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Research paper

Functional effects of diphosphomimetic mutations at cAbl-mediated phosphorylation sites on Rad51 recombinase activity

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ABSTRACT

Homologous Recombination enables faithful repair of the deleterious double strand breaks of DNA. This pathway relies on Rad51 to catalyze homologous DNA strand exchange. Rad51 is known to be phosphorylated in a sequential manner on Y315 and then on Y54, but the effect of such phosphorylation on Rad51 function remains poorly understood. We have developed a phosphomimetic model in order to study all the phosphorylation states. With the purified phosphomimetic proteins we performed *in vitro* assays to determine the activity of Rad51. Here we demonstrate the inhibitory effect of the double phosphomimetic mutant and suggest that it may be due to a defect in nucleofilament formation.

1. Introduction

Human cells are subjected to around 10^5 DNA lesions per cell per day [1,2]. Since genome integrity is necessary for cell survival, regulation of DNA damage response is essential to repair efficiently each kind of damage, thus avoiding potential modification and mutation of the DNA. Among the different types of DNA damage, double strand breaks (DSB) are the most lethal [1,2]. DSB can be faithfully repaired only by the Homologous Recombination (HR) repair pathway [3].

In eukaryotes, HR mechanism is based on homologous strand exchange performed by the evolutionary conserved Rad51 [3]. The process of HR can be divided into three mains steps: 1) presynaptic phase during which DNA DSBs are resected by a nuclease from the MRN complex (MRE11-RAD50-NSB1) to generate 3′-protruding ends which are then protected from further resection by Replication Protein A (RPA). This ssDNA-binding factor removes secondary structures from the ssDNA and is subsequently replaced by Rad51 [4]. Rad51 is recruited onto ssDNA to form a nucleofilament called presynaptic filament with the aid of protein partners such as Rad52 and BRCA2. It is noteworthy that the attenuation of ATP hydrolysis or the use of a non-hydrolyzable ATP analogue enhance the stability of this presynaptic filament [5] 2) The synaptic phase corresponds to homologous DNA pairing. The Rad51 nucleofilament is involved in the search for a homologous DNA sequence. Once a homologous sequence is localized, the Rad51 filament invades the duplex DNA and generates a displacement of the homologous DNA strand to form a D-loop. 3) During the final post-synaptic step the Rad51 nucleofilament catalyzes the strand exchange and helicases such as BLM perform resolution of the DNA intermediate structure resulting in crossover or non-crossover products. Rad51 ATPase activity is required for the disassembly of the filament and Rad51 release from DNA that leads to the turnover of Rad51 protein [5,6].

HR is based on the DNA strand exchange activity of Rad51. This makes of Rad51 a potential key protein for HR regulation. Indeed, HR efficiency is driven by the activation or inactivation of Rad51. In the last decade, a number of post-translational modifications (PTM) of Rad51 have been described. Thereby kinase-mediated Rad51 phosphorylation may be involved in cell cycle regulation, chemoresistance and DNA damage response (DDR).

Rad51 is phosphorylated on T309 by Chk1 kinase [7] and the absence of this phosphorylation increases cell sensitivity to DNA damage. Phosphorylation on T309 could be necessary for the formation of Rad51 nuclear foci on DNA Breaks. Recently a sequential phosphorylation of Rad51 on S14 by CK2 kinase and then on T13 by Plk1 kinase has been proven [8]. This double phosphorylation seems necessary for the recruitment of Rad51 to DNA damage sites.

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Abbreviations

AU	Arbitrary Units
BSA	bovine serum albumin
CD	Circular Dichroism
Chk1	Checkpoint kinase 1
CK2	Casein kinase 2
DDR	DNA damage response
DSB	double strand break
EcRecA	Escherichia coli RecA protein
EDTA	ethylenediaminetetraacetic acid
HR	homologous recombination
HsRad51	Homo sapiens Rad51 protein
Plk1	polo-like kinase 1
PTM	post-translational modifications
RPA	replication protein A
ssDNA	single strand DNA
TNP-ATP	(2′-(or-3′)-O-(Trinitrophenyl) Adenosine 5′-Triphosphate, Trisodium Salt)
WT	wild type

Rad51 is also phosphorylated by c-Abl on two tyrosines, Y54 and Y315. We have previously shown that these Abl-mediated phosphorylations were related through a sequential phosphorylation mechanism. C-Abl kinase first phosphorylates Rad51 on Y315, which favors the second phosphorylation at position Y54 [9]. Yuan et al. [10] have shown that phosphorylation on Y54 could have an inhibiting effect on Rad51-mediated DNA strand exchange, whereas phosphorylation on Y315 could favor the interaction between Rad51 and Rad52 and promote the Rad51 activity [11].

Numerous studies have been conducted to investigate the cellular effect of Abl-mediated Rad51 phosphorylation [12,13]. However, the direct biochemical consequences of these phosphorylations on Rad51 activity are still unknown.

In this study, we investigated the impact of tyrosine phosphorylation at positions 54 and 315 on Rad51 recombinase activity by generating mono- and di-phosphomimetic mutated Rad51. The phosphomimetic approach consists in replacing a tyrosine residue by a glutamic acid residue in order to mimic the negative charge encountered after phosphorylation.

Here, we describe the *in vitro* comparison of wild type Rad51 (Rad51-WT) with the phosphomimetic proteins Rad51 Y54E, Rad51 Y315E and Rad51 Y54/315E. For the first time we demonstrate that the di-phosphomimetic form results in total loss of Rad51 activity from the oligomerization step to final DNA strand exchange step.

Based on our results we propose that the sequential phosphorylation of Rad51 by c-Abl may play a pivotal role in the regulation of HR by modulating the first step of recombinase activity.

2. Materials and methods

2.1. Production and purification of Rad51 proteins

The cDNA of His-tagged Rad51 was cloned in pET15a vector (Novagen). Rad51 Y54E, Rad51 Y315E and Rad51-Y54/315E were generated by site-directed mutagenesis using overlapping oligonucleotides. DNA sequencing of the mutant clones were checked and validated. Rad51-WT, Rad51, RAD51-Y54E, Rad51-Y315E and Rad51-Y54/315E were then over-expressed in *Escherichia coli* BL21-DE3 strain at 37 °C. His-tagged Rad51-proteins were purified on a NiNTA resin (Invitrogen). Imidazole used for purification was removed by dialysis. Protein concentrations were determined by BCA assay, Bradford assay and capillary electrophoresis (Agilent Protein Microchip). The purity of proteins was analysed by SDS-PAGE followed by silver staining and by capillary electrophoresis (Agilent Protein Microchip). All His-tagged Rad51 proteins were kept at −80 °C prior to use.

2.2. Circular dichroism measurement

Circular dichroism (CD) spectra were measured using a J-810 CD spectrophotometer (Jasco) equipped with a Peltier temperature controller. Protein samples at 1 mg/ml concentration were placed into a dismantled cuvette, 0,1 mm layer thickness (Hellma). The spectra was averaged on three independent experiments to increase the signal to noise ratio. Measurement conditions were as following: 2 nm bandwidth; interval 0.1 nm; response time 0.5 s; temperature at 20 °C; wavelength range from 200 to 260 nm. All the spectra were corrected by subtracting the spectrum of the buffer.

2.3. DNA strand exchange assay

His-tagged Rad51 from 0.5 to 3 μM was incubated with 1 μM 58mer ssDNA-IRD800 in buffer (Tris HCl pH 8 20 mM; MgCl2 20 mM; Glycerol 2%; BSA 100 μg/mL; ATP 1 mM; DTT 1 mM) at 37 °C for 20 min. 2.5 μM of 32mer homologous dsDNA was then added for 1 h at 37 °C [14]. Finally, the reaction was stopped by SDS 0.5% and proteinase K 50 μg/mL. DNA products were separated by electrophoresis on 15% polyacrylamide gel. Labelled DNA was detected and quantified by Odyssey scanner at 800 nm.

2.4. D-loop assay

His-tagged Rad51 from 0.5 to 4 μM was incubated with 1 μM 100mer ssDNA-IRD700 (GGG CGA ATT GGG CCC GAC GTC GCA TGC TCC TCT AGA CTC GAG GAA TTC GGT ACC CCG GGT TCG AAA TCG ATA AGC TTA CAG TCT CCA TTT AAA GGA CAA G) at 37 °C in the presence of ATP 1 mM, Tris HCl pH7,5 25 mM, DTT 1 mM, $CaCl_2$ 1 mM. Then homologous supercoiled pPB4.3 (300 μM) was added. After 15 min at 37 °C, the reaction was stopped by the addition of 1% SDS and 1 mg/mL proteinase-K for 20 min. Then DNA products were separated by 1% agarose gel electrophoresis and analysed using Odyssey Infrared Imager (LI-COR) at 700 nm. The scanner software allowed the quantification of the signal of each D-loop band, and then the intensity average from at least three experiments was plotted in a diagram.

2.5. Blitz® DNA binding assay

Biotinylated DNA (polydT 58mer, 100 μM) was bound on a streptavidin coated biosensor (tip). Rad51 proteins (2 μM) were prepared in reaction buffer made of PBS buffer 1X and ATP 1 mM. Kinetics were divided in three steps: firstly, baseline of the buffer is measured for 10s, then the association step between ssDNA and Rad51 at 2 μM is monitored for 120s and finally the dissociation step of the bound Rad51 is monitored for 30s in buffer. Tip was regenerated in NaOH 50 mM twice for 40s before reuse.

2.6. ATP hydrolysis and binding assay

His-tagged Rad51 ATPase activity was determined by titrating the release of Pi. His-tagged Rad51 (4 μM) was incubated with ssDNA polydT (10 μM) in reaction buffer (Tris HCl 20 mM, NaCl 50 mM, MgCl2 2 mM, DTT 1 mM, BSA 0.1 mg/mL, EDTA 0,05 mM) for 30 min at 37 °C. Then ATP 1 mM was added for 40 min. The