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DNA-PKcs and ATM Co-Regulate DNA Double-Strand Break Repair

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Abstract

DNA double-strand breaks (DSBs) are repaired by nonhomologous end-joining (NHEJ) and homologous recombination (HR). The NHEJ/HR decision is under complex regulation and involves DNA-dependent protein kinase (DNA-PKcs). HR is elevated in DNA-PKcs null cells, but suppressed by DNA-PKcs kinase inhibitors, suggesting that kinase-inactive DNA-PKcs (DNA-PKcs-KR) would suppress HR. Here we use a direct repeat assay to monitor HR repair of DSBs induced by *I-SceI* nuclease. Surprisingly, DSB-induced HR in DNA-PKcs-KR cells was 2- to 3-fold above the elevated HR level of DNA-PKcs null cells, and ~4- to 7-fold above cells expressing wild-type DNA-PKcs. The hyperrecombination in DNA-PKcs-KR cells compared to DNA-PKcs null cells was also apparent as increased resistance to DNA crosslinks induced by mitomycin C. ATM phosphorylates many HR proteins, and ATM is expressed at a low level in cells lacking DNA-PKcs, but restored to wild-type level in cells expressing DNA-PKcs-KR. Several clusters of phosphorylation sites in DNA-PKcs, including the T2609 cluster, which is phosphorylated by DNA-PKcs and ATM, regulate access of repair factors to broken ends. Our results indicate that ATM-dependent phosphorylation of DNA-PKcs-KR contributes to the hyperrecombination phenotype. Interestingly, DNA-PKcs null cells showed more persistent ionizing radiation-induced RAD51 foci (but lower HR levels) compared to DNA-PKcs-KR cells, consistent with HR completion requiring RAD51 turnover. ATM may promote RAD51 turnover, suggesting a second (not mutually exclusive) mechanism by which restored ATM contributes to hyperrecombination in DNA-PKcs-KR cells. We propose a model in which DNA-PKcs and ATM coordinately regulate DSB repair by NHEJ and HR.

1. Introduction

Mammalian cells have integrated DNA damage response systems that sense DNA damage, activate signaling cascades, and effect repair. Defects in these systems lead to genome instability and predispose to cancer and other diseases. DNA double-strand breaks (DSBs) are a serious type of damage that can result in mutations, chromosome aberrations, and cell

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death. DSBs are produced by exogenous and endogenous factors such as ionizing radiation (IR) and nucleases, respectively, and are repaired by two major pathways, homologous recombination (HR) and nonhomologous end-joining (NHEJ). NHEJ is predominant in G0 and G1, and HR activity increases during S and G2 [1]. Proteins that mediate HR and NHEJ are largely distinct, but some have been implicated in both pathways. For example, defects in the yeast Mre11/Rad50/Xrs2 complex (MRE11/RAD50/NBS1 - MRN in mammals) affect both HR and NHEJ, presumably reflecting the Mre11 regulation of end-processing and Rad50 end-tethering functions. DNA-dependent protein kinase (DNA-PK), comprising the catalytic subunit (DNA-PKcs), Ku70, and Ku80, is a key NHEJ factor, but it also influences HR [2-7]. However, the precise role of DNA-PKcs in HR regulation has been unclear.

DNA-PKcs is a phosphatidylinositol-3-kinase (PIK) family member recruited to DSBs by Ku and its kinase activity is stimulated ~10-fold upon binding to Ku-bound DNA ends [8]. DNA-PKcs promotes NHEJ and phosphorylates itself and other DNA damage response and repair proteins. DNA-PKcs kinase activity is critical for NHEJ [9]. Numerous *in vitro* phosphorylation targets of DNA-PKcs have been identified including Ku70, Ku80, XRCC4, Artemis, RPA, and p53. There is evidence that DNA-PKcs phosphorylates several targets *in vivo*, including itself, RPA, WRN, and Artemis [5,10-14], although the functional significance of these phosphorylated targets, other than DNA-PKcs itself, remains questionable [15].

ATM is another PIK family member with critical roles in DNA damage responses and cancer suppression. In undamaged cells, ATM is an inactive dimer, but damage causes *trans*-autophosphorylation, TIP60-dependent acetylation, and dissociation into active monomers [16]. ATM phosphorylates DNA repair, checkpoint, and cell cycle control proteins, including p53, NBS1, MDM2, SMC1, CHK2, BRCA1, c-Abl, and H2AX [17-20]. ATM defective cells are sensitive to IR and show defects in DSB repair by NHEJ that are independent of the checkpoint defect [21-24]. Several lines of evidence suggest that ATM promotes DSB repair by HR [22,25-27]. Thus, both ATM and DNA-PKcs influence HR, and crosstalk between these kinases is suggested by their common H2AX target [28], the reduction in ATM levels upon RNAi down-regulation of DNA-PKcs [29], and by evidence that ATM phosphorylates DNA-PKcs after DNA damage [30].

DNA damage triggers phosphorylation of DNA-PKcs at several clusters of serine and threonine residues (including T2609, S2056, and T3950) that regulates DNA-PKcs function [15]. Phosphorylation of the T2609 cluster induces a conformational shift that promotes DNA-PKcs disassociation from broken ends, presumably increasing access of other repair factors to broken ends [31-37]. Other phosphorylation sites within DNA-PKcs may be even more important than the T2609 cluster for DNA-PKcs dissociation [15]. Nonetheless, mutations that prevent T2609 cluster phosphorylation markedly suppress HR stimulated by I-SceI nuclease-induced DSBs (referred hereafter as “DSB-induced HR”) [4]. There is some debate about whether the T2609 cluster is phosphorylated by ATM, DNA-PKcs or both kinases. T2609 phosphorylation was originally defined using *in vitro* assays [38]. Meek et al. [39] concluded that *in vivo*, both T2609 and S2056 clusters were primarily targets of autophosphorylation. However, in that study DNA-PKcs phosphorylation was analyzed in cell extracts, or with purified DNA-PKcs, after cells were treated with DNA damaging agents, raising the possibility that the observed autophosphorylation occurred *in vitro*. Another *in vitro* study showed that NHEJ required DNA-PKcs autophosphorylation, but was independent of ATM [40]. In contrast, a genetic analysis indicated that ATM plays an important role in T2609 cluster phosphorylation *in vivo* [30].

DSB-induced HR is increased in the absence of DNA-PKcs, Ku70, or XRCC4, which could reflect passive shunting of DSBs from NHEJ to HR [2,7]. However, chemical inhibition of DNA-PKcs, mutation of the T2609 cluster, and splice variants that lack the kinase domain, all reduce DSB-induced HR [3,4], indicating that DNA-PKcs actively regulates repair pathway choice. Because chemical inhibition of DNA-PKcs reduces DSB-induced HR, we hypothesized that HR would also be reduced in cells expressing catalytically inactive DNA-PKcs. We tested this in cells with integrated direct repeat HR substrates, which are reasonable models given that the human genome comprises ~50% repeated elements, and HR between repeated elements is linked to dozens of human diseases and is likely a key evolutionary driver [41,42]. We examined DSB-induced HR in cells expressing the catalytically inactive DNA-PKcs K3752R (KR) mutant. The K3752 residue lies in subdomain II outside the ATP binding pocket, but is nonetheless required for ATP binding and kinase activity [43], and DNA-PKcs-KR is NHEJ defective [9]. Surprisingly, we found that DSB-induced HR was ~2- to 3-fold higher in cells expressing DNA-PKcs-KR than in cells lacking DNA-PKcs, and ~4- to 7-fold higher than cells with wild-type DNA-PKcs. Because NHEJ is similarly defective in cells that lack DNA-PKcs or express DNA-PKcs-KR, the higher HR level in cells with DNA-PKcs-KR cannot be explained by passive shunting of DSBs from NHEJ toward HR, but rather that HR is directly or indirectly enhanced in cells expressing DNA-PKcs-KR. This enhanced HR was strongly suppressed by chemical inhibitors of ATM kinase whereas HR in cells lacking DNA-PKcs was unaffected by ATM inhibition. Based on these and other results we suggest a model in which ATM regulates HR by both DNA-PKcs-dependent and -independent mechanisms.

2. Materials and Methods

2.1. Cell lines

CHO V3 derivatives with HR substrates and complemented with DNA-PKcs were described previously [2]. Complementation with DNA-PKcs-KR was performed using similar procedures, and cells were cultured as described [44]. VD-7 cells complemented with DNA-PKcs harboring alanine mutations of the T2609 cluster were described previously [4].

2.2. IR sensitivity and HR assays

All assays employed unsynchronized cells harvested from cultures that were ~65-75% confluent. Sensitivity to γ -rays was determined as described [2]. DSB-induced HR frequencies were determined as described [44]. Briefly, 4×10^5 cells were seeded into 35 mm dishes, incubated for 16 h, and lipofected with 2 μ g of pCMV3xnl(I-SceI) to induce DSBs, or 2 μ g of negative control vector pCMV(I-SceI⁻). Twenty-four h later, 2×10^4 cells were seeded to each of three 10 cm dishes, and after an additional 24 h, G418 (600 μ g/ml, 100% active) was added. Cell viability was determined in parallel by seeding 500 cells per 10 cm dish in nonselective medium. Colonies were stained with 0.1% crystal violet in methanol and scored 10 days later. HR frequencies were calculated as the number of G418-resistant colonies per viable cell plated in selective medium. Recombinant products were analyzed by PCR, restriction mapping, and Southern hybridization to distinguish gene conversions from deletions, and to generate gene conversion tract spectra as described [44,45]. Transfection efficiency was assessed by flow cytometry with a GFP-I-SceI expression vector [46]. Global HR capacity was measured with a mitomycin C (MMC) sensitivity assay. Appropriate numbers of cells were seeded to 10 cm dishes, incubated for 4 hours to allow attachment, exposed to 0-25 ng/ml MMC for 24 hr, then growth media was replaced with fresh growth media, and colonies were stained and scored 10 days later.

2.3. Protein detection

Commercial antibodies included DNA-PKcs (NA57 and PC127, Calbiochem), ATM (AHP397, SeroTec and ab17995, Abcam), ATM phosphoserine 1981 (39529, Active Motif), RAD51 (PC130, Calbiochem), RPA (NA19, Calbiochem), and β -actin (CP01, Calbiochem). Antibodies to DNA-PKcs phospho-T2609 were described previously [38]. HRP-conjugated secondary anti-mouse IgG (NA931) and anti-rabbit IgG (NA934) were from Amersham Biosciences. FITC-conjugated secondary anti-rabbit IgG (Alexa Fluor-488; A11441) and anti-mouse IgG (Alexa Fluor-568; A11031,) were from Molecular Probes.

Whole cell extracts were prepared from $\sim 1.5 \times 10^7$ cells using M-PER mammalian protein extraction reagent supplemented with Halt protease inhibitor cocktail mix and Halt phosphatase inhibitor cocktail mix (Thermo Scientific). Lysates were clarified by centrifugation and protein was quantified by Bradford assay (Bio-Rad). Protein (25-100 μ g) was combined with 2 $\times$ Laemmli loading buffer (150 mM Tris-HCl, pH 7.0, 10% β -mercaptoethanol, 20% glycerol, 3% SDS, and 0.01% bromophenol blue), boiled for 5 min, resolved on a 5-15% SDS-polyacrylamide gel, and transferred to PVDF membranes using 48 mM Tris-HCl, pH 7.4, 390 mM glycine, 0.1% SDS, and 5% methanol. For ATM Westerns, methanol and SDS were omitted from the transfer buffer. The membranes were incubated with primary antibody, stained with HRP-conjugated secondary antibodies, and signals were developed by chemiluminescence (SuperSignal Westpico Chemiluminescence reagent, Thermo Scientific).

For immunofluorescent detection, cells were grown on cover slips to $\sim 45\%$ confluency, exposed to γ -rays or mock-treated, fixed at indicated times with 3% paraformaldehyde in PBS (pH 7.4) or 100% methanol, permeabilize with 0.1% Triton X-100 in PBS, and incubated with primary antibodies (prepared in 3% BSA in PBS). Primary antibodies were detected with Alexa-488 or Alexa 568 conjugated secondary antibodies (Molecular Probes), and cover slips were mounted in Vectashield mounting medium (Vector Laboratories, CA) and photographed with a Zeiss Axioscope fluorescence microscope equipped with a Zeiss AxioCam. Images were processed using Adobe Photoshop, or CorelDraw software. In all cases, identical software processing steps were performed on control and experimental images.

2.4. Statistical analysis

All statistical analyses were performed by using two-tailed Students *t* tests with Prism 5.0 software.

3. Results

3.1. Enhanced DSB-induced HR with kinase-inactive DNA-PKcs

To investigate DNA-PKcs roles in DSB-induced HR we expressed human DNA-PKcs in derivatives of CHO V3 (DNA-PKcs null) cells with single integrated copies of one of two related HR substrates with *neo* direct repeats, termed VD13 and V24 (Fig. 1A) [2]. I-*SceI* induced DSBs stimulate HR repair yielding *neo*⁺ products that confer resistance to G418 by gene conversion with or without associated crossovers or by single-strand annealing [47]. I-*SceI* DSBs are useful for identifying proteins that influence HR efficiency and/or outcome, but they do not fully mimic IR-induced DSBs which must be processed to produce ligatable/extendable ends, nor other damage produced by IR including single-strand breaks, base damage, and complex (multi-lesion) damage sites.

The VD13 and V24 cell lines were transfected with vectors for stable expression of wild-type human DNA-PKcs, or kinase-inactive DNA-PKcs-KR. Transfectants expressing

similar levels of wild-type DNA-PKcs (designated “-C1”) or DNA-PKcs-KR (“-KR3, -KR4, -KR6”) were identified by Western blot (Fig. 1B). As shown previously [2,9], wild-type DNA-PKcs restored radioresistance to near wild-type levels (despite variation in DNA-PKcs expression levels), but DNA-PKcs-KR cells remained radiosensitive (Fig. 1C and data not shown). The topoisomerase inhibitor camptothecin (CPT) creates replication stress and replication-dependent phosphorylation of RPA by DNA-PKcs [10,48]. This response was seen in wild-type (AA8) and wild-type complemented VD13-C1 cells, but not in DNA-PKcs null (VD13) or DNA-PKcs-KR cells (Fig. 1D and data not shown). In the original demonstration of DNA-PKcs-dependent RPA phosphorylation after CPT, only ~50% of RPA was phosphorylated [10], whereas we observed essentially 100% phosphorylation in wild-type cells, reflecting the 10-fold higher CPT concentration in the present study. The radiosensitivity and defective RPA phosphorylation response confirm the kinase defect of DNA-PKcs-KR.

As seen previously [2], complementing VD13 and V24 cells with wild-type DNA-PKcs reduced DSB-induced HR by 2- to 3-fold (Fig. 2). Surprisingly, VD13 derivatives with DNA-PKcs-KR had DSB-induced HR levels ~2- to 3-fold above DNA-PKcs null cells, and ~4- to 7-fold above wild-type (Fig. 2A). Similarly, DSB-induced HR was nearly 2-fold higher in V24 cells expressing DNA-PKcs-KR than null V24, and ~7-fold higher than wild-type (Fig. 2A). All cell lines showed equivalent transfection efficiencies with a GFP vector (Fig. 2B), indicating that the enhancement of DSB-induced HR by DNA-PKcs-KR is a general effect. To determine whether DNA-PKcs influences late HR stages, DSB-induced HR product spectra were generated. In wild-type cells, DSB-induced HR yields primarily continuous, short-tract, bidirectional gene conversions without crossovers (averaging 237 bp) [44]. Southern blot analysis of 19 and 24 DSB-induced HR products from VD13-KR4 and V24-KR3, respectively, showed that all 43 arose by gene conversion without crossovers. Conversion tracts from 30 V24-KR3 products were similar to wild-type: most were continuous and bidirectional, although average length was somewhat longer (354 bp). Thus, DNA-PKcs-KR affects HR efficiency, but not HR outcome, indicating that DNA-PKcs influences HR initiation, but not late HR stages.

Because NHEJ is the primary determinant of survival after IR [8,49], it is not surprising that the several-fold increase in HR in DNA-PKcs-KR does not increase IR resistance (Fig. 1A). Sensitivity to DNA crosslinking agents, such as MMC, is a highly sensitive measure of HR capacity because interstrand crosslink repair is strongly dependent on HR [50]. As shown in Fig. 2C, DNA-PKcs-KR cells were significantly more resistant to MMC than DNA-PKcs null cells. Together, these results indicate that DNA-PKcs-KR cells have a general hyperrecombination phenotype.

To further investigate the role of DNA-PKcs in HR regulation, we monitored RAD51 nuclear foci after IR [51]. These foci mark sites where RAD51 nucleoprotein filaments are formed, a necessary early HR step. RAD51 filaments search for and invade homologous sequences, and although filament formation and strand invasion are necessary early steps, studies with yeast and mammalian cells indicate that RAD51 turnover (“disassembly”) is important to complete the HR process [52-55]. Thus, RAD51 foci mark sites of HR initiation, but not necessarily successful completion. RAD51 foci were scored in wild-type and DNA-PKcs mutant cells 6, 12, and 24 hr after exposure to 5 Gy of γ -rays, and in untreated controls (Fig. 3). In untreated cells, there were slightly more RAD51 foci in DNA-PKcs null cells than wild-type or DNA-PKcs-KR cells, potentially reflecting defective NHEJ as well as other factors since DNA-PKcs-KR and null cells share a similar NHEJ defect. In this experiment, all three genetic backgrounds showed a peak in the percentage of cells with RAD51 foci 6 hr after IR, and this declined over time. Wild-type cells had significantly fewer IR-induced RAD51 foci and foci resolved more quickly, reflecting fully

functional NHEJ and HR systems. RAD51 persisted far longer in both DNA-PKcs null and DNA-PKcs-KR cells. Interestingly, although the two DNA-PKcs defective cell types had similar percentages of cells with IR induced RAD51 foci (Fig. 3B), DNA-PKcs-KR cells had significantly fewer foci per cell than null cells (Fig. 3C). Because DNA-PKcs-KR and null cells have comparable NHEJ defects, this difference cannot be explained by differential NHEJ capacity. Instead, these results are consistent with DNA-PKcs-KR cells having a greater propensity to complete HR (i.e., resolve RAD51 foci), and are also consistent with their hyperrecombination phenotype (Fig. 2A, C).

3.2. DNA-PKcs is recruited to DSBs independently of its kinase activity

DNA-PKcs could regulate HR by direct action at DSBs, or indirectly through protein-protein interactions. However, any effect that requires a high level of kinase activity would likely require DNA ends since DNA-PKcs is fully activated by DNA end-binding [8]. To further explore HR regulation by DNA-PKcs, we monitored DNA-PKcs focus formation after irradiation with 5 Gy γ -rays. DNA-PKcs foci were apparent in irradiated wild-type control (AA8) cells, in DNA-PKcs null cells complemented with wild-type DNA-PKcs or with DNA-PKcs-KR, but not in cells lacking DNA-PKcs (Fig. 4A). Irradiated cells expressing wild-type or DNA-PKcs-KR each had ~15 foci per cell whereas unirradiated and DNA-PKcs null cells had 0-1 foci (Fig. 4B). Fewer DNA-PKcs foci were detected than the >150 DSBs expected after 5 Gy IR, perhaps because DNA-PKcs may accumulate at detectable levels at a small fraction of DSBs. We conclude that DNA-PKcs recruitment to IR-induced DSBs occurs independently of its kinase activity, consistent with the results with laser-induced damage [56], raising the possibility that DNA-PKcs regulates HR at least in part through its presence at DNA ends.

3.3. DNA-PKcs regulates ATM levels independently of DNA-PKcs kinase activity

We next examined potential roles of ATM in the hyperrecombination phenotype of DNA-PKcs-KR cells because ATM is implicated in HR regulation, and ATM protein level decreases rapidly when DNA-PKcs is reduced by siRNA [29]. To test whether DNA-PKcs regulation of ATM levels depends on DNA-PKcs kinase activity, we measured total ATM levels and ATM activation by IR (S1981 phosphorylation) [16] in wild-type, DNA-PKcs null, and DNA-PKcs-KR cells (Fig. 5). Consistent with Peng et al. [29], ATM was reduced 3-fold in DNA-PKcs null cells, but ATM was largely restored by complementation with either wild-type DNA-PKcs or DNA-PKcs-KR (Fig. 5A, B). Cells expressing wild-type DNA-PKcs or DNA-PKcs-KR showed robust ATM S1981 phosphorylation after IR, but this response was attenuated by 3-fold in DNA-PKcs null cells (Fig. 5C, D), correlating well with ATM protein levels. Thus, ATM protein level depends on DNA-PKcs, but this regulation is independent of DNA-PKcs kinase activity.

3.4. Chemical inhibition of ATM reduces DSB-induced HR, phosphorylation of the DNA-PKcs T2609 cluster, and IR-induced RAD51 foci

Low concentrations of caffeine inhibit the kinase activity of ATM kinase, and higher concentrations also inhibit ATR and DNA-PKcs [57]. Caffeine sensitizes cells to the lethal effects of IR by inactivating the ATM-dependent G2/M DNA damage checkpoint [58]. Caffeine also suppresses both DSB-induced HR, and IR-induced RAD51 focus formation, but these effects are independent of the checkpoint defect [59]. However, no prior evidence directly linked caffeine-suppression of DSB-induced HR with ATM inhibition. To explore this possibility, we measured DSB-induced HR in cells treated with the highly specific ATM inhibitor KU55933 [60]. DSB-induced HR was reduced 4-fold by KU55933 in cells expressing wild-type DNA-PKcs or DNA-PKcs-KR, both of which express normal ATM levels, but only 2-fold in DNA-PKcs null cells with reduced ATM expression (Fig. 6). Similar results were obtained with CGK733 (Fig. 6), another ATM inhibitor [61]. The

marked reduction in DSB-induced HR in DNA-PKcs-KR cells is clear evidence that these inhibitors suppress HR independently of DNA-PKcs kinase inhibition. Note that absolute HR levels varied by ~2-fold in the KU55933 and CGK733 experiments reflecting different transfection efficiencies on different days. Similar trends were observed with caffeine, a relatively non-specific ATM inhibitor (supplementary Fig. S1). At the doses used, none of the ATM inhibitors had a significant effect on cell viability in these genetic backgrounds (data not shown). The lesser effects of the ATM inhibitors in DNA-PKcs null cells is consistent with the lower ATM protein level in these cells (Fig. 5A, B).

The DNA-PKcs T2609 cluster can be phosphorylated by DNA-PKcs itself and by ATM [30,38,62,63]. Phosphorylation of the T2609 cluster (and other sites) can increase access of repair factors to DNA ends by causing conformation shifts and/or DNA-PKcs dissociation from broken ends [3,4,15]. Because autophosphorylation of the T2609 cluster is not possible in DNA-PKcs-KR cells, we investigated ATM-dependent phosphorylation of the T2609 cluster as a potential contributor to the DNA-PKcs-KR hyperrecombination phenotype. We measured T2609 phosphorylation (pT2609) and RAD51 foci in irradiated wild-type and DNA-PKcs-KR mutant cells in the presence or absence of the ATM inhibitor KU55933. The pT2609 antibody detects pT2609 on Western blots [30], but also detects another protein phosphorylated after IR, revealed as a low-level signal (and generally smaller foci) in cells expressing mutant DNA-PKcs in which the T2609 cluster residues are changed to alanine (ABCDE; Fig. 7A) or lacking DNA-PKcs (data not shown). To quantitate pT2609 foci, the average number of foci in the ABCDE mutant was subtracted from values obtained with cells expressing wild-type DNA-PKcs or DNA-PKcs-KR. In wild-type cells, pT2609 foci were strongly induced 1 hr after IR (Fig. 7A), with >20% of cells showing >10 foci (Fig. 7B). This signal decreased to background levels by 6 hr after IR (data not shown). IR also strongly induced pT2609 in DNA-PKcs-KR cells; this is an ATM-dependent event as it was completely eliminated by the ATM inhibitor KU55933 (Fig. 7A, B). KU55933 did not completely eliminate pT2609 foci in wild-type cells, consistent with residual DNA-PKcs autophosphorylation (data not shown). Similar results were obtained with caffeine (data not shown).

Interestingly, KU55933 had identical effects on RAD51 foci, reducing RAD51 foci in wild-type cells and eliminating them in DNA-PKcs-KR cells (Fig. 7C, D). Again, similar results were seen with CGK733 (data not shown), as reported with caffeine [59]. The differential effects of ATM inhibition in wild-type vs. DNA-PKcs-KR cells can be explained by residual DNA-PKcs autophosphorylation of T2609 in wild-type, a pathway that is blocked in the DNA-PKcs-KR mutant. Note also that RAD51 foci were unaffected by KU55933 in cells lacking DNA-PKcs, consistent with the low level of ATM in these cells. The results in Figs. 6 and 7 demonstrate a strong correlation among IR-induced pT2609, IR-induced RAD51 foci, and DSB-induced HR.

4. Discussion

DNA-PKcs has emerged as a key regulator of both NHEJ and HR. DNA-PKcs plays critical roles in NHEJ which repairs 70-85% of nuclease-induced DSBs in log-phase mammalian cells [3,64]. Thus, the 2- to 5-fold increase in DSB-induced HR in cells lacking DNA-PKcs, Ku, or XRCC4 is consistent with passive shunting of DSBs toward HR [2,6,7]. However, chemical inhibition of DNA-PKcs, and DNA-PKcs isoforms lacking the kinase domain, suppress DSB-induced HR [3,4], indicating that DNA-PKcs regulation of HR is not solely due to passive shunting. The current data demonstrating that a DNA-PKcs kinase-inactive point mutant increases DSB-induced HR and MMC resistance above levels observed in DNA-PKcs null cells further supports the idea that DNA-PKcs actively regulates NHEJ and HR, and reveals additional complexity in this regulation. NHEJ factors are recruited to laser

induced DSBs more rapidly than HR factors but there is a significant period of time when both sets of factors are present at damage sites [65], providing an opportunity for crosstalk. Our study suggests two general ways that DNA-PKcs regulates DSB-induced HR: through modulation of ATM levels (and ATM activity), and through end accessibility to repair factors.

4.1. Indirect regulation of HR by DNA-PKcs through ATM

In addition to its well-characterized checkpoint signaling functions, ATM has been implicated in several aspects of HR. ATM inhibitors reduce DSB-induced HR and block IR-induced RAD51 foci (Figs. 6, 7, S1) [3,59]. Note that ATM inhibitors reduce HR in cells expressing wild-type DNA-PKcs and DNA-PKcs-KR, but not in DNA-PKcs null cells (Fig. 6), which have low ATM levels (Fig. 5). The fact that ATM can phosphorylate the DNA-PKcs T2609 cluster [30], and that this modification, along with other phosphorylated residues in DNA-PKcs, regulates end accessibility (see below), suggests a mechanism by which ATM regulates HR through DNA-PKcs modification, contributing to the hyperrecombination phenotype of DNA-PKcs-KR cells. Genetic and molecular analyses indicate that ATM promotes HR repair of DSBs that arise independently of replication (i.e., from IR or I-SceI) [25,26]. Part of this ATM role is phosphorylation of H2AX because DSB-induced HR is reduced several-fold in H2AX^{-/-} cells [27]. Although two reports suggested ATM does not promote DSB-induced HR [66,67], this may reflect redundancy with DNA-PKcs since DNA-PKcs can phosphorylate both itself, including the T2609 cluster [15], and H2AX [68]. Also, defects in mouse ATM and DMC1, a meiosis-specific RAD51 homolog, cause very similar meiotic repair defects [69].

Among known or suspected ATM targets, at least 12 are implicated in HR [70]. Importantly, six of these targets are also phosphorylated by DNA-PKcs (Fig. 8A). Moreover, ATM levels are controlled by DNA-PKcs in many cell types [29] (this study) and DNA-PKcs and ATM each phosphorylate the DNA-PKcs T2609 cluster that is important for NHEJ [30,38,62] and for HR [4], indicating that these proteins coordinately regulate DSB repair. We show here that DNA-PKcs controls ATM levels independently of DNA-PKcs kinase activity, indicating that ATM stabilization does not depend on DNA-PKcs-mediated phosphorylation of ATM or other targets. Also, the level of activated ATM after IR is normal in cells expressing DNA-PKcs-KR, but not in DNA-PKcs null cells (Fig. 5C, D). The restoration of ATM protein by DNA-PKcs-KR is likely a major contributor to the hyperrecombination phenotype of DNA-PKcs-KR cells. A key ATM target is the c-Abl kinase, which regulates RAD51 activity. Defects in c-Abl or ATM delay IR-induced RAD51 focus formation [71], suggesting that these proteins act early during RAD51 filament formation. Importantly, c-Abl phosphorylation of RAD51 reduces RAD51 affinity for single-stranded DNA, so this modification may drive RAD51 filament disassembly, a key late-stage process in the maturation of HR intermediates to HR products [52-55]. In DNA-PKcs null cells, ATM levels are reduced but not eliminated, and IR-induced RAD51 foci are readily detected (Fig. 3). In the DNA-PKcs-KR mutant, with normal ATM levels, IR also induces RAD51 foci but they are not as persistent as those in DNA-PKcs null cells (Fig. 3C). One interpretation of these results is that longer-lived RAD51 foci in DNA-PKcs null cells represent sites where HR intermediates fail to mature to products, whereas more quickly resolved RAD51 foci in DNA-PKcs-KR cells reflect successful completion of HR. Interestingly, ATM defective cells show increased IR-induced RAD51 foci [72], which indicates that RAD51 foci form independently of ATM, and consistent with the idea that ATM promotes RAD51 turnover. Given that many HR proteins are targeted by ATM (Fig. 8A), this is but one example of how restoration of ATM in DNA-PKcs-KR cells might contribute to the hyperrecombination phenotype of DNA-PKcs-KR cells.

4.2. HR regulation by DNA-PKcs through end accessibility

Phosphorylation of the DNA-PKcs T2609 and S2056 clusters regulates accessibility of broken ends to repair factors [5,31-37]. In DNA-PKcs-KR, the S2056 cluster is not phosphorylated because this cluster is subject only to autophosphorylation [30]. Mutations in the S2056 cluster that block phosphorylation increase DSB-induced HR above the wild-type level, but not above the level seen in DNA-PKcs null cells [5], unlike DNA-PKcs-KR which does increase HR above that in null cells (Fig. 2). Thus, the higher HR levels with DNA-PKcs-KR cannot be fully explained by the absence of S2056 cluster phosphorylation, but probably also reflects the absence of NHEJ competition since NHEJ strongly depends on DNA-PKcs kinase activity [9] but only weakly depends on S2056 cluster phosphorylation [5]. Because the T2609 cluster is phosphorylated *in vivo* by ATM [30], ATM may play an important role in this (and perhaps other) DNA-PKcs modifications that regulate end accessibility by releasing DNA-PKcs or effecting conformational changes, particularly when DNA-PKcs autophosphorylation is blocked in DNA-PKcs-KR cells. This view is supported by the marked reduction in T2609 phosphorylation in DNA-PKcs-KR cells treated with ATM inhibitors (Fig. 7A, B). Although ATM-mediated modifications of DNA-PKcs-KR may be sufficient to allow HR to proceed, ATM cannot replace DNA-PKcs kinase activity during NHEJ [9].

DNA-PKcs-KR was shown to dissociate more slowly from laser-induced DSBs than wild-type DNA-PKcs, perhaps reflecting slower kinetics of ATM-dependent phosphorylation vs. autophosphorylation, and this was proposed to inhibit NHEJ [56]. However, slow dissociation of DNA-PKcs-KR may not reduce DSB-induced HR since HR occurs more slowly than NHEJ, and minor delays may not be detected in DSB-induced HR assays since HR products are selected two days after DSB induction. It is also possible that DNA-PKcs dynamics differ at *I-SceI* vs. laser-induced DSBs.

HR levels increase when cells progress from G1 to S/G2 when sister chromatids become available as HR repair templates. If T2609 phosphorylation is required for DNA-PKcs dissociation from ends before end processing for HR, it is curious that IR-induced T2609 phosphorylation is lower in S phase than G1 phase [63]. DNA-PKcs recruitment to DSBs may be attenuated in S phase. This would reduce T2609 phosphorylation because DNA-PKcs is likely to be phosphorylated only when bound to DSBs. Alternatively, T2609 may be phosphorylated in S phase at undetectable, but sufficient levels to increase end accessibility, or accessibility may increase at later times. The reduction in T2609 phosphorylation in S phase was observed 30 min after IR [63] whereas HR occurs over several hours. As above, T2609 phosphorylation may also differ between IR- and *I-SceI*-induced DSBs. In any case, our data indicate that T2609 is phosphorylated in DNA-PKcs-KR cells, this is blocked by ATM inhibitors, and blocking T2609 phosphorylation correlates with reduced RAD51 foci and reduced HR (Figs. 6 and 7).

4.3. Co-regulation of DSB repair by DNA-PKcs and ATM

The regulation of ATM levels by DNA-PKcs and phosphorylation of DNA-PKcs by ATM suggest that these proteins function in an integrated manner to regulate DSB repair by NHEJ and HR. In Fig. 8 we propose a model for co-regulation of NHEJ and HR by DNA-PKcs and ATM. In wild-type cells, DNA-PKcs promotes NHEJ which suppresses HR; this involves auto- and/or ATM-dependent phosphorylation of DNA-PKcs increasing end accessibility (shown as DNA-PKcs release in Fig. 8B). Because ATM targets many HR proteins (Fig. 8A), the low ATM level in DNA-PKcs null cells, and its restoration by DNA-PKcs-KR, can account for the higher DSB-induced HR levels in DNA-PKcs-KR cells. In both cases, the competing NHEJ pathway is inactive, but in the absence of DNA-PKcs only a moderate increase in HR is seen because the ATM level is low (Fig. 8C). In cells expressing DNA-

PKcs-KR, NHEJ is also inactive but ATM is expressed at a normal level and activated normally by damage, thus ATM can exert its full positive effect(s) on DSB-induced HR (Fig. 8D). ATM inhibitors like KU55933 block HR in wild-type and DNA-PKcs-KR cells (Fig. 8B, D), but not DNA-PKcs null cells (Fig. 8C). Included in this model are DNA-PKcs- and ATM-dependent phosphorylation of c-Abl, which is proposed to disassemble RAD51 filaments (light grey ovals) during a late HR stage. This is but one example of how ATM might enhance HR in DNA-PKcs-KR cells compared to DNA-PKcs null cells. Note also that c-Abl phosphorylates DNA-PKcs (not shown in Fig. 8), and probably DNA-PKcs-KR as well, providing a feedback loop [73].

In conclusion, our results indicate that DSB repair pathway choice and efficiency are regulated by DNA-PKcs and ATM, and that ATM promotes HR repair of DSBs arising independently of replication by phosphorylating DNA-PKcs and HR factors. DSB repair by NHEJ and HR contribute to genome stability, both pathways are likely to play significant roles in tumor resistance to chemo- and radiotherapy, and targeting these pathways may improve the efficacy of cancer therapy. The manipulation of HR and NHEJ pathways also holds promise for improving gene targeting efficiency in laboratory and gene therapy settings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

DSB	double-strand break
HR	homologous recombination
NHEJ	nonhomologous end-joining
PIK	phosphatidylinositol-3-kinase
IR	ionizing radiation

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Fig. 1.

Characterization of V3 derivatives expressing kinase-inactive DNA-PKcs. (A) HR substrates. The upstream *neo* is driven by the mouse mammary tumor virus promoter and is inactivated by insertion of an I-SceI recognition site; the downstream *neo* has wild-type coding sequence but lacks a promoter. HR substrates in VD13 and V24 are identical except the substrate in V24 has 12 silent restriction fragment length polymorphisms at ~100 bp intervals (*neo12*) for mapping conversion tracts. (B) Expression of DNA-PKcs in V3 derivatives. Western blotting with anti-DNA-PKcs antibodies confirms expression of DNA-PKcs or DNA-PKcs-KR. CHO AA8 cells are wild-type controls and β-actin serves as loading control. (C) DNA-PKcs-KR does not confer radioresistance. Cell survival after IR treatment measured as relative plating efficiency (average +SD) for V24 and its derivatives V24-C1 and V24-KR3 is shown. VD13 and its derivatives gave similar results (not shown). (D) DNA-PKcs-KR fails to phosphorylate RPA after camptothecin (CPT). Western blot of protein extracts from cells treated with 10 μM CPT or mock-treated for 1 hr.

Fig. 2.

DNA-PKcs-KR enhances HR. (A) DSB-induced HR frequencies are shown for cells expressing wild-type DNA-PKcs, DNA-PKcs-KR, or null (Δ). Values are averages ($\pm$ SD) for 3-5 determinations. Double asterisks indicate significant differences at $P \leq 0.01$ (t tests). (B) GFP transfection efficiencies measured by flow cytometry. (C) Percent survival as a function of MMC concentration in wild-type (AA8 and VD13-C1), DNA-PKcs-KR (VD13-KR4), and null (VD13) cells. Values are averages ($\pm$ SD) for 3 determinations.

Fig. 3.

IR-induced RAD51 foci resolve slowly in DNA-PKcs defective cells. (A) Representative immunofluorescent images of wild-type (AA8), DNA-PKcs null (VD13) and DNA-PKcs-KR (VD13-KR4) cells mock treated or treated with 5 Gy IR. RAD51 was detected with anti-RAD51 antibodies (red) 6, 12, or 24 hr after IR. Nuclei were stained blue (DAPI). Magnification = 40 $\times$. (B, C) Percentage of cells with 1 or more RAD51 foci, and number of RAD51 foci per cell. Values are averages $\pm$ SEM for three sets of images, with an average of 105 cells scored per data point. * indicates $P < 0.05$; ** indicates $P < 0.01$.

Fig. 4.

DNA-PKcs is recruited to DSBs independently of its kinase activity. (A) Representative immunofluorescence images of DNA-PKcs foci 1 hr after IR, detected with anti-DNA-PKcs antibodies. Nuclei are stained with DAPI (blue) and DNA-PKcs is stained red.

Magnification = 100 $\times$. (B) Average number of DNA-PKcs foci per cell ($\pm$ SEM); an average of 17 cells were scored per condition.

Fig. 5.

Robust IR-induced ATM S1981 phosphorylation in wild-type and DNA-PKcs-KR mutant cells, but not in DNA-PKcs null cells. (A) Representative Western blot of ATM in cells expressing wild-type DNA-PKcs, DNA-PKcs-KR, or null; β -actin is the loading control. (B) Quantitation of ATM protein levels by scanning densitometry of three blots including the blot shown in panel A. For each blot, ATM levels were first normalized to actin levels, then the averages of normalized values (+SEM) for the three blots were plotted as a percentage of AA8 values. ** indicates $P \leq 0.01$. (C) Representative images of activated ATM (pATM) detected with a phospho-S1981-specific antibody. Cells were fixed 1 hour after IR. Magnification = 40 $\times$. (D) Percentage of cells with at least one phospho-S1981 focus. Values are averages (+SEM) for 3-6 images; an average of 40 cells were scored per condition. ** indicates $P \leq 0.0011$.

Fig. 6.

Suppression of DSB-induced HR by ATM inhibitors is proportional to ATM protein levels. The ATM inhibitors KU55933 or CGK733 were present for 30 min before transfection with the I-SceI expression vector, absent during transfection, and present for 12 hr post-transfection, then medium was replaced with selective (G418) medium and cells were incubated for 10 days before staining with crystal violet. Plotted are average HR frequencies (+SD) for 2-3 determinations per strain.

Fig. 7.

IR-induced phosphorylation of the DNA-PKcs T2609 cluster correlates with RAD51 focus formation. (A) Representative images of cells probed with antibodies against pT2609. Cells expressing wild-type DNA-PKcs, DNA-PKcs-KR, or the ABCDE mutant were untreated or treated with 20 μ M KU55933 for 30 min prior to IR or mock exposure, fixed 1 hr later, and probed for pT2609. Magnification = 40 $\times$. (B) Quantitative analysis of pT2609 foci in wild-type and DNA-PKcs-KR cells. The average number of foci in the ABCDE mutant was subtracted from wild-type or DNA-PKcs-KR values with equivalent treatment to correct for non-specific foci. Foci were scored in an average of 112 nuclei per treatment. (C) Representative images of IR-induced RAD51 foci in DNA-PKcs null or derivatives expressing wild-type DNA-PKcs or DNA-PKcs-KR. Cells were treated with 20 μ M KU55933 for 30 min prior to IR or mock exposure, and fixed 6 hr later and probed with anti-RAD51 antibodies (red). Nuclei were stained with DAPI (blue). Magnification = 40 $\times$. (D) Frequency distributions of IR-induced RAD51 foci based on scoring an average of 208 cells per condition.

Fig. 8.

Model for DNA-PKcs and ATM regulation of NHEJ and HR. See text for details.