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Overexpression of Rad51 protein stimulates homologous recombination and increases resistance of mammalian cells to ionizing radiation

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ABSTRACT

Rad51 proteins share both structural and functional homologies with the bacterial recombinase RecA. The human Rad51 (HsRad51) is able to catalyse strand exchange between homologous DNA molecules *in vitro*. However the biological functions of Rad51 in mammals are largely unknown. In order to address this question, we have cloned hamster Rad51 cDNA and overexpressed the corresponding protein in CHO cells. We found that 2–3-fold overexpression of the protein stimulated the homologous recombination between integrated genes by 20-fold indicating that Rad51 is a functional and key enzyme of an intrachromosomal recombination pathway. Cells overexpressing Rad51 were resistant to ionizing radiation when irradiated in late S/G_2 phase of the cell cycle. This suggests that Rad51 participate in the repair of double-strand breaks most likely by homologous recombination involving sister chromatids formed after the S phase.

INTRODUCTION

Homologous recombination is a fundamental process shared by organisms from bacteria to man. In *Escherichia coli*, recombination is catalysed by RecA protein and is involved in horizontal genetic transfer as well as repair of DNA damage such as DNA crosslinks, strand breaks and post-replication gaps (1). In eukaryotes it is well established that homologous recombination is essential during meiosis for correct segregation of homologous chromosomes and as a source of genetic diversity. But the role of recombination in DNA repair and tolerance is not well defined in higher eukaryotes. However, several results support the idea that homologous recombination plays a role in DNA repair in mammals (2). First, treatment of cells with various genotoxic agents increases the homologous recombination frequency between selectable genes (3–5). Second, hamster cells defective in the DNA-dependent protein kinase (DNA-PK) repair pathway of double-strand breaks (DSBs), are much more resistant to ionizing radiation when exposed in late S/G_2 than in G_1/early S phases of the cell cycle (6). This indicates a possible role of homologous recombination between newly-formed sister chromatids in DSB repair. Finally, the identification, from yeast to man, of *E.coli* RecA protein homologues, called Rad51, has greatly opened the research field of homologous recombination in eukaryotes (7).

The RecA homologue from *Saccharomyces cerevisiae* (ScRad51) shares both sequence and functional homologies with the bacterial protein (8–10). Like RecA, ScRad51 is able to catalyse *in vitro* strand exchange between homologous DNAs, though less efficiently (11,12). The reaction is stimulated by the addition of yeast proteins Rad55, Rad57, Rad52 and replication protein A (RP-A) (13–16). *In vivo*, ScRad51 is necessary for DSB repair occurring during mitotic and meiotic recombination, or following treatment with ionizing radiation and methyl methane sulfonate (MMS) (10,17,18). Like RecA, ScRad51 is transcriptionally induced after genotoxic treatment (8–10). cDNAs coding for RecA homologous proteins have also been isolated from many higher eukaryotes (19–21). Like ScRad51, the human HsRad51 protein has been shown by electron microscopy observation to form protein filaments on DNA which are similar to those formed by RecA (22,23). Furthermore, HsRad51 catalyses strand exchange reaction *in vitro* (24–26). As in yeast, this reaction is slow and requires additional factors such as human RP-A and HsRad52 (24,25,27). *In vivo*, Rad51 has been shown to associate with mouse meiotic chromosomes undergoing homologous recombination, and to relocalise to nuclear foci in cells treated by genotoxic agents (28,29). These results are consistent with possible roles of mammalian Rad51 in meiotic recombination as well as DNA repair. Rad51 is essential for mouse embryonic development and cell growth, suggesting a gain of function of the mammalian protein compared to RecA and ScRad51 (30,31). Accordingly, Rad51 has recently been shown to fulfil an essential role in the repair of endogeneous chromosome breaks in proliferating chicken B lymphocytes (32).

In order to investigate the recombination and DNA repair functions of Rad51 protein, we have cloned the Chinese hamster *Cricetulus griseus* corresponding cDNA (*CgRAD51*). The encoded protein CgRad51 was 98% identical to HsRad51. As Rad51 is an essential protein the development of overexpressing cells appeared to be a powerful tool to explore Rad51 functions. We show here that a 2–3-fold overexpression of CgRad51 in CHO cells stimulated 20-fold the spontaneous homologous recombination between chromosomal integrated genes. Interestingly, CgRad51 overproducing cells were resistant to ionizing radiation when irradiated specifically in late S/G_2 phase of the cell cycle. These

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results indicate a key role of CgRad51 in both homologous recombination and ionizing radiation resistance.

MATERIALS AND METHODS

Bacteria and mammalian cell culture

Bacteria were grown in LB broth supplemented when required with zeocin (Cayla) at 20 µg/ml. AA8 Chinese Hamster Ovary (CHO) fibroblasts were obtained from the American Type Culture Collection (ATCC). These cells were grown in L-glutamine containing α-MEM medium (Gibco) supplemented with 10% foetal calf serum (Gibco), penicillin G-Streptomycin sulphate (Sigma).

Genotoxic treatments and cell survival curves

Cells were trypsinized, counted and 1×10^6 cells were plated in 10 cm dishes. Forty eight hours later, exponentially growing cells were incubated with fresh medium containing various concentrations of either MMS, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) obtained from Aldrich; mitomycin C (MMC) or *cis*-diamminedichloroplatinum(II) (cisplatin) obtained from Sigma. UV irradiation was done using a germicidal lamp emitting predominantly at 254 nm. γ-irradiation was carried out at CERT ONERA (Toulouse) with a ^{60}Co source at a dose rate of 2 Grays/min. At different times following these treatments cells were collected for protein, RNA or cell cycle analysis.

For survival measurements, cells were trypsinized after genotoxic treatment, counted, and various cell dilutions were seeded in 6 cm dishes. Cells were grown for 7–10 days, fixed, stained with crystal violet and colonies were counted.

Cell cycle analysis and aphidicolin synchronisation

To analyse the cell cycle distribution, cellular nuclear DNA was labelled with propidium iodide according to the Vindelov procedure (33) and analysed on a Becton Dickinson FACScan. To synchronise the cells in late S/G_2, 25 cm^2 flasks containing 9×10^5 cells were incubated in α-MEM containing 1 µg/ml aphidicolin (Sigma) for 14 h. The cells were then washed three times with 6 ml of medium and post-incubated 6–7 h before irradiation.

Cloning and sequencing of hamster RAD51 cDNA

Total RNAs were extracted from exponentially growing AA8 cells with a standard guanidin method (34). Poly(A) mRNA were purified using a Pharmacia Biotech mRNA Purification Kit™. Corresponding cDNAs were synthesised using a Pharmacia T primed first strand kit™. Two degenerated oligonucleotides including the two regions corresponding to the start and stop codons of mouse and human Rad51 proteins were designed, RAD51 sense: 5′-ATGGCNATGCARATGCARCTTGA-3′ and RAD51 antisense: 5′-CGCGGATCCTCYTTNGCRTCNCCN-ACNCCRTC-3′, where N is A+G+C+T, R is A+G and Y is C+T. These oligonucleotides served to PCR amplify a 1 kb fragment, using *Pfu* polymerase purchased from Stratagene for high fidelity of DNA synthesis. This fragment was subcloned in a pBluescript II KS (+/–) vector (pBS) purchased from Stratagene and sequenced on both strands. To sequence the 5′ and 3′ non-translated regions two pairs of internal oligonucleotides were designed, race5.1: GCAACAGCCTCCACCGTATG; race5.2: TAAGGATCCAGCTTCTTCTAATTTCTTC and race3.1: ACCAACCAGGTAGTAGCCCA; race3.2: TAAGGATCCGAAGAGGGGAGACCAGAATCT. RACE PCR was performed using a Clontech Marathon kit™. Two amplified products were subcloned in a pBS vector and sequenced. These sequences were then used to amplify a cDNA containing the whole opened reading frame (ORF) which was then cloned in a pBS vector to generate a pBSRad51 vector.

EMBL accession number of the hamster *RAD51* mRNA (*CgRAD51*) and of the deduced protein (CgRad51) sequences is Y08202.

Expression and recombination vectors, DNA transfection

CgRad51-overexpressing plasmid pZeoRad51 was constructed by PCR-amplifying the cDNA of CgRad51 from the pBSRad51 matrix with primers: TTTGGTACCATGGCTATGCAGATGCAGCTT and TTCTTCGAATCAGTCCTTGGCATCTCCTAC. The upstream oligonucleotide was designed in order to get an adenine at –3 position from the first ATG, which has been shown to strongly enhance gene expression (35). After a *Asp*718/*Bst*BI double cut, the PCR product was inserted into a pZeoSV1 vector (Cayla VECT001) opened by the same endonucleasic digestion. pZeoSV1 gives high stable expression of proteins under the control of enhancer/promoter sequences from SV40 early region. This shuttle vector carries the *Sh ble* gene conferring resistance to zeocin and used as dominant marker for both *E.coli* and mammalian cell lines. pLrec plasmid contains a *neo* gene for selection of stable transfectants on G418 and two mutant bacterial *lacZ* genes, tandemly arranged. One copy, namely 5′Δ*lacZ*, is deleted in 5′ while the other, namely 3′*mut lacZ*, is disrupted by a *Xho*I linker insertion. pNeoA carries an hygromycin resistance gene surrounded by two mutant *neo* genes in tandem. Both pLrec and pNeoA plasmids were kindly provided by Dr S. Meyn (36,37). CHO parental cells were transfected using the DMSO/polybrene shock procedure (38) by incubating cells with DNA/polybrene mixture for 6 h before treating them for 4 min with 30% DMSO. After a 62-h phenotypic expression, cells were diluted 1/10 then incubated in α-MEM medium supplemented with either 500 µg/ml zeocin, 600 µg/ml G418 (Sigma) or 350 µg/ml hygromycin (Clontech). Well isolated clones were picked after a 10–15-day selection. Stably transfected CHO cells were maintained in medium supplemented with 250 µg/ml zeocin, 350 µg/ml G418 or 250 µg/ml hygromycin. Recombination events were detected by β-galactosidase production in confluent layer of pLrec transfected cells, fixed with 2% formaldehyde, 0.2% glutaraldehyde and incubated with 1 mg/ml Xgal (Biosynth AG), 2 mM $MgCl_2$, 4 mM K ferricyanide, 4 mM K ferrocyanide during 6 h at 37°C. Recombination rates were derived from the number of positive LacZ cells or G418 resistant colonies detected and the total number of cells screened by using the fluctuation analysis tables provided by Capizzi and Jameson (39).

Southern blot analysis

Genomic DNA was purified from the cells using a standard procedure (40). These DNAs were restricted with *Bam*HI enzyme and separated on a 0.7% agarose gel. DNA was transferred to a positively charged nylon membrane from Oncor, and prehybridised with 50% formamide, 10% dextran sulfate, 1% SDS and blocking reagents (Oncor). Genomic bands containing each *lacZ* gene were revealed using a 1.1 kb ^{32}P-labelled pLrec *Sac*I–*Cla*I fragment containing part of the *lacZ* gene. The correct integration

of the vector generated a 4.2 kb fragment containing the 5′Δ*lacZ* copy and one or several larger junction fragments containing the 3′*mut lacZ* copy along with genomic DNA (Fig. 2). ^{32}P signals were revealed on a Storm scanner from Molecular Dynamics.

Western blot analysis

Twenty µg of cell extracts were electrophoresed in a 10% SDS–polyacrylamide gel. Proteins were transferred electrophoretically to PVDF membrane supplied by Millipore. Rad51 and actin proteins were revealed by incubating the membrane with purified rabbit polyclonal anti-human Rad51 at a dilution of 1/1000 (a generous gift of Dr C. Radding) and monoclonal anti-actin at a dilution of 1/10 000 (Chemicon International Inc.) respectively. These antibodies were revealed by alkaline phosphatase coupled antibodies (Sigma Immuno Chemicals) for chemifluorescence detection using the Amersham detection reagent. Protein expression was revealed by a Molecular Dynamics Storm scanner and quantified with the Image QuantR 4.2A software.

RESULTS

Cloning and expression of the hamster Rad51 cDNA

To study the role of Rad51 in repair and homologous recombination, we chose to use Chinese hamster cells as a model to take advantage of the variety of existing DNA repair mutants allowing future genetic studies. To clone the hamster Rad51 cDNA (*CgRAD51*) we designed degenerated oligonucleotides based on the sequences of the human and mouse Rad51 proteins, and amplified a 1 kb fragment by PCR. The product was subcloned in a pBS vector and sequenced. The 5′ and 3′ non-translated regions were obtained by RACE PCR, subcloned in a pBS vector and sequenced. The whole cDNA contains a potential ORF coding a 339 amino acid protein, with >98% identity with other mammalian Rad51 proteins. The CgRad51 differs by only 4 and 6 amino acids with the human and mouse proteins, respectively (data not shown). A *Nde*I–*Hin*dIII fragment of the cDNA was used to hybridise total and poly(A) RNAs extracted from AA8 cells, revealing two mRNA products, a major band migrating at 1.8 kb and corresponding probably to the mature mRNA, and a minor band migrating around 6.8 kb which could represent a pre-mRNA (data not shown). The corresponding protein detected by western blot had an estimated molecular weight of 40 kDa, identical to that observed in HeLa extracts (data not shown). The western blot was revealed with rabbit antibodies against HsRad51, taking advantage of the high identity of the two proteins.

CgRad51 is not induced after genotoxic treatment

In bacteria and yeast, RecA and ScRad51 are transcriptionally induced after genotoxic treatment. To determine the response of CgRad51 to such treatments, we exposed proliferating AA8 cells to various doses of the following DNA damaging agents: UV, γ-rays, MMS, MNNG, cisplatin and MMC. At different times after these treatments, we quantified CgRad51 by western blot. Even though these agents gave rise to various lesions and lead to cell arrest in various phases of the cell cycle, no accumulation of CgRad51 was detected (data not shown).

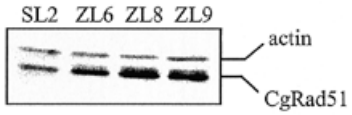

Figure 1. Western blot detection of Rad51 expression in a pZeoSV1 transfected (SL2), and in three pZeoRad51 transfected clones (ZL6, ZL8, ZL9).

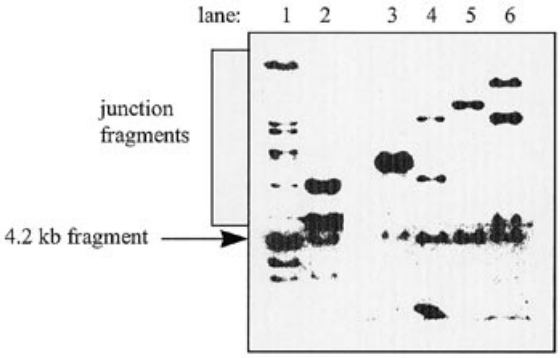

Figure 2. Southern blot of total *Bam*HI restricted genomic DNA from control and overexpressing clones probed with a ^{32}P-labelled *Sac*I–*Cla*I fragment of pLrec. Lanes 1 and 2: control clones (SL1, SL2); lanes 3–6: overexpressing clones (ZL5, ZL6, ZL8, ZL9). The 4.2 kb fragment contains the 5′Δ*lacZ* copy and the junction fragments contain the 3′*mut lacZ*.

Overexpression of Rad51 stimulates *in vivo* homologous recombination between two adjacent homologous alleles

In order to understand the biological roles of Rad51 protein in CHO cells, we cloned the cDNA in the pZeoSV1 vector, under the control of an SV40 enhancer promoter sequence. Extracts were prepared from cells stably transfected with either pZeoRad51 or pZeoSV1, as a control, and analysed by western blot (Fig. 1). Rad51 overexpression was determined in different clones after normalisation to the endogenous actin (Table 1). The mean overexpression ratio was ~3 ± 1.

Rad51 catalyses *in vitro* strand exchange. We therefore expected that an overexpression of the protein would influence *in vivo* homologous recombination. We thus transfected recombinase-overexpressing clones with a reporter vector for recombination, pLrec, carrying two different alleles of *E.coli lacZ* gene as recombination substrates surrounding a *neo* gene for selection of stable transformants (36). Correct integration of both recombination substrates was controlled by Southern blotting using a radiolabelled probe targeting both *lacZ* alleles (Fig. 2). We determined the proportions of cells expressing β-galactosidase by X-gal staining pZeoSV1- or pZeoRad51-transfected cells having integrated pLrec (Fig. 3). The number of blue cells is directly correlated with the frequency of mitotic intrachromosomal recombination (Table 1). The mean recombination rate of control cells (2.7×10^{-5}) was similar to those measured by other groups using vectors reporting intrachromosomal recombination in mouse (36,41), monkey (42) or hamster cells (5,43). pZeoRad51-transfected cells presented an increase in homologous recombination of ~20-fold (mean recombination rate of 59×10^{-5}) compared to cells transfected with the control pZeoSV1 plasmid (Table 1). The results were confirmed by stably transfecting pZeoSV1 and pZeoRad51 cells, with the pNeoA vector in which the recombination substrates are two copies of differently mutated *neo* genes giving rise to G418 resistant clones upon recombination. In

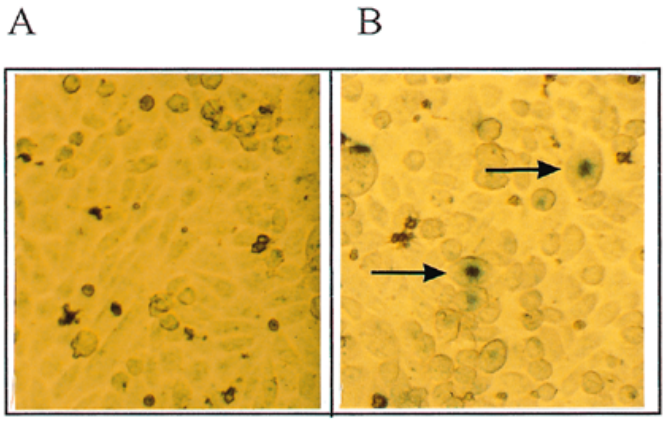

Figure 3. Detection of recombination events by β-galactosidase production. Cells were fixed, then incubated with X-Gal. (**A**) Control cells. (**B**) Rad51 overexpressing cells. Arrows indicate positive blue cells.

this system, homologous recombination rates in either over-expressing or control clones were comparable to those obtained with the pLrec system and the pZeoRad51 clones showed a 25-fold stimulation of recombination (Table 1).

Overexpression of CgRad51 confers a specific G_2 resistance to ionizing radiation killing

The repair of DSBs is primarily carried out by the Ku–DNA–PK repair pathway in mammalian cells while in yeast it is driven by proteins of the Rad52 epistasis group (44). However, there are some indications that homologous recombination could also be involved in DSB repair in mammalian cells (2,44). We therefore asked whether CgRad51 overexpression influences cell resistance to ionizing radiation. When an asynchronous CHO population was irradiated, no significant difference in the survival of both cell types was observed, except at high doses for which overexpressing cells were slightly less sensitive (Fig. 4E). In asynchronous population, cells were mostly in G_1 and S phases (Fig. 4A and B). Because homologous recombination is thought to act more frequently once sister chromatids are formed, we synchronised the same clones with aphidicolin. This base analogue blocks the cells in early S phase. Six to seven hours after release of this block, cells were mainly in late S and G_2 phases (Fig. 4C and D). Microscopic observation of the cells stained with DAPI showed that the proportion of mitotic cells remained under 8% in these conditions (data not shown). As shown in Figure 4F, in G_2 phase CgRad51 overexpressing cells were more resistant to ionizing radiation killing than control cells, suggesting that a recombination process has taken place leading to a better survival.

DISCUSSION

In this study, we cloned the cDNA coding the hamster Rad51 protein and found that the deduced protein was >98% identical to the human and mouse homologues, thereby confirming the high conservation of the protein among mammals. We could not detect any accumulation of CgRad51 after various genotoxic treatments. Similarly, in human cells, Rad51 has been shown to relocalize in nuclear foci rather than to accumulate after such treatment (28). It appears then that Rad51 regulation could be different in mammalian cells compared with *Saccharomyces cerevisiae* and *E.coli*, ScRad51 and RecA proteins being induced by DNA damage. A high constitutive level of CgRad51 in CHO cells could explain the absence of induction. Consistent with this hypothesis, it has been shown recently that immortal human cells possess more Rad51 than primary cells, suggesting then that a high amount of this protein could be a general feature of immortal cell lines (45).

Table 1. Intrachromosomal recombination rates in Rad51-overexpressing CHO cells

CHO transfected with[a]	Clone names	Rad51 overexpression ratio[b]	Recombination rate = $\times 10^{-5}$ recombinants/cell generation
pLrec + pZeoSV1	SL1	1	3
	SL2	1	2.5
pNeoA + pZeoSV1	SN1	1	0.8
	SN2	1	3.5
pLrec + pZeoRad51	ZL5	ND[c]	34
	ZL6	2	63
	ZL8	3	50
	ZL9	3	89
pNeoA + pZeoRad51	ZN5	2	60
	ZN8	3	57

[a]Parental cells were stably transfected with either plasmids pLrec or pNeoA and pZeoSV1 or pZeoRad51 as described in Materials and Methods.
[b]Overexpression ratios of Rad51 were determined by comparing immunodetected protein levels from extracts of cells transfected by pZeoRad51 and pZeoSV1.
[c]Not determined.

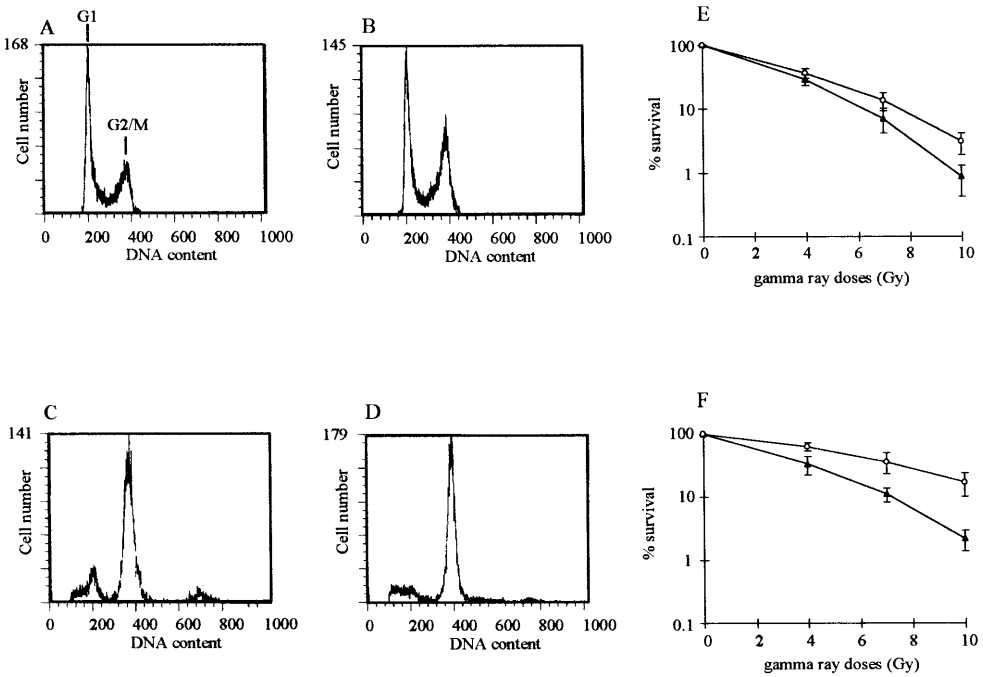

Figure 4. Survival to ionizing radiation treatment of asynchronous and late S/G_2 synchronised Rad51 overexpressing and control cells. (A–D) Cell cycle distribution of control and overexpressing cells at the time of irradiation: (**A**) asynchronous control cells; (**B**) asynchronous overexpressing cells; (**C**) synchronised control cells; (**D**) synchronised overexpressing cells. (**E**) γ-ray survival of two asynchronous representative clones; a control (closed triangles) and a CgRad51 overproducer (open circles). (**F**) γ-ray survival of the same cells synchronised in late S/G_2. The survival curves correspond to three independent experiments in which three dishes have been counted per dose.

To investigate the role of Rad51 in homologous recombination and DNA repair, we analysed the consequences of overexpressing the protein in CHO cells. A 2–4-fold overexpression of Rad51 increased the rate of intrachromosomal recombination by a factor of 20 in hamster cells. In addition, CgRad51 was able to promote *in vivo* recombination in a Δ*recA E.coli* strain when expressed from the RecA promoter sequence, though less efficiently than RecA itself (data not shown). Taken together these results demonstrate clearly that Rad51 is a key protein for *in vivo* homologous recombination. Accordingly, a recent study reported that Rad51 is required for SV40 large T antigen induction of recombination in immortalised human cells (45). *In vitro*, human Rad51, purified from recombinant bacteria extracts, has been shown to efficiently promote DNA strand exchange only in the presence of protein cofactors such as hRPA and HsRad52 (25,27). Hence, other proteins are likely required for efficient recombination in the cell. We report that a relatively low level of overexpression of Rad51 induces a high rate of recombination *in vivo*. This implies that the intracellular pool of cofactors is not limiting and that the additional amount of Rad51 is able to recruit an efficient recombinosome.

The repair of DSBs is primarily mediated by the DNA–PK pathway (44,46). However, there is evidence that homologous recombination can contribute to survival when cells are irradiated in late S/G_2 phase of the cell cycle (44). When asynchronous CgRad51 cells were irradiated, only a slight resistance compared to the control was observed at high doses (Fig. 4E). This improved survival may reflect a protective role of the increased pool of CgRad51 for the late S/G_2 cell fraction overexpressing the protein (Fig. 4C and D). Strong evidence for a role of Rad51 in survival clearly appeared when both cell lines were irradiated in late S/G_2 phase (Fig. 4F). The CgRad51 overproducer was significantly more resistant than the parental cell line when cells had just undergone DNA replication. This suggests that if the proper substrates (i.e., sister chromatids) are present, recombination repair of DSBs can occur. In support of this hypothesis, it has previously been reported that in *S.cerevisiae*, sister chromatids are preferred over homologous chromosomes as substrates for homologous recombination repair of X-ray damage (47). In yeast, the Rad52 epistasis group including Rad51 participate in DSB repair by recombination (10,17,18,48). Recently, overexpression of HsRad52 in monkey cells has also been reported to stimulate spontaneous homologous recombination and enhance resistance to gamma rays, though to a lower extent than that observed in this work, maybe due to a non-synchronisation of the cells (42). Thus, the radioresistance associated with the overproduction of Rad51 and Rad52 proteins provides evidence for the existence of a DSB repair pathway by homologous recombination in mammalian cells depending on both proteins and operating after DNA replication. It is then conceivable that other lesions such as post-replication gaps or DNA crosslinks could be substrates of this recombination repair process.

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