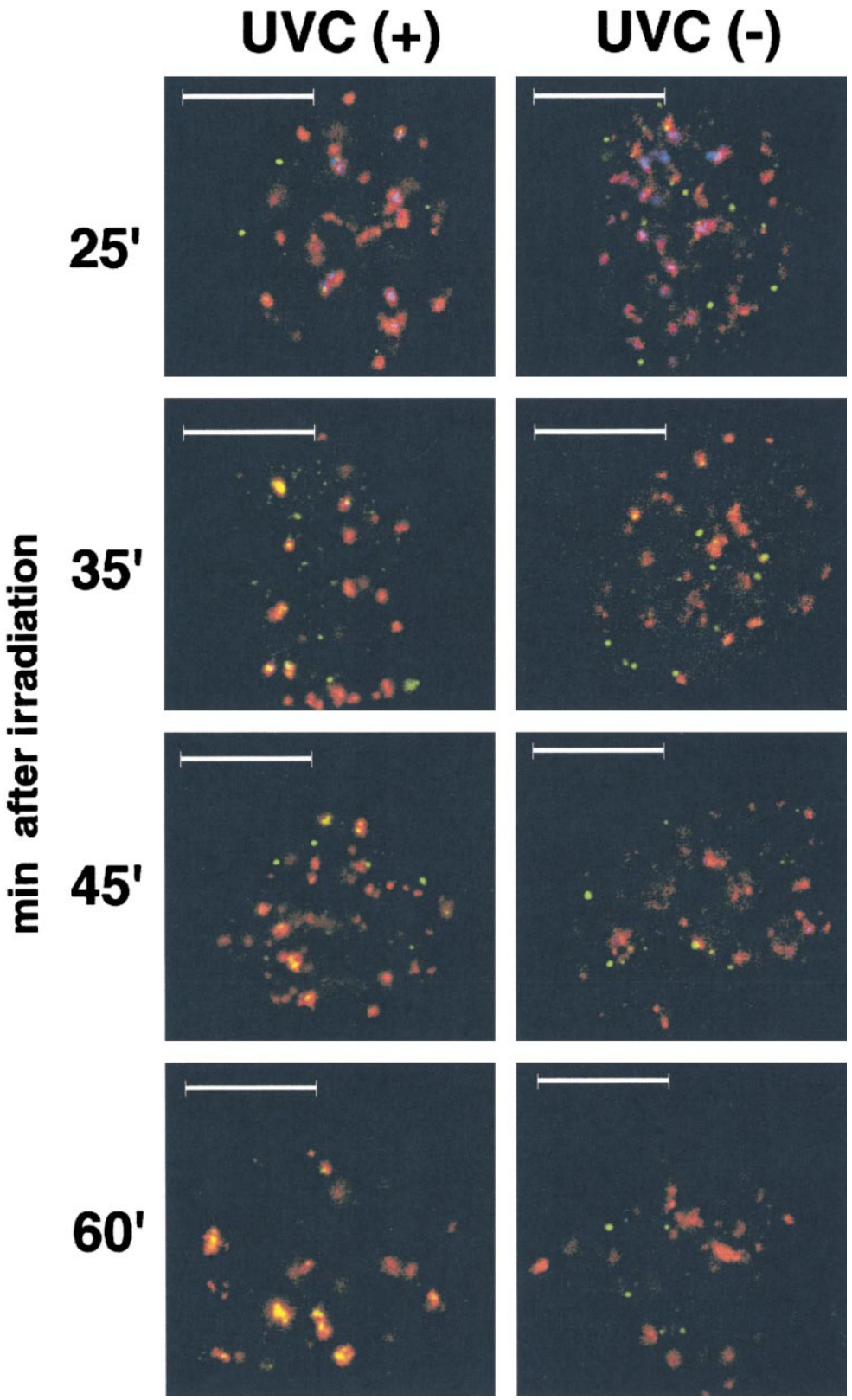

Figure 2. Recruitment of Rad51 to sites of DNA damage after whole cell UVC irradiation. GM02063 cells were first labeled with IdU for 60 min. Immediately after being replaced in normal medium, cells were subjected to UVC irradiation at 10 J/m^2. Cells were fixed 25, 35, 45, and 60 min after irradiation. Rad51, IdU, and CldU are visualized in green, red, and blue, respectively. Colocalization of Rad51 and IdU yields merged yellow signals. Bars, 10 μm.

nificantly until 60 min after irradiation in this experiment (Table II), the result of this experiment indicates a major redistribution of Rad51 to IdU-labeled chromatin containing UVC-induced DNA breaks. This result fully confirms the results of our microirradiation experiments.

Association of Rad51 Foci with Postreplicative Chromatin in Nonirradiated Human Fibroblasts

A recent study revealed a spontaneous accumulation of chromosome gaps and breaks in a Rad51-deficient chicken B lymphocyte line not exposed to any DNA damaging

Table II. Colocalization of Rad51 Foci with IdU Signals Observed at Different Times after UVC Irradiation (+) in Comparison to Nonirradiated Cells (−)

Min	% of Rad51 foci showing colocalization with IdU signals		Ratio
	UVC(+)	UVC(−)	UVC(+)/UVC(−)
25	25.7	5.2	4.9
	(11.9)	(9.7)	
35	30.9	10.1	3.0
	(10.3)	(10.4)	
45	24.2	9.6	2.5
	(10.6)	(7.3)	
60	30.2	9.0	4.0
	(7.5)	(10.6)	

20 nuclei were analyzed in each experiment. Average numbers of Rad51 foci in one nucleus are given in parentheses.

agents (Sonoda et al., 1998), suggesting that Rad51 is not only essential for exogenously induced DNA damage but also is involved in the repair of endogenously arising damage. During the course of the microbeam experiments described above, colocalization of ssDNA with Rad51 foci was occasionally noted in BrdU-labeled, unirradiated cells (Fig. 1 B). This observation suggested that Rad51 foci in nonirradiated cells might not be present only as storage sites, but might instead be directly involved in recombinational repair of endogenously occurring DNA damage. The following experiment was performed to study the colocalization of Rad51 foci with chromatin foci of nonirradiated cells in more detail. We wished to know whether Rad51 associates preferentially with chromatin in a postreplicative state or with replicating chromatin, or indiscriminately with both types of chromatin. To visualize simultaneously replicating chromatin and chromatin in a postreplicative state, a double labeling scheme with the halogenated thymidine analogues IdU and CldU was applied to GM02063 cells in the exponential growth phase (Aten et al., 1992). Cells were first incubated in medium containing IdU for 60 min, then incubated in normal medium for 10 min, and thereafter were incubated in medium containing CldU for 15 min. Labeling times and other experimental conditions were empirically defined such that the number of labeled pixels representing IdU-labeled chromatin matched the number of pixels representing CldU-labeled chromatin (see Materials and Methods). In this experiment, chromatin foci labeled exclusively with IdU represented chromatin in the postreplicative state, whereas CldU-labeled chromatin foci were actively engaged in DNA replication (Fig. 3, A–D). Cells were kept in the dark to avoid any damage by exposure to the ultraviolet spectrum of daylight. (For possible damage resulting from the incorporation of halogenated thymidine analogues alone even in the absence of irradiation see Discussion.) Two threshold levels were applied for segmentation of IdU and CldU signals (Fig. 3 E). The average numbers of both IdU- and CldU-positive pixels counted in median nuclear light optical section of 20 nuclei were 1,600 pixels at low thresholds, and 480 pixels at high thresholds, respectively. For both thresholds we found a strong preference of Rad51-positive pixels to colocalize with IdU-positive pixels (i.e., with postreplicative chromatin; Fig. 3 F). If Rad51 foci were distributed randomly, one would expect the same percentages of Rad51 foci colocalizing with postreplicative and replicating chromatin. At the low threshold level the percentage of Rad51 foci colocalizing with IdU-labeled chromatin aggregates was 12.6%, whereas 2.9% colocalized with CldU-positive aggregates ($P < 0.0001$, Fig. 3 F, a). Using the high level, the percentages were 6.4% and 0.5%, respectively ($P < 0.0005$, Fig. 3 F, b). These results argue for a 4–13-fold higher preference of Rad51 to postreplicative chromatin as compared with replicating chromatin. We assume that the data support a 13-fold rather than a 4-fold difference for two reasons: (i) The analysis performed at the low threshold level probably included a higher number of pixels which were misclassified as IdU- or CldU-positive pixels but in fact belonged to background pixels. These background pixels should diminish the true difference. (ii) Some of the CldU-positive pixels reflecting replicating chromatin may in fact belong to chromatin that was already in a postreplicative state at the end of the second pulse, indicating that the chromatin fraction attributed to replicating chromatin was an upper estimate.

Discussion

In this report we provide strong evidence for the recruitment of Rad51 to sites of DNA damage. In microirradiation experiments we observed the dose-dependent disappearance of Rad51 foci in nonirradiated nuclear areas resulting in an accumulation of Rad51 exclusively at microirradiated nuclear sites. In whole cell UVC irradiation experiments, we observed the redistribution of Rad51 foci to chromatin foci sensitized by the incorporation of IdU. Recently, the binding of Rad51 to ssDNA was observed in γ-irradiated human cells (Raderschall et al., 1999). In accordance with this finding, we noted the formation of ssDNA at microirradiated nuclear sites in BrdU-labeled cells. From whole cell UVA irradiation ($\lambda = 334$ nm) experiments (Peak et al., 1987; Peak and Peak, 1990), we can estimate that ~80 SSBs and ~0.3 DSBs should be produced in the microirradiated nuclear area (focal diameter <1 μm) of a non-BrdU–labeled cell. Sensitization of DNA to UVA irradiation by the substitution of thymidine with BrdU has an estimated yield of 1,000 SSBs and 10 DSBs in the microirradiated part of BrdU-labeled nuclei (Limoli and Ward, 1993). DSBs were probably produced only in BrdU-labeled microirradiated cells, whereas SSBs were produced in significant numbers at microirradiation sites of both BrdU-labeled and unlabeled cells. Rad51 accumulation was not observed after microirradiation of BrdU-labeled nuclei with energies lower than 1 MJ/m^2. In nuclei microirradiated with energies higher than 25 MJ/m^2, Rad51 accumulation was observed even in the absence of BrdU within 30 min after microirradiation (data not shown). This may be due to the direct formation of DSBs by cutting effects of microirradiation with high energy densities (Greulich and Leitz, 1994) or to the formation of closely adjacent SSBs. The results of our UVA microbeam and UVC whole cell irradiation experiments are compatible with a role for Rad51 foci in recombinational repair of DSBs produced in DNA sensitized to UV irradiation by the incorporation of halogenated thymidine analogues.

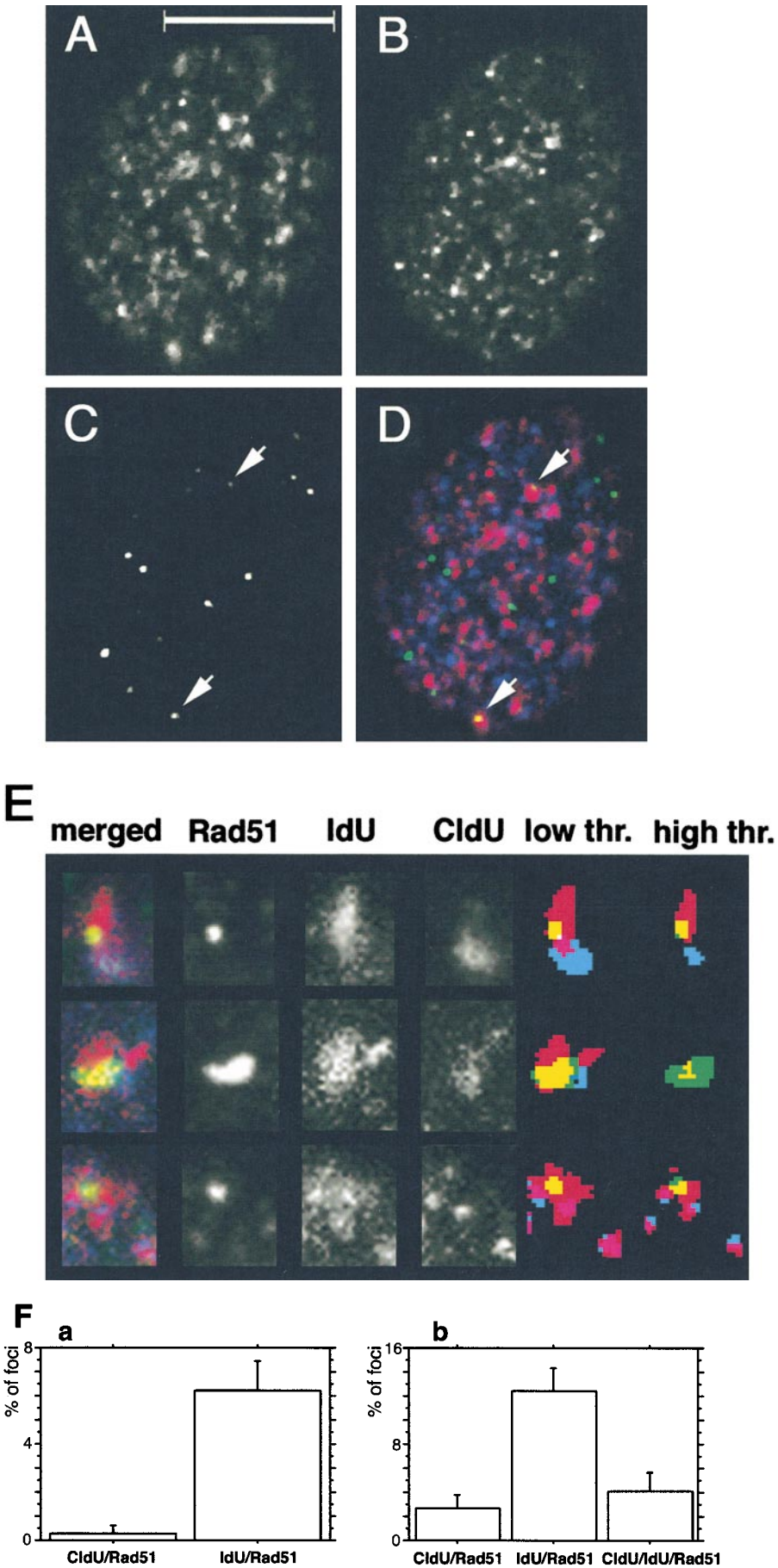

Figure 3. Assembly of Rad51 in postreplicative chromatin. IdU and CldU signals show chromatin sites in a postreplicative state and ongoing replicating state, respectively. Rad51, IdU, and CldU are visualized in green, red, and blue, respectively. Arrows indicate Rad51 foci showing colocalization with IdU signals. (A) Raw image showing IdU-labeled chromatin in a confocal nuclear section. (B) Raw image showing CldU-labeled chromatin in the same nuclear section. (C) Raw image of Rad51 foci noted in this section. (D) Merged image of IdU, CldU, and Rad51 signals. (E) Three examples of Rad51-, IdU-, and CldU-labeled chromatin at higher magnification. The first vertical row shows merged images, and the second to fourth vertical rows show individual Rad51, IdU, and CldU images, respectively, without thresholding. The fifth and sixth vertical rows show the merged images after low and high thresholding. Rad51 foci demonstrated in the first and third horizontal lanes clearly colocalize with postreplicative chromatin at both low and high thresholds. The Rad51 focus in the middle horizontal lane was classified to colocalize with postreplicative chromatin at the low threshold but not at the high threshold. Bar, 10 μm. (F) Bar plots of the percentages of Rad51 foci showing colocalization with IdU and CldU signals. (a) Using high thresholds and (b) using low thresholds. Triple colocalizations of Rad51, IdU, and CldU signals were noted only at low thresholds. Error bars denote the SEM.

Recombinational repair requires the close spatial association of the damaged DNA strand with an undamaged homologous counterpart. In human fibroblasts, such a requirement is fulfilled by postreplicative chromatin during the S phase and G2 but not at other stages of the cell cycle. We have shown that homologous chromosomes occupy distinct territories in human cell nuclei (Lichter et al., 1988). In various human cell types studied so far, such as fibroblasts and lymphocytes, we and others did not observe nonrandom homologous chromosome associations (Cremer et al., 1993; Lesko et al., 1995; Dietzel et al., 1998; Nagele et al., 1999; our unpublished data). Even in the case of randomly occurring spatial associations of two homologous chromosome territories, the adjacent parts were generally provided by nonhomologous segments (Tanabe, H., and T. Cremer, unpublished data). Accordingly, major chromatin movements would be required in G1 nuclei to locate homologous regions sufficiently close to each other as a necessary condition of recombinational repair. Microirradiation experiments performed with Chinese hamster fibroblasts did not reveal major movements of microirradiated chromatin during a postirradiation incubation period of several hours (Cremer et al., 1982). Although the extent of chromatin movements in human cell types requires further investigation (Zink et al., 1998; Bornfleth et al., 1999), current observations suggest that the topological conditions for recombinational repair are not fulfilled in nuclei of human fibroblasts during G1. Possible exceptions may include disperse repetitive elements that are present on many chromosomes, or very rare situations where homologous segments of a pair of chromosomes become spatially associated by chance. In agreement with these topological hindrances for recombinational repair in G1, we and others never observed Rad51 foci in G1 cell nuclei (Tashiro et al., 1996; Scully et al., 1997b). This situation changes decisively with the formation of sister chromatids during S phase. We hypothesize that spatially closely associated sister chromatids or segments thereof become available in postreplicative chromatin and provide an essential condition for Rad51 to fulfill its role in recombinational repair. This hypothesis is supported by our finding that Rad51 foci in nonirradiated cells (irradiated cells have not yet been studied in this regard) colocalized in a significantly higher frequency with postreplicative chromatin foci than with actively replicating foci. In human fibroblasts the requirement of homologous DNA strands in a close neighborhood is only fulfilled in postreplicative chromatin.

A caveat in the interpretation of the experiments presented here has to be taken into consideration. The preferential association of Rad51 with postreplicative, IdU-labeled chromatin was observed in unirradiated cells after double labeling with IdU and CldU. CldU was given as a second pulse to visualize replicating chromatin foci as well. Although the purpose of this double labeling strategy was solely for the differential visualization of postreplicative and replicative chromatin, we cannot exclude that the incorporation of halogenated thymidine analogues induced damage considerably above the level of other endogenously occurring damage that requires Rad51-dependent repair processes. The observation that incorporation of halogenated thymidine analogues induces a small increase in sister chromatid exchanges adds to this concern (Wolff and Perry, 1974). If Rad51 is required in the repair of such damage, our experimental schedule (first pulse IdU, second pulse CldU) could allow for more time for Rad51 to redistribute to damage formed in earlier labeled chromatin. This possibility cannot be ruled out by a reverse labeling protocol (first pulse with CldU, second pulse with IdU). A bias in the recruitment of Rad51 to IdU-labeled, postreplicative chromatin foci as compared with CldU-labeled actively replicating foci could be enhanced, if IdU incorporation yielded more damage than CldU incorporation. Note that we used a longer pulse with IdU (1 h) than with CldU (15 min) to achieve a similar signal intensity for both IdU- and CldU-labeled chromatin (see Materials and Methods). Again, this problem cannot be solved by reverse labeling protocols, since a 1-h pulse with IdU is not suitable to label actively replicating chromatin foci. In conclusion, although our experiments unequivocally demonstrate the recruitment of Rad51 to damaged chromatin, the evidence that Rad51 is preferentially redistributed to postreplicative chromatin is circumstantial and needs to be corroborated in future studies.

It is known that DNA replication/transcription requires numerous proteins, which exist as preassembled complexes in mammalian nuclei (Lamond and Earnshaw, 1998). In addition to Rad51 foci, which act as recombinational repair complexes, probably at sites of DSBs in postreplicative chromatin, we consider the possibility that Rad51 foci also provide storage sites of preformed repair protein complexes. At present it is not clear whether such storage complexes are located at random sites of the interchromatin space or if they are located in association with specific chromosome segments. Recent studies (in yeast) have shown that some proteins involved in DSB repair redistribute from the telomeres and become more diffusely localized throughout the nucleoplasm, presumably interacting with sites of DNA damage (Mills et al., 1999). It remains to be seen to what extent Rad51 foci observed in nonirradiated cell nuclei represent such storage sites or sites of recombinational repair of endogenously occurring DNA damage.

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