Localization and dynamic relocalization of mammalian Rad52 during the cell cycle and in response to DNA damage

Yilun Liu*, Ming-jie Li*†, Eva Y-H.P. Lee‡ and Nancy Maizels*§

The importance of *RAD52* in establishment and maintenance of genomic structure has been established by genetic experiments in the yeast *Saccharomyces cerevisiae*, where mutation of *RAD52* has been shown to diminish DNA repair and recombination of a variety of markers, including the rDNA [1–3]. Biochemical analysis has shown that yeast and mammalian Rad52 proteins have some identical functions *in vitro* [4–6], but targeted deletion of Rad52 in vertebrates has little effect on repair and recombination [7,8]. These results raise the question of whether mammalian Rad52 does indeed function in recombination and/or repair. Here we show that Rad52 is distributed throughout the nucleoplasm in actively cycling mammalian cells and is localized specifically to the nucleoli in S phase. In response to ionizing radiation, Rad52 relocalizes to form distinctive foci which are distributed throughout the nucleus and which colocalize with Rad50 foci in the DNA damage response. These data suggest that rDNA recombination and DNA repair are functions shared by mammalian Rad52 and its *S. cerevisiae* homolog, and provide evidence for the coordinated action of Rad50 and Rad52 in DNA repair.

Addresses: *Department of Molecular Biophysics and Biochemistry and §Genetics, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06520-8024, USA. ‡Institute of Biotechnology/Center for Molecular Medicine, University of Texas Health Science Center, 15355 Lambda Drive, San Antonio, Texas 78245, USA.

Present address: †Department of Hematology, City of Hope National Medical Center, 1500 East Duarte Road, Duarte, California 91010, USA.

Correspondence: Nancy Maizels
E-mail: nancy.maizels@yale.edu

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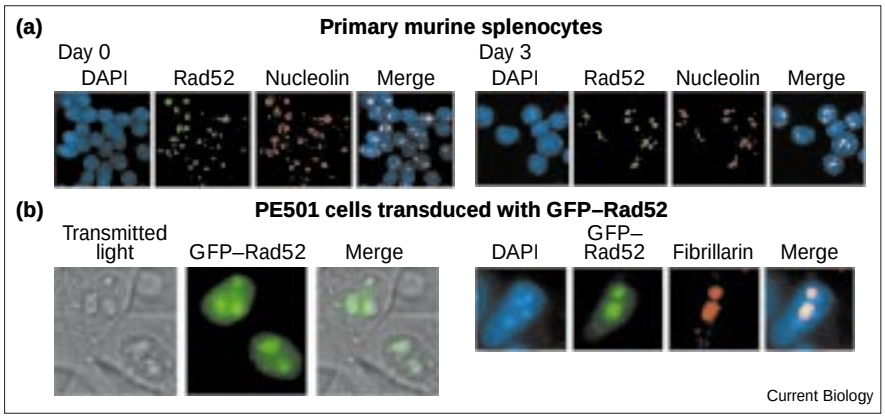
Results and discussion

The subcellular distribution of Rad52 was studied using antibodies to the endogenous protein and by visualizing the localization of a fusion protein comprising the green fluorescent protein and Rad52 (GFP–Rad52). Immunofluorescence microscopy identified prominent Rad52 foci in primary murine spleen cells stained with anti-Rad52 antibodies. Rad52 foci were present within the nucleus of essentially every cell, both before activation and following culture *in vitro* with the polyclonal mitogen, lipopolysaccharide (LPS; Figure 1a). Double staining with antibodies against Rad52, and antibodies against the abundant nucleolar protein, nucleolin, showed that Rad52 localized to the nucleoli in both quiescent (left) and activated (right) primary cells. The green GFP–Rad52 signal was visualized by fluorescence microscopy of stable transductants of the murine fibroblast line, PE501. The compact, granular nucleoli in the same cells were visualized by transmitted light microscopy; when the transmitted light and fluorescent images were merged, GFP–Rad52 was found to be concentrated predominately within the nucleoli (Figure 1b, left). Nucleolar localization of GFP–Rad52 was further verified by staining with anti-fibrillarin antibodies (Figure 1b, right).

We established that Rad52 relocalizes in response to cell-cycle controls by monitoring GFP–Rad52 localization in PE501 cells that had been transduced with GFP–Rad52 and then synchronized with nocodozole, which causes M-phase arrest. During the first two hours after removal of nocodozole, most cells were in M phase (Figure 2a). As the nuclear envelope disassembles during M phase, it was not surprising to find GFP–Rad52 throughout the cells at this time (Figure 2b). Between 2–8 hours after removal of nocodozole, most cells were in G_1, and GFP–Rad52 localized to the nucleoplasm in more than 98% of cells analyzed at this time. At this stage of the cell cycle, GFP–Rad52 appeared to be excluded from the nucleoli, which were typically visible as dark holes in both the DAPI and GFP–Rad52 staining patterns. The peak of DNA synthesis occurred at 11 hours after release from the nocodozole block (S phase; Figure 2a). The number of cells in which GFP–Rad52 localized to the nucleoli increased dramatically at this time: during S phase, GFP–Rad52 was localized to the nucleoli in about 80% of all cells emitting a green fluorescent signal (Figure 2b). Thus, the localization of Rad52 underwent dramatic changes in response to cell-cycle controls. In G_1, GFP–Rad52 was evenly distributed throughout the nucleus; during S phase, when DNA synthesis occurred, Rad52 localized to the nucleoli; and during M phase, when the nuclear envelope had broken down, the Rad52 signal was evident throughout the cell.

Nucleolar localization of Rad52 was evident not only in S phase but also in quiescent (G_0) cells. Rad52 may be

Figure 1



Rad52 localizes to the nucleolus. (a) Examples of resting (day 0, left) and LPS-activated (day 3, right) murine primary splenocytes stained with 4′,6-diamidino-2-phenylindole (DAPI, blue) and immunostained with anti-Rad52 (green) and anti-nucleolin (red) antibodies. In the merged images, yellow signals indicate colocalization of Rad52 and nucleolin. (b) Examples of PE501 cells tranduced with GFP–Rad52. Nucleoli were visualized by transmitted light microscopy (left), or fluorescence microscopy (right) subsequent to anti-fibrillarin antibody staining (red). The green GFP–Rad52 signal was concentrated predominantly in nucleoli.

sequestered in the nucleoli of quiescent cells, poised to function in recombination upon re-entry into the cell cycle or in response to DNA damage. The fact that Rad52 localized to the nucleolus in both S phase and in quiescent cells explains why Rad52 localized to the nucleoli in essentially all cells in the primary splenocyte culture (Figure 1a). Cells in this population are either quiescent or carrying out very rapid cell division, with most of the cell cycle devoted to S phase. In contrast, in asynchronous cultures of transformed cell lines, most cells are in G_1, consistent with the nucleoplasmic localization of Rad52 in the majority of cells in such cultures.

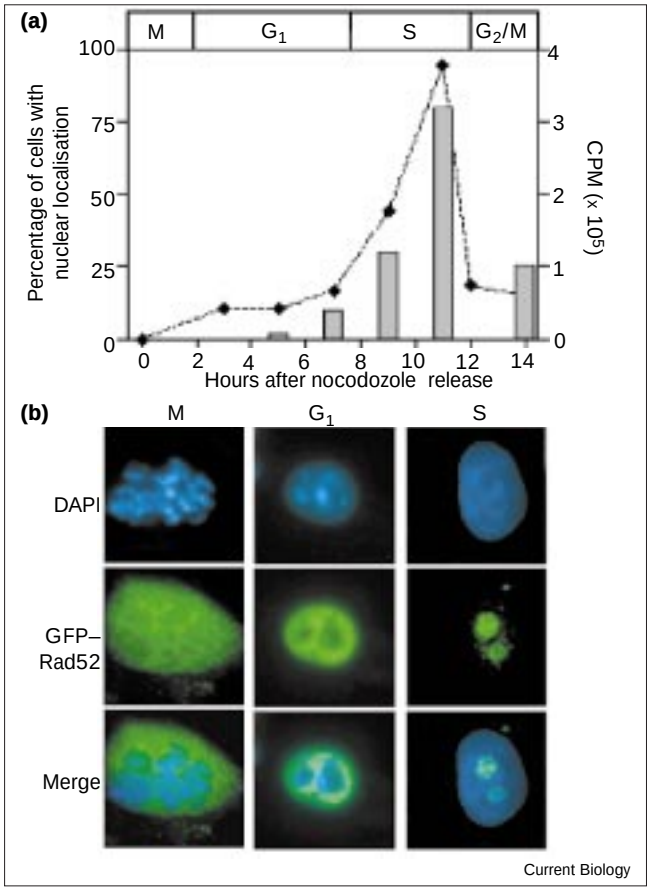


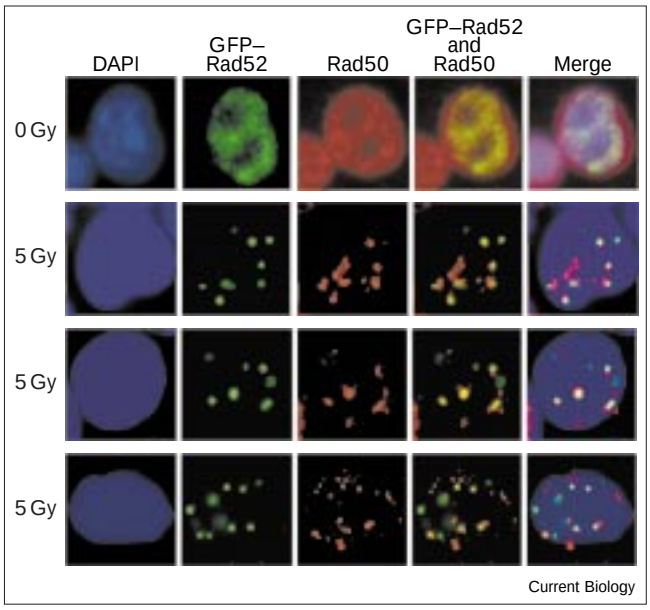
Figure 2

Dynamic relocalization of GFP–Rad52 during the cell cycle. (a) Murine fibroblast PE501 cells transduced with GFP–Rad52, and synchronized with nocodozole, were monitored by [^{3}H]thymidine labeling (line graph) to establish the timing of the S phase and by microscopy to localize GFP–Rad52. Bars indicate the percentage of cells in which GFP–Rad52 was localized to the nucleoli, relative to all cells that expressed GFP–Rad52. (b) Examples of GFP–Rad52-transduced PE501 cells seen at M, G_1 and S phases of the cell cycle. The merged images show colocalization of the DAPI stain (blue) and GFP–Rad52 signal (green).

Regulated localization of Rad52 to the nucleoli during S phase is likely to reflect participation of Rad52 in rDNA recombination. In *S. cerevisiae*, Rad52 is essential for rDNA recombination, and DNA replication is required for Rad52-dependent Holliday junction formation [2]. Our data suggest that the function of mammalian Rad52 in rDNA recombination may be similarly coordinated with DNA replication.

Colocalization of Rad52 and Rad50 in response to DNA damage provided evidence of Rad52 function in repair. In asynchronous cultures of untreated cells, GFP–Rad52 was evenly distributed throughout the nucleus; but within 8 hours following treatment with ionizing radiation (5 Grays, 5 Gy), GFP–Rad52 had become redistributed among many distinct foci scattered throughout the nucleus (Figure 3). Similar relocalization was evident following treatment with methylmethanesulfate or hydroxyurea (data not shown). The mammalian recombination/repair factors Rad50 and Mre11 form foci in response to ionizing radiation, apparently by migrating to regions of DNA damage [9–11]. Staining with anti-Rad50 antibodies

Figure 3



Colocalization of GFP–Rad52 and Rad50 foci induced by ionizing radiation. GFP–Rad52-transduced murine PD31 pre-B cells were either not irradiated (top panels), or irradiated with 5 Gy (and visualized 24 h later), stained with DAPI (blue) or anti-Rad50 antibody (red). The GFP–Rad52 fluorescent signal (green) and merged images are indicated.

showed that the majority of Rad52 and Rad50 foci colocalized. This provides additional evidence for Rad52 function in a pathway of ionizing-radiation-induced double-strand break repair that is recombination-dependent [12]. There were also significant numbers of Rad52 foci that did not colocalize with Rad50 foci, and vice versa. This raises the possibility that Rad52 may also be involved in Rad50-independent pathways of DNA repair.

Materials and methods

Cloning

To facilitate visualization and to avoid any possible artefacts due to antibody cross-reactivity, we generated a construct in which Rad52 was fused to GFP (reviewed in [13]). Sequences encoding the GFP tag were inserted at the 5′ end of the murine *Rad52* cDNA because other tags (GST, His_6 and the Myc epitope) expressed at the amino terminus of Rad52 have been shown not to interfere with Rad52 function *in vitro* and *in vivo* [4,14,15]. The GFP–Rad52 construct was carried in pLX-GR52, a retroviral shuttle vector that also carried the gene for neomycin resistance as a positive selection marker. These genes were flanked by retroviral packaging signals and coexpressed under control of the retroviral LTR. To generate pLX-GR52, a *Rad52* cDNA was first amplified from total RNA isolated from murine primary B cells by reverse transcription (RT) followed by PCR (RT-PCR), using a primer complementary to the 3′ untranslated region (UTR) for reverse transcription (5′-AGAGTTCAGCATGAGCCAAGG-3′) and two additional primers for PCR (5′-*ATG*GCTGGGCCTGAAGAAGCAG-3′ and 5′-*TCA*GGATGGATCTAGTTTCC-3′). The 1263 bp PCR product, which spans the murine *Rad52* gene (GenBank accession number U12135) from the ATG start codon through the TGA stop codon (italicized in the respective primers), was then cloned into the PCR cloning vector, pGEM-T (Promega). The sequence of the *Rad52* cDNA was confirmed by DNA sequencing throughout its length. Plasmid pAFS144, which contains sequences encoding GFP fused to the *Escherichia coli lac*I gene [16,17], generously provided by A. Straight, UC San Francisco, was modified by excising *lac*I by *Eco*RI and *Bam*HI digestion, and replacing it with a 34 bp polylinker with *Eco*RI–*Bam*HI ends and internal *Apa*I and *Sal*I cleavage sites. The *Rad52* cDNA was digested with *Apa*I and *Sal*I, which cut within the pGEM-T polylinker at sites flanking the PCR cloning site, to release the *Rad52* gene, which was then cloned into the corresponding sites of the modified pAFS144 plasmid to generate an in-frame GFP–Rad52 fusion. The GFP–Rad52 cassette was then excised with *Xho*I and *Bam*HI and transferred to the corresponding sites of the retroviral vector, LXSN, derived from the MoMLV and MoMSV viruses [18], generously provided by A.D. Miller and G.J. Rosman, Fred Hutchinson Cancer Research Center, Seattle.

Cell culture

Primary splenocytes were cultured as described previously [19,20]. Briefly, spleens were removed from 8–12-week-old BALB/c mice (Jackson Laboratory), disaggregated, and the cells washed twice with RPMI 1640 (Gibco) and then treated with 144 mM NH_4Cl, 17 mM Tris–HCl pH 7.4, for 5 min to lyse erythrocytes. Recovered lymphocytes were washed once with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) and cultured for 3 days in RPMI 1640 supplemented with 10% fetal bovine serum, 20 μM β-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin and 40 μg/ml LPS (Sigma). LPS activates only primary B lymphocytes to proliferate, so whereas at day 0 of culture approximately 70% of the cells are B cells and 30% are T cells, most of the cells surviving after 3 days of culture are B cells. PD31, a murine pre-B-cell line, was cultured under the same conditions but without added LPS. The murine fibroblast cell line, PE501, derived from NIH3T3 TK cells [18], was cultured in DMEM (Gibco), 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were cultured at 37°C in 5% CO_2.

DNA transfection and selection of stable cell lines

To generate retroviral virions carrying the LXSN-GR52 genomic RNA, cells of the PE501 packaging line (5×10^5 cells per 25 cm^2) were transfected with 5 μg pLX-GR52 DNA by incubation for 24 h with DNA treated with calcium phosphate (Gibco). The transfection medium was then removed and PE501 stable transfectants were selected by growth in medium containing 1 mg/ml G418; this medium was changed every 24 h for the first 4 days after transfection to remove dead cells and every 2–3 days thereafter to maintain stable transductants. The murine pre-B-cell line, PD31, was infected with LXSN-GR52 by overnight co-culture with PE501/GFP–Rad52 stable transfectants in the presence of 8 μg/ml polybrene (Sigma). PD31 pre-B cells, which grow in suspension, were separated from the adherent PE501 fibroblast cells by aspiration, and PD31 cells stably transduced with GFP–Rad52 were selected by culture in 1 mg/ml G418, as above.

Cell staining and immunofluorescence microscopy

To visualize nucleolar structure using transmitted light, cells were grown on chamber slides, washed once with PBS and examined without further fixation. For staining with anti-Rad52 or anti-nucleolin antibodies, primary splenocytes were prepared as described previously [20], washed once with PBS and resuspended at 4×10^5 cells per ml, and 0.5 ml aliquots were then centrifuged onto glass slides at 800 rpm for 4 min in a Cytospin 3 (Shandon). Cells were fixed in 3.7% paraformaldehyde for 30 min at room temperature, then in methanol for 30 min at –20°C. The slides were incubated with rat anti-human recombinant Rad52 antiserum (generously provided by E. Golub and C. Radding, Yale University) and rabbit anti-human recombinant nucleolin (residues 284–709) antiserum [20] for 30 min at room temperature (1:50 dilution in PBS with 1% bovine serum albumin (BSA)). After three washes with PBS, 10 min per wash, the slides were incubated

with the secondary antibody, which was either fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG (Pierce; 1:50 dilution in PBS, 1% BSA) or Texas-red-conjugated anti-rabbit Ig (Pierce; 1:100 dilution in PBS, 1% BSA), washed three times with PBS, counterstained with DAPI and mounted in antifade solution. For fibrillarin staining, PE501 cells transduced with GFP–Rad52 were grown on tissue culture chamber slides (Nalge Nunc International). Cells were fixed as described above, then incubated with mouse anti-fibrillarin monoclonal antibodies (72B9, generously provided by J.A. Steitz, Yale University) at 1:100 dilution for 40 min in PBS containing 1% BSA, washed twice with PBS for 10 min per wash, then incubated for 40 min with Texas-red-conjugated anti-mouse Ig (Pierce; 1:100 dilution in PBS, 1% BSA). Slides were washed again and treated with DAPI and antifade as described above. Rad50 immunostaining was carried out with culture supernatant [21] diluted 1:2, with other procedures as described above except that slides were incubated overnight at 4°C. In all cases where secondary antibodies were used in staining, controls were carried out to show that the secondary antibody alone gave no signal.

Cell synchronization and treatment with ionizing radiation

For nocodozole synchronization, PE501 cells stably transfected with GFP–Rad-52 (3×10^4 cells per cm^2) were incubated in growth medium containing 100 ng/ml nocodozole (Sigma) for 15 h, then released from cell-cycle arrest by washing twice with 4 ml pre-warmed DMEM followed by culture in medium lacking nocodozole. For hydroxyurea synchronization, 1 mM hydroxyurea (Sigma) was added to the culture for 15 h and removed as described above. Cell-cycle progression was monitored by labeling with 10 μCi/ml [^{3}H]thymidine (76 Ci/mmole; Amersham) for 1 h, then washing cells with PBS, and harvesting by trypsinization followed by centrifugation. To assay [^{3}H]thymidine incorporation, 5×10^4 cells were lysed by incubation for 20 min in 0.5 ml 10 mM EDTA, 0.1 N NaOH, 0.1% SDS at 70°C, then 0.5 ml 20% trichloroacetic acid was added, the lysate incubated on ice for 20 min and the precipitate collected and incorporated label measured by scintillation counting. PD31 cells transduced with GFP–Rad52 were cultured at 5×10^5 cells per ml for 1 h before treatment with ionizing radiation, then irradiated at 5 Gy using a cesium (^{137}Cs) irradiator at 4.28 Gy/min, and visualized 24 h later.

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