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CK2 phosphorylation of XRCC1 facilitates dissociation from DNA and single-strand break formation during base excision repair

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ABSTRACT

CK2 phosphorylates the scaffold protein XRCC1, which is required for efficient DNA single-strand break (SSB) repair. Here, we express an XRCC1 protein (XRCC1^{ckm}) that cannot be phosphorylated by CK2 in XRCC1 mutated EM9 cells and show that the role of this post-translational modification gives distinct phenotypes in SSB repair and base excision repair (BER). Interestingly, we find that fewer SSBs are formed during BER after treatment with the alkylating agent dimethyl sulfate (DMS) in EM9 cells expressing XRCC1^{ckm} (CKM cells) or following inhibition with the CK2 inhibitor 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT). We also show that XRCC1^{ckm} protein has a higher affinity for DNA than wild type XRCC1 protein and resides in an immobile fraction on DNA, in particular after damage. We propose a model whereby the increased affinity for DNA sequesters XRCC1^{ckm} and the repair enzymes associated with it, at the repair site, which retards kinetics of BER. In conclusion, our results indicate that phosphorylation of XRCC1 by CK2 facilitates the BER incision step, likely by promoting dissociation from DNA.

1. Introduction

With an estimated 10^4 spontaneous depurinations of the DNA in one cell during a single day, it is apparent that a cell is constantly in need of efficient repair [1]. DNA base damages can vary in size, complexity and toxicity and consequently they require specific sensors to be detected in the DNA. The monofunctional N-methylpurine-DNA glycosylase (MPG) is the primary sensor for the methylated DNA bases produced by DMS [2]. MPG will excise the damaged base from the DNA and the remaining apurinic/apyrimidinic (AP) site requires APE1 for incision of the DNA backbone [3], as opposed to the direct single strand breaks (SSBs) that are produced by reactive oxygen species. A direct SSB is formed when an oxidizing agent reacts with the sugar-phosphate backbone of the DNA and, as opposed to the enzymatically formed SSB intermediate produced during BER, can have very complex ends. These ends might need processing to ensure proper nucleotide insertion and ligation, which is done by damage-specific enzymes such as polynucleotide kinase (PNKP), Pol β, aprataxin and APE1 [4–7]. According to current models, the pathway to repair a direct SSB will converge with the pathway of BER only to diverge again into short patch repair or long patch repair depending on which DNA polymerase is chosen to replace the missing nucleotide.

The scaffold protein XRCC1 is a key player in both BER and SSB repair as it interacts with and stimulates enzymes throughout both processes, e.g. Pol β [8,9], PNKP [10], APE1 [11] and PARP-1 [12]. XRCC1 has not been found to display any enzymatic activity of its own but it has been shown to interact with and stimulate the activity of multiple glycosylases *in vitro*, such as hOGG1, NEIL1 and MPG [13–15]. More importantly, XRCC1 is known to stabilize LigIIIα [16] by forming a protein complex that is required for efficient and fast rejoining of SSBs through short patch repair [17]. XRCC1 has several serine/threonine residues within its linker region (spanning amino acids 403–538), located between two BRCT domains. These residues are phosphorylated *in vivo* by the kinase CK2 [18], which is an essential and pleiotropic enzyme, with a vast repertoire of substrates [19]. Many of these substrates are involved in cell cycle regulation [20–23] or apoptosis [24–27] and several human cancers display an elevated activity of CK2 [28–30]. Additionally, the dysregulated expression of CK2 was shown to cause lymphoma in a transgenic mouse model [31]. The kinase is also known to directly facilitate DNA strand break repair by the interactions of CK2-

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phosphorylated XRCC1 with the end-processing enzymes PNKP and Aprataxin [10,18]. As mentioned earlier, XRCC1 is a key protein in both BER and the repair of direct SSBs and the post-translational modification of XRCC1 by CK2 is known to stabilize the protein and to stimulate the repair of direct SSBs [32]. Here, we investigate the effects of CK2 phosphorylation of XRCC1 in BER by monitoring the repair of alkylation damage in the DNA.

2. Materials and methods

2.1. Cell cultures

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 9% fetal calf serum and penicillin-streptomycin (90 units/ml) at 37 °C in an atmosphere containing 5% CO_2. The cell lines used in this study are based on the *XRCC1*-deficient Chinese hamster ovary (CHO) cell line EM9, transfected with empty vector (EM9-V), his-tagged *XRCC1* gene (EM9-XH) or *XRCC1*^{ckm} gene (EM9-CKM) as described in [10]. EM9-CKM cells were maintained in G418 (1.5 mg/ml) to select for cells with the *XRCC1*^{ckm} construct.

2.2. Proteins

The bacterial and mammalian expression vectors for decahistidine-tagged XRCC1^{wt} and XRCC1^{ckm} were kindly provided by Prof. K.W. Caldecott. Histidine-tagged human XRCC1^{wt} and XRCC1^{ckm} were purified from CHO cells (EM9-XH and EM9-CKM respectively) on Ni-NTA agarose (Qiagen, Crawley, UK) as recommended by the manufacturer.

2.3. Repair assay

The DNA repair assay was carried out as described earlier [2] with the following alterations: exposure to DNA damaging agent and the following steps were performed in the presence of the PARP inhibitor 1,8-napthalimide (50 μM) [33] and/or CK2 inhibitor 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT) (10 μM) [34] in specified samples. Additionally, in samples where CK2 was inhibited, DMAT was included during the 1 h incubation in DMEM prior to treatment with damaging agent. In order to study remaining damages, PARP inhibitor (50 μM, in pre-warmed DMEM) was added as indicated, 2 h after terminating the treatment with DMS.

To quantify the level of SSBs with the ADU (alkaline DNA unwinding) assay the cells were washed twice with 0.15 M NaCl and lysed in DNA unwinding solution (0.15 M NaCl, 30 mM NaOH) at room temperature, with the exception of the experiment with PARP inhibitor added after 2 h of repair, in which the unwinding was performed on ice. The unwinding of DNA was continued for 30 min in darkness and was subsequently neutralized by the addition of 20 mM NaH_2PO_4. The DNA was then fragmented by sonication (15 s, Branson sonifier B-12, with micro tip), after which SDS (final concentration of 0.24%) was added. The samples were kept frozen for at least 12 h at −20 °C. Separation of double-stranded (ds) and single-stranded (ss) DNA was performed on hydroxyl apatite columns, kept at 60 °C, as described earlier [35].

2.4. Immunofluorescence

Cells were seeded at a density of 1–3 × 10^5 cells per cover slip and cultured for 24 h prior to treatment. Treatment with a DNA damaging agent was performed for 15 min on ice (1 mM DMS or 1 mM H_2O_2, diluted in HBSS^{++}) and was terminated by washing the cells with DMEM. Fresh DMEM was added to the cells, which were incubated for 4 h at 37 °C to allow time for repair. The cells were washed with PBS and fixated on the cover slips with 3% paraformaldehyde in 0.1% PBS-T (PBS with 0.1% Triton X-100) for 20 min. Incubations with BSA, secondary antibody and ToPro, were all preceded by 2 × 10 min washes with 0.1% PBS-T and permeabilization in 0.3% PBS-T for 10 min. To prevent unspecific binding of antibodies, blocking was performed with 3% BSA (dissolved in PBS) for 40 min. Primary antibody (rabbit polyclonal anti-RAD51, H-92, Santa Cruz) was added to the cells at a dilution of 1:1000, followed by incubation at 4 °C over night. The cells were then incubated with secondary antibody (Alexa 555 donkey anti-rabbit IgG antibody, Molecular Probes) for 1 h at room temperature at a dilution of 1:1000. Staining of the DNA was achieved with 2.5 μM ToPro (Molecular Probes) diluted in PBS. The cover slips were mounted with ProLong Gold Antifade Reagent (Molecular Probes). Images were acquired with a Zeiss laser scanning 510 inverted confocal microscope using a planachromat 63×/NA 1.4 oil immersion objective and excitation wavelengths of 543 and 633 nm. A RAD51-positive cell was classified as a nucleus containing 10 foci or more, 300 nuclei were counted on each slide.

2.5. hprt-mutation assay

The *hprt* assay monitors mutational and acute toxic effects of compounds by analyzing cloning of surviving cells 24 h after treatment. The number of cells mutated in the *hprt* gene was analyzed by selection after 7 days of growth to express the mutated phenotype [36]. The experiments were initiated by seeding of 2.5 × 10^5 cells in 25 cm^2 flasks which were cultivated at 37 °C in 5% CO_2 overnight in DMEM, and thereafter the cells were exposed to different concentrations of DMS, dissolved in DMSO and added to 4 ml HBSS^{++}. The treatments were terminated after 4 h, the HBSS^{++} was replaced by DMEM and the cells were incubated as described above. Following one day of recovery, the cells were reseeded for expression growth and clonogenic survival. Clonogenic survival was determined by seeding 250 cells in Petri dishes (10 cm), which were incubated for 7 days and finally fixed in methylene blue dissolved in methanol (4 g/l). For the expression, two 75 cm^2 flasks per treatment group were seeded with 3 × 10^5 cells and reseeded after incubation for 3 days and then left to grow for 4 additional days. After incubation the flasks containing cells treated with the same concentration of DMS were pooled and seeded for cloning and selection in Petri dishes, with 6-TG (5 μg/ml), and incubated for 7 days followed by fixation as described above. Analysis was performed by calculating the survival as a percentage of control and as an average of mutated cells per 10^5 cloning cells.

2.6. Cell fractionation of soluble and chromatin bound fractions

Cells were fractionated as described previously [37]. Briefly, cells were harvested by scraping and the cell pellets were resuspended in two packed cell volumes of Buffer A (10 mM HEPES-KOH (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 0.5% Triton X-100, 1 mM PMSF, 10 mM MG132, 10 μl/ml phosphatase inhibitors (Set I and Set II, Calbiochem), 1 μg/ml each of pepstatin, aprotinin, chemostatin and leupeptin) and kept on ice for 5 min. Following centrifugation at 1300 × *g* for 4 min, the supernatant was transferred to a new Eppendorf tube and designated as soluble protein fraction (S). The nuclear pellet was washed twice with Buffer A and resuspended in two packed cell volumes of Buffer B (3 mM EDTA, 1 mM PMSF, 10 mM MG132, 10 μl/ml phosphatase inhibitors (Set I and Set II, Calbiochem), 1 μg/ml each of pepstatin, aprotinin, chemostatin and leupeptin) and incubated on ice for 10 min before centrifugation at 1700 × *g* for 4 min. The pelleted material was washed twice more with