Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 344 (2006) 206–213
BBRC
www.elsevier.com/locate/ybbrc

A novel interation of nucleolin with Rad51 ☆

Ananya De [a,1], Sarah L. Donahue [a,2], Azah Tabah [a], Nancy E. Castro [a], Naomi Mraz [b], Jennifer L. Cruise [b], Colin Campbell [a,*]

[a] *Department of Pharmacology, The University of Minnesota Medical School, Minneapolis, MN 55455, USA*
[b] *Department of Biology, The University of St. Thomas, St. Paul, MN 55105, USA*

Received 13 March 2006
Available online 29 March 2006

Abstract

Nucleolin associates with various DNA repair, recombination, and replication proteins, and possesses DNA helicase, strand annealing, and strand pairing activities. Examination of nuclear protein extracts from human somatic cells revealed that nucleolin and Rad51 co-immunoprecipitate. Furthermore, purified recombinant Rad51 associates with in vitro transcribed and translated nucleolin. Electroporation-mediated introduction of anti-nucleolin antibody resulted in a 10- to 20-fold reduction in intra-plasmid homologous recombination activity in human fibrosarcoma cells. Additionally, introduction of anti-nucleolin antibody sensitized cells to death induced by the topoisomerase II inhibitor, amsacrine. Introduction of anti-Rad51 antibody also reduced intra-plasmid homologous recombination activity and induced hypersensitivity to amsacrine-induced cell death. Co-introduction of anti-nucleolin and anti-Rad51 antibodies did not produce additive effects on homologous recombination or on cellular sensitivity to amsacrine. The association of the two proteins raises the intriguing possibility that nucleolin binding to Rad51 may function to regulate homologous recombinational repair of chromosomal DNA.

Keywords: Nucleolin; Rad51; Homologous recombination; Protein interaction; DNA repair

The nucleolus is the subdomain where ribosomal RNA synthesis occur [1]. Recent proteomic analysis reveals that over 400 distinct proteins are present in nucleolus-enriched fractions [2,3]. The abundant nucleolar polypeptide nucleolin is believed to be essential for ribosome biosynthesis [4]. Consistent with this hypothesis yeast clones lacking the nucleolin homolog NSR1 have a reduced complement of ribosomes [5]. An extensive array of biochemical functions has been ascribed to nucleolin [4,6,7]. The protein binds DNA and RNA, and has DNA and RNA helicase activity. Nucleolin is a DNA-dependent ATPase and is capable of auto-degradation. Partially purified nucleolin isolated from a human tumor cell line possesses homologous DNA strand pairing activity [8]. Homologous DNA pairing activity is associated with a recombinant protein comprising the carboxyl terminal two-thirds of nucleolin expressed in *Escherichia coli*[8]. Nucleolin is believed to play a role in regulating cell growth and DNA replication. In addition, the protein is subjected to extensive post-translational modification [6,7].

Studies have revealed that nucleolin associates with a number of other proteins. For example, it was shown to be a component of LR1, a DNA-binding complex that regulates transcription in activated B cells [9]. Nucleolin is part of a B cell specific DNA recombination complex [10]. In addition, it was recently shown that nucleolin, along with

☆ *Abbreviations:* IgY, immunoglobulin Y; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; BSA, bovine serum albumin.

* Corresponding author. Fax: +1 612 625 4808.
E-mail address: campb034@umn.edu (C. Campbell).
[1] Present address: Department of Obstetrics and Gynecology, Stanford University School of Medicine, USA.
[2] Present address: Department of Microbiology and Immunology, Vanderbilt University Medical Center, USA.

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.03.113

topoisomerase I, controls the holoenzyme cohesion of the SV40 large T antigen helicase at DNA replication forks in vitro [11]. Furthermore, nucleolin binds to the human telomerase reverse transcriptase catalytic subunit hTERT [12].

The DNA helicase activity, homologous strand pairing activity, ability to renature complementary DNA strands, binding to topoisomerase I, and association with a number of DNA recombination complexes raise the possibility that nucleolin may regulate or modulate homologous recombinational repair of DNA. While the molecular biology of recombinational DNA repair in mammalian somatic cells remains poorly understood, it is widely believed that the RecA homolog Rad51 plays a major role [13]. It has been shown that the tumor suppressor protein p53 regulates the DNA strand-transferase activity of Rad51 [14,15]. Interestingly, it has recently been shown that the Rad51-binding replication protein A [16] and p53 physically associate with nucleolin [17,18]. We therefore postulated that the Rad51 and nucleolin proteins might also associate in vivo, and that this association could play a role in regulating Rad51-dependent homologous recombination activity in mammalian somatic cells.

The results presented below demonstrate that nucleolin is present in a complex with Rad51 in nuclear protein extracts prepared from mammalian somatic cells. Our data are consistent with the interpretation that nucleolin and Rad51 participate in a common pathway of homologous recombinational repair. The finding that the two proteins associate with each other raises the intriguing possibility that nucleolin may function to regulate Rad51's DNA repair activity.

Experimental procedures

Human cells. HT1080 human sarcoma-derived cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in humidified 5% CO_2 at 37 °C, in Dulbecco's modified Eagle's media with 10% fetal bovine serum. Nuclear protein extracts were prepared as described [19].

Antibodies. Chicken anti-nucleolin antibody was prepared as follows. A maltose-binding protein fusion containing residues 284–709 of human nucleolin was expressed in *E. coli* from plasmid pMalNuc (kindly provided by L. Hanakahi, see [19]), and purified on amylose resin (New England BioLabs, Beverly, MA) and by SDS–polyacrylamide gel electrophoresis. Laying hens were immunized with 100 μg protein. Preimmune IgY was purified as described [20] from eggs collected before the initial injection, and anti-nucleolin IgY from eggs collected 4 weeks after the initial injection. Affinity-purified anti-nucleolin IgY was prepared as described [21]. Briefly, recombinant his-tagged mouse nucleolin (exons 5–14, kind gift of Renato Aguilera, see [22]) was expressed in *E. coli* and loaded onto a nickel-agarose affinity chromatography column (Qiagen, Valencia, CA). This immobilized nucleolin was then used as an affinity column to purify the anti-nucleolin IgY preparation described above. Affinity-purified chicken anti-nucleolin was used in all experiments. Additional antibodies used were: rabbit polyclonal anti-nucleolin (C23) and rabbit polyclonal anti-Rad51 (both from Santa Cruz Biotechnology, Santa Cruz, CA).

Co-immunoprecipitation. Protein extracts were incubated in the presence or absence of specific antibody at 4 °C for 2 h. Subsequently, 25 μl of a 50% slurry of protein G–agarose beads was added and the mixture was incubated overnight at 4 °C. Immune complexes were sedimented and washed 4 times with buffer containing 20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM ethylene diamine, 0.1% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, and a protease inhibitor cocktail. Samples were resuspended in PAGE loading buffer and Western blot analysis was performed as described below.

Co-immunoprecipitation of in vitro transcribed and translated proteins. cDNA encoding p53 or nucleolin was used to perform polymerase chain reactions using Ready-To-Go-PCR beads (Amersham, Piscataway, NJ). The reaction products were incorporated into the pCR2.1 TA-cloning vector (Invitrogen, Carlsbad, CA), and the resulting constructs confirmed by DNA sequencing. Plasmids were linearized and 1–2 μg of each was used separately in a T7-coupled rabbit reticulocyte lysate in vitro transcription/translation system (Promega, Madison, WI) performed in the presence of ^{35}S-radiolabeled methionine (Amersham) for 90 min at 30 °C. Co-immunoprecipitation reactions were set up by incubating the p53 or nucleolin transcription/translation products with 1 μg of recombinant Rad51 protein commercially obtained from Abnova (Taiwan) in co-immunoprecipitation buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 0.1% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol) and rocked at 4 °C for 1–2 h. Anti-Rad51 antibody (5 μg) was added and another 1-h incubation was performed before the addition of 10 μl of a 50% slurry of protein G–agarose beads. This mixture was incubated overnight at 4 °C on a rocker platform. The following day, the beads were sedimented and washed three times with co-immunoprecipitation buffer. The washed beads were boiled for 5 min in loading buffer containing mercaptoethanol and resolved by SDS–PAGE on a 4–20% gradient gel (Bio-Rad). The gel was fixed in 10% acetic acid, dried, and exposed to a phosphoimager screen.

Western blot analysis. Protein extracts were resolved by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked in Tris-buffered saline (TBS) containing 5% bovine serum albumin (BSA) for 1 h at room temperature. Antibodies (diluted in TBS) were incubated with membranes overnight at 4 °C. Membranes were subsequently washed and appropriate second antibodies (anti-chicken or anti-rabbit) conjugated with alkaline phosphatase (1:5000 dilution; Sigma–Aldrich, St. Louis, MO) were added. After 1 h incubation at room temperature, membranes were again washed as above. After the final wash, membranes were incubated with alkaline phosphatase substrate (SigmaFast, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium, Sigma) and the color reaction stopped by rinsing with water.

In vivo intra-plasmid homologous recombination. Homologous recombination in intact cells was assayed as previously described [23]. Briefly, 20 μg of the substrate plasmid pSV2neoDR:DL(D) [24] was electroporated into 10^7 HT1080 cells in ice-cold serum-free media together with 5 μg pRSVEdl884 [25]. Electroporation was performed using a BTX (Holliston, MA) ECM 600 device set for 300 V and 960 μF (4 mm gap, 0.2 mL reaction volume). The latter plasmid encodes the SV40 large-T antigen, expression of which drives replication of plasmids that (like pSV2neo-DR:DL(D)) contain an SV40 origin of replication; the pRSVEdl884 plasmid itself lacks such an origin of replication. Immediately following electroporation, cells were plated and maintained for 48 h. Plasmid DNA was recovered from the cells [26] digested with the restriction enzyme *Dpn* I to eliminate plasmids that had not replicated in mammalian cells, and the DNA electroporated into the DH10B strain of *E. coli*. The plasmid pSV2neoDR:DL(D) confers resistance to ampicillin, however, appropriate homologous recombination between the two heteroalleles of the neomycin phosphotransferase gene on the plasmid confers resistance to both ampicillin and kanamycin. Therefore, the frequency with which the substrate plasmid undergoes homologous recombination in the mammalian cells can be determined by dividing the number of bacterial colonies that are resistant to both ampicillin and kanamycin by the total number of bacterial colonies that are resistant to ampicillin. The homologous recombination frequency is expressed as a percentage after multiplying this value by 100. Control experiments reveal that the frequency of spontaneous homologous recombination catalyzed by the recombination-deficient host bacterial strain is several orders of magnitude lower than the

frequency with which recombination occurs upon transfection of the plasmid into mammalian somatic cells (data not shown, but see [24]).

Antibody-mediated sensitivity to amsacrine. Cellular sensitivity to drug-induced cell death was determined using the sulforhodamine B assay [27], modified as previously described [28]. Approximately 2×10^6 cells were electroporated as described above in the presence or absence of 0.4 μg of antibody. Immediately following electroporation, 1×10^5 cells/well were plated in 24-well dishes and allowed to recover for 24 h in drug-free media. Cultures were then exposed to media containing various concentrations of amsacrine (Sigma) dissolved in dimethyl sulfoxide, or vehicle alone; after 96 h of incubation, cells were exposed to sulphorhodamine B and percent cell survival was estimated using the optical density at 564 nm, relative to that of cultures not exposed to amsacrine.

Ultraviolet light sensitivity. Sensitivity to ultraviolet light was determined using the sulphorhodamine B assay. Cells were electroporated as described above in the presence or absence of antibody, plated and allowed to recover for 12–16 h. Subsequently, growth media were removed and cells were exposed to 0, 10, 20, 50, or 100 J/m^2 ultraviolet light; normal growth media were immediately replaced. Cells were maintained for a further 96 h before measuring cell survival. For each treatment, the optical density at 564 nm was measured and used to estimate percent survival, compared to cultures not exposed to ultraviolet light.

Antibody-mediated cell hypersensitivity to PvuII. Cellular sensitivity to *Pvu*II-induced cell death was determined using the sulforhodamine B assay. Approximately 2×10^6 cells were electroporated as described above in the presence or absence of 0.4 μg of antibody and the presence or absence of 10 U *Pvu*II. Immediately following electroporation, 1×10^5 cells/well were plated in 24-well dishes and allowed to recover for 24 h in serum-containing media. After 96 h of incubation, cell survival was estimated using sulforhodamine B. For each treatment, the optical density at 564 nm was measured and used to estimate percent survival, compared to cells that had been electroporated in the absence of *Pvu*II.

Results

We previously observed an intriguing correlation between the concentration of nucleolin and the frequency with which plasmid DNA molecules underwent homologous recombination, both in vitro and in intact cells [29]. Based on these findings and on other published findings linking nucleolin to DNA recombination activity (see Introduction), we hypothesized that nucleolin could participate in or facilitate homologous recombination in mammalian somatic cells.

To determine whether nucleolin and Rad51 associate in human somatic cells, protein extracts were prepared from the human fibrosarcoma cell line HT1080 cells and subjected to immunoprecipitation and Western blotting using anti-Rad51 or anti-nucleolin antibodies. As Fig. 1A shows, both antibodies precipitated Rad51 protein. Crucially, no Rad51 immunoreactivity was detected in precipitates formed in the absence of specific antibody. Fig. 1B illustrates the results of the converse experiment, in which both anti-nucleolin and anti-Rad51 antibodies immunoprecipitated nucleolin. These findings indicate that Rad51 and nucleolin associate in nuclear protein extracts.

To determine whether these proteins could also interact in vitro radioactive recombinant nucleolin was generated using a coupled in vitro transcription/translation reaction, and this material was incubated with unlabeled purified Rad51 protein. Coomassie blue-stained SDS–polyacrylamide gels reveal that the purified recombinant Rad51 protein was devoid of detectable protein contaminants (data not shown). Previous results have demonstrated that recombinant nucleolin and p53 proteins directly interact in vitro [17,18]. To confirm that the commercially obtained Rad51 possessed biological activity, we examined whether the recombinant Rad51 protein precipitated radioactive p53 protein only when recombinant Rad51 protein was present. The data in Fig. 2A reveal that radioactive p53 was precipitated by anti-Rad51 antibody only when purified Rad51 was present in the immunoprecipitation reaction.

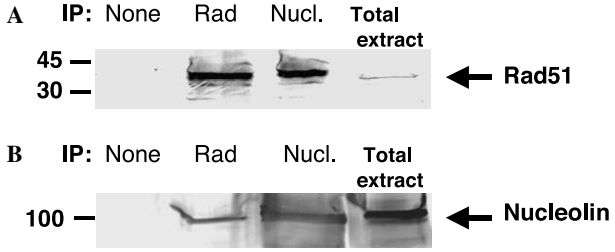

Fig. 1. Nucleolin and Rad51 co-immunoprecipitate from nuclear protein extracts. Immunoprecipitation reactions (IP) were performed on 50 μg of nuclear protein extract using the indicated antibodies. Precipitated material was resolved by SDS–polyacrylamide gel electrophoresis and Western blot analysis performed using antibody specific for Rad51 (A) or nucleolin (B). Arrows depict the electrophoretic mobilities of Rad51 (A) and nucleolin (B) proteins. Samples of untreated nuclear extracts (10 μg) were resolved alongside immunoprecipitates to serve as positive controls.

We then examined the interaction of recombinant Rad51 and radiolabeled nucleolin. The results in Fig. 2B illustrate that anti-Rad51 antibody could precipitate in vitro transcribed and translated nucleolin when recombinant Rad51 was present, but not when it was absent. These data support the conclusion that Rad51 and nucleolin are capable of interacting in vitro. While we cannot rule out the formal possibility that this interaction is mediated by one or more components present in the in vitro transcription/translation reaction, this seems unlikely, and suggests that the two proteins are capable of directly interacting.

The finding that Rad51 and nucleolin interact raises the possibility that the two proteins may cooperate in a pathway of recombinational repair. We have previously shown that electroporation-mediated introduction of specific antibodies can be used to test whether a particular antigen plays an essential role in intracellular DNA repair or recombination [23,32]. We therefore used co-electroporation of antibodies specific for either nucleolin or Rad51 to test the hypothesis that the two proteins cooperate in a recombinational DNA repair pathway. We examined the frequency with which the introduced plasmid substrate pSV2DR:DL(D) underwent homologous recombination (depicted schematically in Fig. 3A). As Fig. 3B depicts, intra-plasmid homologous recombination occurred within the pSV2DR:DL(D) plasmid at a basal frequency of 0.64% (lane '−'). Expressed another way, these data indicated that of every 10,000 plasmids recovered, 64 had undergone a homologous recombination event that

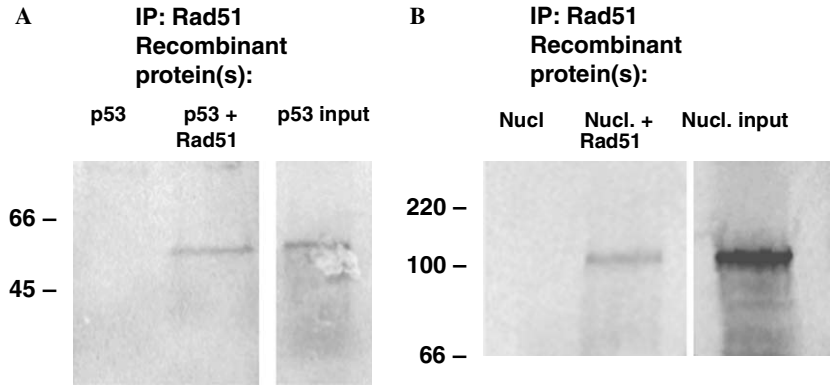

Fig. 2. Nucleolin and p53 interact with Rad51 in vitro. (A) [^{35}S]Methionine-labeled recombinant p53 generated by in vitro transcription/translation was used in Rad51 immunoprecipitation reactions performed in the absence (p53) or presence (p53 + Rad51) of purified recombinant Rad51. The precipitated material was resolved by SDS–polyacrylamide gel electrophoresis, and radioactivity was detected using a phosphorimager screen. The lane marked p53 input contains in vitro transcribed and translated p53 that was directly resolved by SDS–polyacrylamide gel electrophoresis. The amount of p53 protein loaded on this lane is equivalent to the amount of p53 used in the immunoprecipitation experiments depicted in the two other lanes. (B) [^{35}S]Methionine-labeled recombinant nucleolin generated by in vitro transcription/translation was used in immunoprecipitation reactions performed in the absence (Nucl.) or presence (Nucl. + Rad51) of 1 μg purified recombinant Rad51. The lane marked Nucl. input contains in vitro transcribed and translated nucleolin that was directly resolved by SDS–polyacrylamide gel electrophoresis. The amount of nucleolin protein loaded in this lane is equivalent to the amount used in the immunoprecipitation experiments depicted in the two other lanes.

reconstituted a functional neomycin phosphotransferase gene. This value is in close agreement with intra-plasmid homologous recombination frequencies previously reported for human cell lines in culture [29,33]. Co-introduction of non-specific immunoglobulin (Fig. 3B, lanes C1 and C2) or pre-immune immunoglobulin (Fig. 3B, lane P) did not significantly alter the frequency of intra-plasmid homologous recombination. In contrast, co-introduction of affinity-purified anti-nucleolin antibody reduced the frequency of intra-plasmid homologous recombination by 13-fold, to 0.049% (Fig. 3B, lane N1). When a commercially obtained anti-nucleolin antibody was used the intra-plasmid homologous recombination frequency was 0.023%, a reduction of nearly 30-fold, compared to controls (Fig. 3B, lane N2).

We had previously observed that introduction of anti-Rad51 antibody reduced the frequency of intra-plasmid homologous recombination in Rad51-overexpressing Fanconi anemia cells [23], and in an immortalized human tumor cell line that overexpressed Rad51 (data not shown). It was therefore not surprising to observe that as is depicted in Fig. 3B (lane 51), introduction of anti-Rad51 antibody reduced the frequency of intra-plasmid homologous recombination in HT1080 cells by nearly 10-fold to 0.074%. We noted that the magnitude of inhibition observed in the presence of the anti-Rad51 antibody was similar to that seen in the presence of either of the two anti-nucleolin antibodies, raising the possibility that the two proteins cooperate in a common pathway of homologous recombination.

We have observed that approximately 90% of HT1080 cells observed 24–48 h post-electroporation are transfected (data not shown). We therefore reasoned that electroporation in the presence of two different antibody molecules should result in the co-introduction of both antibodies into a large percentage of cells. Control experiments were performed in which HT1080 cells were electroporated with 0.4 μg each of two different labeled antibodies, one of which was labeled with fluorescein and the other of which was labeled with rhodamine. Approximately 90% of the cells examined 24 h post-electroporation contained detectable levels of fluorescent antibody. Greater than 95% of these cells displayed fluorescence from both antibodies. Based on these findings, we reasoned that it would be possible to utilize a co-electroporation strategy to gain insight into whether the nucleolin and Rad51 proteins were cooperating in a common pathway of homologous recombination.

We compared the frequency of intra-plasmid homologous recombination in cells treated with control antibody, or antibody specific for Rad51, nucleolin, or both. As the results in Fig. 3C reveal, in all cases, introduction of antibodies specific for Rad51 (lane 51) or nucleolin (lane N2) significantly reduced the frequency of homologous recombination ($P < 0.001$, χ^2), compared to that observed in the absence of antibody (lane '-'). The frequency of intra-plasmid homologous obtained when anti-Rad51 and anti-nucleolin antibodies were co-introduced along with the recombination substrate (lane B) was not significantly different from that observed when either of the two antibodies was introduced separately. The residual levels of homologous recombination activity present under these conditions were sufficiently above background levels that additional inhibitory effects associated with co-introduction of the two antibodies would have been detectable.

A previous report indicated that recombinational repair mediated via Rad51 plays an important role in cell survival following treatment with a type II topoisomerase inhibitor [34]. We therefore examined the effect of introduced antibodies on survival of cells treated with 100 nM amsacrine, a type II topoisomerase inhibitor. Fig. 4A shows that

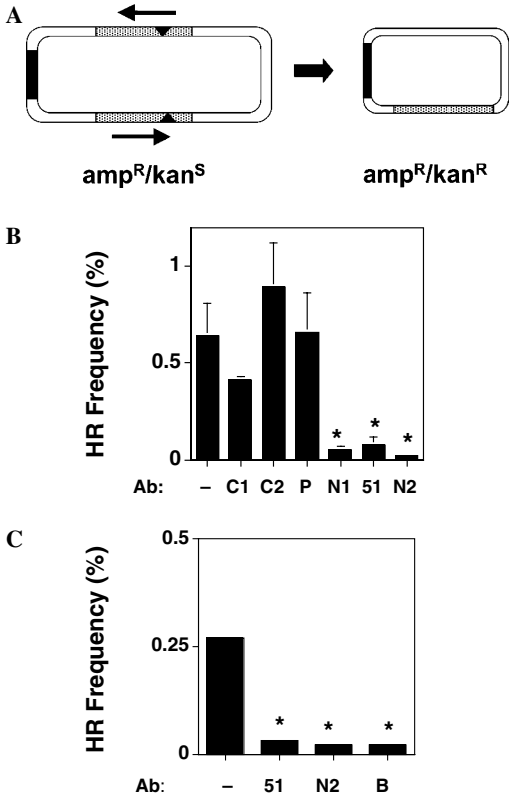

Fig. 3. Homologous recombination activity is reduced in the presence of anti-nucleolin and anti-Rad51 antibodies. The frequency of homologous recombination was determined in human HT1080 cells in the presence or absence of antibodies using an extrachromosomal plasmid-based assay system. (A) Schematic illustration of the plasmid pSV2neoDR:DL(D) and a potential recombinant product plasmid. The non-recombinant plasmid harbors an intact β-lactamase gene (filled rectangle), capable of conferring ampicillin resistance (ampR) on sensitive bacteria, and two non-functional heteroalleles of the neomycin phosphotransferase gene (shaded rectangles). These non-reverting alleles harbor intragenic non-overlapping deletions that span essential portions of the coding region of the gene (filled triangles). The arrows depict the transcriptional orientation of the neomycin phosphotransferase genes. Homologous recombination between these two alleles can reconstitute a functional neomycin phosphotransferase resistance gene (indicated by the shaded rectangle lacking a triangle), capable of conferring kanamycin resistance (kanR) on sensitive bacteria. See Experimental procedures, and Results for additional details. (B) pSV2neo DR:DL(D) was electroporated into HT1080 cells in the presence and absence of 4μg of antibody, as indicated, and the homologous recombination frequency was calculated as described in the methods section. Treatments were: no antibody (−); rabbit non-specific immunoglobulin (C1), chicken non-specific antibody (C2); pre-immune chicken immunoglobulin (P); chicken affinity-purified anti-nucleolin antibody (N1); rabbit anti-Rad51 antibody (R); rabbit anti-nucleolin antibody (N2). The results represent the mean plus or minus the SEM from four or more independent experiments. $^*P < 0.0025$, t test. There was no significant difference amongst the levels of residual homologous recombination detected in cells exposed to non-specific antisera ($P > 0.2$ or higher). (C) Intra-cellular homologous recombination was measured in the absence of no antibody (−); rabbit anti-Rad51 antibody (51); rabbit anti-Nucleolin antibody (N2); both anti-Rad51 and anti-Nucleolin antibody (B). In all cases 0.4 μg of antibody was used. The data represent the average of two or more experiments. $^*P < 0.001$, χ^2.

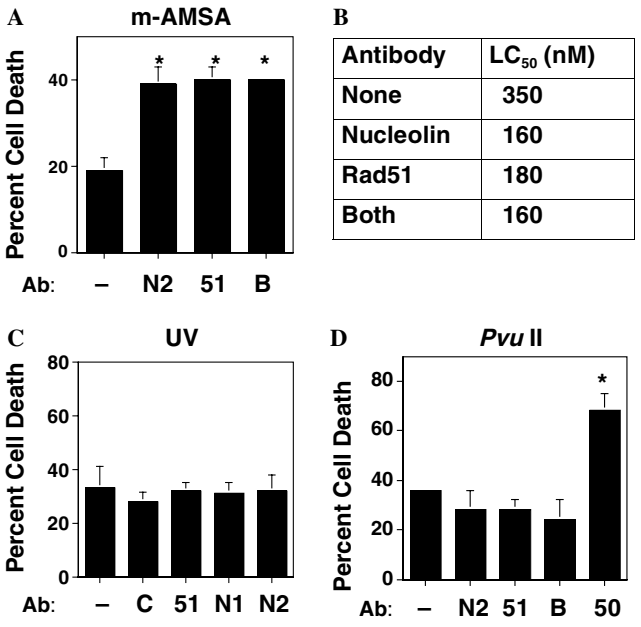

Fig. 4. Introduction of anti-nucleolin or anti-Rad51 antibodies sensitizes cells to m-AMSA, but not to ultraviolet radiation or electroporated *Pvu*II. (A) Human HT1080 cells were electroporated in the presence or absence of 0.4 μg antibodies as indicated below, and subsequently exposed to100 nM amsacrine, and cell viability was determined as described in Experimental procedures. (−) No antibody; (N2) rabbit anti-nucleolin antibody; (R) rabbit anti-Rad51 antibody; (B) anti-nucleolin antibody plus anti-Rad51 antibody. Data represent the average ± SEM of three or more independent experiments. Sensitivity to cell death induced by m-AMSA was significantly reduced by the presence of specific antibodies compared to that obtained in the absence of antibody ($P < 0.01$, t test). (B) The concentration of amsacrine necessary to kill 50% of cells (LC_{50}) was calculated from a series of cytotoxicity experiments ($N = 4$) performed using a drug concentration range of 50–350 nM. In all cases, antibody treatment significantly reduced the LC_{50} value ($P < 0.01$, t test). (C) The effects of antibodies on ultraviolet radiation-induced cell death. (−) No antibody; (C) rabbit non-specific antibody; (R) rabbit anti-Rad51 antibody; (N1) chicken anti-nucleolin antibody; (N2) rabbit anti-nucleolin antibody. Data represent the average ± SEM of three or more independent experiments. Antibody treatment did not significantly alter cellular sensitivity to ultraviolet radiation-induced cell death. (D) The effect of co-electroporation of antibodies on cell death induced by *Pvu*II (20U). (−) No antibody; (N2) rabbit anti-Nucleolin antibody; (R) anti-Rad51 antibody; (B) anti-Rad51 antibody plus anti-nucleolin antibody; (50) anti-Rad50 antibody. Data represent the average ± SD of two or more independent experiments. Only anti-Rad50 antibody exerted a significant effect on the percent cell death. $^*P < 0.01$, t test.

treatment with either anti-nucleolin (lane N2) or anti-Rad51 antibodies (lane 51) significantly increased the sensitivity of cells to the cytotoxic effects of 100 nM amsacrine, compared to cells in which non-specific antibody had been introduced (lane '–'). Again, co-introduction of anti-Rad51 and anti-nucleolin antibodies did not produce an additive effect on amsacrine-induced cell death (Fig. 4A, lane B). A series of concentration-dependence experiments showed that antibody treatment reduced the LC_{50} (drug concentration that kills 50% of cells) of amsacrine by approximately 2-fold, from 350 nM for control antibody-treated cells to 160 nM (anti-nucleolin-treated) and 180 nM (anti-Rad51-treated) cells. The LC_{50} of amsacrine in cells treated with both antibodies was not significantly different from that observed for cells treated with either of

the antibodies separately (Fig. 4B). To confirm that this effect was specific, we also examined the effect of antibody treatment on survival following exposure to ultraviolet radiation, for which there is no evidence implicating recombinational repair. Data presented in Fig. 4C indicate that introduction of neither anti Rad51 (lane 51) nor anti-nucleolin antibody (lanes N1 and N2) had any significant effect on cellular sensitivity to death induced by ultraviolet radiation, compared to cells in which either no antibody (lane '−') or control non-specific antibody (lane C) was introduced.

We also examined the influence of introduction of anti-Rad51 and anti-nucleolin antibodies on cellular survival following introduction of the restriction endonuclease *Pvu*II. The results presented in Fig. 4D reveal that, as in the case with ultraviolet radiation-induced cell death, neither anti-nucleolin (lane N2) nor anti-Rad51 (lane 51) antibody increased cellular sensitivity to *Pvu*II-induced cell death to levels above those observed in control antibody-treated cells (lane '−'). Again, co-introduction of both antibodies had no effect on cellular survival (lane B). As a control, we performed a parallel experiment in which anti-Rad50 antibody, which we had previously determined sensitized mammalian somatic cells to restriction endonuclease-induced cell death [32] was introduced into the cells along with *Pvu*II. As Fig. 4C reveals, this treatment significantly enhanced the cytotoxic effect of *Pvu*II on the cells (lane 50). Taken together, the results in Figs. 3 and 4 are consistent with the interpretation that Rad51 and nucleolin functions are both essential for optimal cellular recombinational DNA repair.

Discussion

The results described above indicate that the Rad51 and nucleolin proteins interact both in vitro and in vivo. The results from the antibody inhibition studies suggest that this interaction may have functional significance with respect to cellular recombinational DNA repair. While the molecular genetics of homologous recombination in mammalian somatic cells is incompletely understood, it is known that the evolutionarily conserved protein Rad51 plays an important and probably central role. A recent paper showed that Rad51 protects mammalian somatic cells from cell death induced by type II topoisomerase inhibitors [34]. It was therefore unsurprising that introduction into HT1080 cells of antibodies specific for Rad51 significantly reduced the frequency of intra-plasmid homologous recombination and sensitized the cells to the cytotoxic effects of amsacrine, without exerting an influence on cellular sensitivity to ultraviolet radiation. Interestingly, we observed that the magnitude of the inhibitory effects of antibodies against Rad51 and nucleolin was comparable, and that there was no additive effect when both antibodies were co-introduced into cells. These findings suggest that both proteins may be involved in a single pathway of homologous recombination.

Our findings add an entry to the growing list of Rad51-binding proteins, a list that includes Rad52 [35], MCD 1 [36], Blm, the Bloom's helicase [37], Ubl1 [38], Xrcc2 [39], Brca2 [40], replication protein A [16], and p53 [14,15], among others. At the same time, Rad51 joins YB-1 [41], topoisomerase I [42], replication protein A [16], and p53 [18] as proteins known to associate with nucleolin. Replication protein A and p53 have each been shown to interact with both nucleolin and Rad51. This is of particular interest, as p53 functions to negatively regulate Rad51's strand-transferase activity [14,15]. It is known that this inhibitory effect of p53 is independent of its G1-S checkpoint or transcriptional activation functions [43]. Instead, it appears that the inhibition is mediated through direct interaction with the Rad51 protein [43], and is dependent upon its ability to interact with, and potentially sequester, replication protein A, a single-strand DNA-binding protein [44]. Replication protein A promotes strand pairing and exchange by human Rad51 [16,45]. The mechanism is thought to involve removal by replication protein A of secondary structure from single-stranded DNA, to allow efficient formation of a Rad51 nucleoprotein filament. In vitro, replication protein A actually competes with Rad51 for binding to single-stranded DNA, and additional proteins of the Rad52 epistasis group—yeast Rad52 itself [46] or human Rad51B and Rad51C [47]—are required to relieve this competition and allow replication protein A to promote homologous recombination. Rad51B and C are indispensable for normal recombination and repair, but they only partially relieve the competitive effect of replication protein A.

A number of recent papers reveal that both heat shock and DNA-damaging agents induce a relocalization of nucleolin from the nucleolus to the nucleoplasm. These studies reveal that nucleolin subsequently binds replication protein A, resulting in a DNA replication blockade. Interestingly, Daniely and Boroweic [48] and Kim et al. [49] report that nucleolin translocation and the formation of the replication protein A/nucleolin complex in vivo are dependent on a physical interaction between nucleolin and p53. These findings are consistent with the hypothesis that regulated interactions between Rad51 and the three Rad51-binding proteins nucleolin, replication protein A, and p53 function as part of a cell checkpoint process that is activated following genotoxic stress. One can imagine that interactions between these various molecules could function to either activate or repress Rad51-mediated repair and/or replication, depending on the nature and extent of DNA damage detected. It is tempting to propose, as others have, that nucleolin is sequestered within the nucleolus and that its regulated release from this site, triggered by recognition of DNA damage, functions to help the cell regulate the complex response to DNA damage that, depending on multiple factors, will lead to cell cycle arrest, activation of DNA repair, or activation of programmed cell death.

Acknowledgments

The authors thank M. Rafferty and M. Riehle for technical assistance. This work was supported in part by grants from the National Cancer Institute (CA70494-01; JLC) and the National Institute on Aging (AG16678; CC). A.D. was supported by a pre-doctoral fellowship from the American Heart Association.

References

[1] Y.W. Lam, L. Trinkle-Mulkahy, A.I. Lamond, The Nucleolus, J. Cell Sci. 118 (2005) 1335–1337.
[2] J.S. Andersen, C.E. Lyon, A.H. Fox, A.K. Leung, Y.W. Lam, H. Steen, M. Mann, A.I. Lamond, Directed proteomic analysis of the human nucleolus, Curr. Biol. 12 (2002) 1–11.
[3] A. Scherl, Y. Coute, C. Deon, A. Calle, K. Kindbeiter, J.C. Sanchez, A. Greco, D. Hochstrasser, J.J. Diaz, Functional proteomic analysis of human nucleolus, Mol. Biol. Cell 13 (2002) 4100–4109.
[4] H. Ginisty, H. Sicard, B. Roger, P. Bouvet, Structure and functions of nucleolin, J. Cell. Sci. 112 (1999) 761–772.
[5] W.C. Lee, D. Zabetakis, T. Melese, NSR1 is required for pre-rRNA processing and form the proper maintenance of steady-state levels of ribosomal subunits, Mol. Cell. Biol. 12 (1992) 3865–3871.
[6] M. Srivastave, H.B. Pollard, Molecular dissection of nucleolin's role in growth and cell proliferation: new insights, FASEB J. 13 (1999) 1911–1922.
[7] R. Tuteja, N. Tuteja, Nucleolin: a multifunctional major nucleolar phosphoprotein, Crit. Rev. Biochem. Mol. Biol. 33 (1998) 407–436.
[8] B. Thyagarajan, R. Lundberg, M. Rafferty, C. Campbell, Nucleolin promotes homologous DNA pairing in vitro, Somat. Cell Mol. Genet. 24 (1998) 263–272.
[9] L.A. Hanakahi, L.A. Dempsey, M.J. Lin, N. Maizels, Nucleolin is one component of the B cell-specific transcription factor and switch region binding progein, LR1, Proc. Natl. Acad. Sci. USA 94 (1997) 3605–3610.
[10] T. Borggrefe, M. Wabl, A.T. Akhmedov, R. Jessberger, A B-cell-specific DNA recombination complex, J. Biol. Chem. 273 (1998) 17025–17035.
[11] S. Seinsoth, H. Uhlmann-Schiffler, H. Stahl, Bidirectional DNA unwinding by a ternary complex of T antigen, nucleolin and topoisomerase I, EMBO Rep. 4 (2003) 263–268.
[12] S. Khurts, K. Masutomi, L. Delgermaa, K. Arai, N. Oishi, H. Mizuno, N. Hayashi, W.C. Hahn, S. Murakami, Nucleolin interacts with telomerase, J. Biol. Chem. 279 (2004) 51508–51515.
[13] W. Henning, H.W. Sturzbecher, Homologous recombination and cell cycle checkpoints: Rad51 in tumour progression and therapy resistance, Toxicol. 193 (2003) 91–109.
[14] S.P. Linke, S. Sengupta, N. Khabie, B.A. Jeffries, S. Buchhop, S. Miska, W. Henning, R. Pedeux, X.W. Wang, L.J. Hofseth, Q. Yang, S.H. Garfield, H.W. Sturzbecher, C.C. Harris, p53 interacts with hRad51 and hRad54, and directly modulates homologous recombination, Cancer Res. 63 (2003) 2596–2605.
[15] D. Yoon, Y. Wang, K. Stapleford, L. Wiesmullar, J. Chen, p53 inhibits strand exchange and replication fork regression promoted by human Rad51, J. Mol. Biol. 336 (2004) 639–654.
[16] E.I. Golub, R.C. Gupta, T. Haaf, M.S. Wold, C.M. Radding, Interaction of human Rad51 recombination protein with single-stranded DNA binding protein RPA, Nucleic Acids Res. 26 (1998) 5388–5393.
[17] Y. Daniely, J.A. Borowiec, Formation of a complex between nucleolin and replication protein A after cell stress prevents initiation of DNA replication, J. Cell Biol. 149 (2000) 799–810.
[18] Y. Daniely, D.D. Dimitrova, J.A. Borowiec, Stress-dependent nucleolin mobilization mediated by p53-nucleolin complex formation, Mol. Cell. Biol. 22 (2002) 6014–6022.
[19] R. Jessberger, P. Berg, Repair of deletions and double strand gaps by homologous recombination in a mammalian in vitro system, Mol. Cell. Biol. 11 (1991) 445–457.
[20] A. Polson, M.B. von Wechmar, G. Fazakerley, Antibodies to proteins from yolk of immunized hens, Immunol. Commun. 9 (1980) 495–514.
[21] J. Gu, C.G. Stephenson, M.J. Iadarola, Recombinant proteins attached to a nickel–NTA column: use in affinity purification of antibodies, BioTechniques 17 (1994) 257–262.
[22] G.A. Miranda, I. Chokler, R. Aguilera, The murine nucleolin protein is an inducible DNA and ATP binding protein which is readily detected in nuclear extracts of lipopolysaccharide-treated splenocytes, Exp. Cell Res. 217 (1995) 294–308.
[23] S.L. Donahue, R. Lundberg, R. Saplis, C. Campbell, Deficient regulation of DNA double-strand break repair in Fanconi anemia fibroblasts, J. Biol. Chem. 278 (2003) 29487–29495.
[24] B. Thyagarajan, C. Campbell, Elevated homologous recombination activity in Fanconi anemia fibroblasts, J. Biol. Chem. 272 (1997) 23328–23333.
[25] F.A. Ray, D.S. Peabody, J.L. Cooper, L.S. Cram, P.M. Kraemer, SV40 T antigen alone drives karyotype instability that precedes neoplastic transformation of human diploid fibroblast, J. Cell Biochem. 42 (1990) 13–31.
[26] B. Hirt, Selective extraction of polyoma DNA from infected mouse cell cultures, J. Mol. Biol. 26 (1967) 365–369.
[27] P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J.T. Warren, H. Bokesch, S. Kenney, M.R. Boyd, New colorimetric cytotoxicity assay for anticancer-drug screening, J. Natl. Cancer Inst. 82 (1999) 1107–1112.
[28] S.L. Donahue, Campbell, A DNA double strand break repair defect in Fanconi anemia fibroblasts, J. Biol. Chem. 277 (2002) 46243–46247.
[29] B. Thyagarajan, J.L. Cruise, C. Campbell, Elevated levels of homologous DNA recombination activity in the regenerating rat liver, Somat. Cell Mol. Genet. 22 (1996) 31–39.
[32] S.L. Donahue, C. Campbell, A Rad50-dependent pathway of DNA repair is deficient in Fanconi anemia fibroblasts, Nucleic Acids Res. 32 (2004) 3248–3257.
[33] G.K. Finn, B. Kurz, R.Z. Cheng, R.J. Shmookler Reis, Homologous plasmid recombination is elevated in immortally transformed cells, Mol. Cell. Biol. 9 (1989) 4009–4017.
[34] L.T. Hansen, C. Lundin, M. Spang-Thomasen, L.N. Petersen, T. Helleday, The role of RAD51 in etoposide (VP16) resistance in small cell lung cancer, Int. J. Cancer 105 (2003) 472–479.
[35] Z. Shen, K.G. Cloud, D.J. Chen, M.S. Park, Specific interactions between the human Rad51 and Rad52 proteins, J. Biol. Chem. 271 (1996) 148–152.
[36] J. Zhang, Z. Ma, A. Treszezamsky, S.N. Powell, MDC1 interacts with Rad51 and facilitates homologous recombination, Nat. Struct. Mol. Biol. 12 (2005) 902–920.
[37] L. Wu, S.L. Davies, N. Levitt, I.D. Hickson, Potential role for the BLM helicase in recombinational repair via a conserved interaction with Rad51, J. Biol. Chem. 276 (2001) 19375–19381.
[38] Z. Shen, P.E. Pardington-Purtymun, J.C. Comeaux, R.K. Moyzis, D.J. Chen, UBL1, a human ubiquitin-like protein associating with human Rad51/Rad52 proteins, Genomics 36 (1996) 271–279.
[39] K.S. Shim, C. Schmutte, G. Tombline, C.D. Heinen, R. Fishel, hXrcc2 enhances ADP/ATP processing and strand exchange by hRad51, J. Biol. Chem. 279 (2004) 30385–30394.
[40] R. Mizuta, J.M. LaSalle, H.-L. Cheng, A. Shinohara, H. Ogawa, N. Copeland, N.A. Jenkins, M. Lalande, F.W. Alt, Rab22 and Rab163/mouse BRCA2: proteins that specifically interact with the Rad51 protein, Proc. Natl. Acad. Sci. USA 94 (1997) 6927–6932.
[41] I. Gaudreault, D. Guay, M. Lebel, YB-1 promotes strand separation in vitro of duplex DNA containing either mispaired bases or cisplatin modifications, exhibits endonucleolytic activities

and binds several DNA repair proteins, Nucleic Acids Res. 32 (2004) 316–327.
[42] A.K. Bharti, M.O. Olson, D.W. Kufe, E.H. Rubin, Identification of a nucleolin binding site in human topoisomerase I, J. Biol. Chem. 271 (1996) 1993–1997.
[43] P. Bertrand, Y. Saintigny, B.S. Lopez, p53's double life: transactivation-independent repression of homologous recombination, Trends Genet. 20 (2004) 235–243.
[44] L.Y. Romanova, H. Willers, M.V. Blagosklonny, S.N. Powell, The interaction of p53 with replication protein A mediates suppression of homologous recombination, Oncogene 23 (2004) 9025–9033.
[45] P. Baumann, S.C. West, The human Rad51 protein: polarity of strand transfer and stimulation by hRP-A, EMBO J. 16 (1997) 5198–5206.
[46] T. Sugiyama, S.C. Kowalczykowski, Rad52 protein associates with replication protein A (RPA)-single stranded DNA to accelerate Rad51-mediated displacement of RPA and presynaptic complex formation, J. Biol. Chem. 277 (2002) 31633–31672.
[47] S. Sigurdsson, S. Van Komen, W. Bussen, D. Schild, J.S. Albala, P. Sung, Mediator function of the human Rad51B-Rad51C complex in Rad51/RPA-catalyzed DNA strand exchange, Genes Dev. 15 (2001) 3308–3315.
[48] Y. Daniely, J.A. Boroweic, Formation of a complex between nucleolin and replication protein A after cell stress prevents initiation of DNA replication, J. Cell Biol. 149 (2000) 799–809.
[49] K. Kim, D.D. Dimitrova, K.M. Carta, A. Saxena, M. Daras, J.A. Borowiec, Novel checkpoint response to genotoxic stress mediated by nucleolin-replication protein A complex formation, Mol. Cell. Biol. 25 (2005) 2463–2474.