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NOTCH1 modulates activity of DNA-PKcs

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ABSTRACT

DNA-dependent protein kinase catalytic subunit (DNA-PKcs) controls one of the most frequently used DNA repair pathways in a cell, the non-homologous end joining (NHEJ) pathway. However, the exact role of DNA-PKcs in NHEJ remains poorly defined. Here we show that NOTCH1 attenuates DNA-PKcs-mediated autophosphorylation, as well as the phosphorylation of its specific substrate XRCC4.

Surprisingly, NOTCH1-expressing cells do not display any significant impairment in the DNA damage repair, nor cellular survival, and remain sensitive to small molecule DNA-PKcs inhibitor. Additionally, *in vitro* DNA-PKcs kinase assay shows that NOTCH1 does not inhibit DNA-PKcs kinase activity, implying that NOTCH1 acts on DNA-PKcs through a different mechanism. Together, our set of results suggests that NOTCH1 is a physiological modulator of DNA-PKcs, and that it can be a useful tool to clarify the mechanisms by which DNA-PKcs governs NHEJ DNA repair.

1. Introduction

DNA double strand breaks (DSBs) are one of the most cytotoxic lesions, therefore their fast and accurate repair is essential. DSBs can be repaired by two main DNA repair mechanisms: non-homologous end joining (NHEJ) and homology directed repair (HDR). Due to the fact that HDR is restricted to the S/G2 phase of the cell cycle and NHEJ is active throughout the cell cycle, the majority of DSBs are repaired by the latter. DNA-dependent protein kinase (DNA-PK) is the central kinase that regulates NHEJ repair. DNA-PK is composed of a catalytic subunit (DNA-PKcs) and DNA binding KU70/80 heterodimer [1]. Despite extensive investigations, the exact role of DNA-PKcs in NHEJ remains unclear. It has been established that DNA-PKcs kinase activity is indispensable for DNA repair by NHEJ, as cells with catalytically inactive or chemically inhibited DNA-PKcs show impaired DNA damage repair [2,3]. Although many specific targets of the kinase activity of DNA-PKcs have been reported, such as KU70/80 heterodimer, XRCC4, LIGASE IV or XLF, so far none of the phosphorylated residues, except its autophosphorylation, have been demonstrated to be necessary for NHEJ in cells [4,5]. Thus, the apparent contradiction between the necessity of DNA-PKcs kinase activity for NHEJ repair and its nonessential role in mediating phosphorylations of NHEJ factors, other than itself, remains unresolved. A method that would allow further clarification of the requirement of DNA-PKcs targets for NHEJ repair, would greatly improve our understanding of the role of DNA-PKcs kinase in the DNA damage repair processes.

NOTCH1 is a trans-membrane receptor that regulates a number of cellular processes like proliferation, differentiation, migration and apoptosis [6]. Upon activation, intracellular part of NOTCH1 (N1IC) translocates to the nucleus to regulate transcription of its target genes [7]. Next to affecting gene expression as a transcription factor, NOTCH1 can act in a non-canonical, transcriptionally independent, manner. Recently we reported that NOTCH1 modulates DNA damage response (DDR) by interacting with ATM kinase and inhibiting its kinase activity [8,9].

Here we report that NOTCH1 receptor has the ability to modulate phosphorylations mediated by the DNA-PKcs kinase, but it does not seem to inhibit DNA-PKcs activity necessary for NHEJ repair.

2. Material and methods

2.1. Cell culture and treatments

HEK293T cells (ICLC), EJ5-GFP U2OS [10], were grown in the DMEM medium supplemented with 10% FBS, penicillin/streptomycin and L-glutamine. CUTLL1 cells [11] were grown in RPMI 1640 10% FBS supplemented with penicillin/streptomycin and L-glutamine. ATM inhibitor (KU60019, Selleck Chemicals) and DNA-PKcs inhibitor

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(NU7441, Tocris Bioscience) were resuspended in DMSO and used at the 5 μM concentration (unless indicated otherwise), 3 h prior the treatments. Cells were subjected to ionizing irradiation (IR) using a Faxitron machine (X-Ray Corporation)(2 Gy/min). For the UV irradiation, a UV crosslinker (UVP) was used.

2.2. Cell culture transfections

Cells were always transfected with expression constructs and corresponding empty vectors in parallel as listed: pcDNA3 and N1ΔE-Flag; pEGFPN1 and N1IC-GFP. HEK293T cells were transfected with standard calcium phosphate method (3 μg of plasmid/well of 6 well plate) for 10 h after which medium was replaced with the fresh one. 24 h after transfections cells were subjected to the indicated treatments. U2OS (EJ5-GFP) cells were transfected with Lipofectamine 2000 (Invitrogen) for 4 h (3 μg of plasmid/well of 6 well plate) according to the manufacturer's protocol.

2.3. Immunoblot analysis, immunoprecipitations and GST-pulldowns

After harvesting cells were lysed in the TEB150 buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA pH 8, 1 mM dithiothreitol (DTT), 0.5% Triton X-100 (v/v), 10% glycerol (v/v), protease inhibitor cocktail set III (Calbiochem) and Benzonase nuclease (Sigma) 1:1000).

For the purpose of immunoblot analysis of NOTCH1-expressing cells HEK293T cells were transfected with N1IC-GFP or EV-GFP with the use of calcium phosphate method (10 μg of the plasmid/10 cm dish), collected 16 h after and subjected to fluorescence-activated cell sorting (FACS). After 10 h recovery cells were subjected to indicated treatments and collected in TEB150 buffer.

Immunoprecipitations were carried out with 1 mg of protein lysate, which was incubated with the indicated antibodies (Supplementary Table 1) over night at 4 °C followed crosslinking with Protein G (Zymed Laboratories)(2 h; 4 °C). At the end beads were washed 3 times with the lysis buffer.

GST-pulldowns were done as follows. 1–2 μg of recombinant GST-DNA-PKcs fragments were incubated for 2 h at 4 °C with either 800 μg of cell lysate (HEK293T cells expressing N1ΔE-Flag) or 2 μg of the human recombinant NOTCH1 (N1IC-Flag). After incubation beads were washed 5 times with TEB150 buffer and subsequently subjected to immunoblot analysis.

For the purpose of XRCC4 phosphorylation dependent electrophoretic mobility shift visualization, collected protein samples were subjected to SDS-PAGE on the 9% Tris-Glycine gel, allowing proteins smaller then 40 kDa to run out from the gel. Next, samples were analysed by immunoblot.

2.4. Immunofluorescence analysis

Cells were fixed in the Methanol/Acetone solution (1:1) for 2 min or 4% solution of PFA in PBS for 10 min. Fixed cells were blocked in PBG solution (0.2% cold-water-fish gelatin and 0.5% BSA in PBS) and immunostained with indicated antibodies (Supplementary Table 1) for 1 h at RT. Secondary antibodies (Jackson Laboratories) conjugated with specific fluorophores were used at 1:400 dilution for 1 h at RT. Nuclei were counterstained with DAPI and mounted on a slide. Images for the analysis were acquired with the use of wide field microscope (Olympus Biosystems Microscope). Representative images were taken with confocal laser microscope (Leica TCS SP2 AOBS). Images were analysed with the use of Cell profiler software (2.1.1)[12].

2.5. Recombinant protein purification

Human N1IC was purified from Hi5 insect cells, as previously described [9]. Human GST-tagged DNA-PKcs fragments [13] were purified from *Escherichia coli* BL21 competent bacteria with the use of Gluthatione sepharose beads (GE Healthcare). Shortly, bacteria were induced with 0.2 mM IPTG (OD = 0.6-0.8) over night at 20 °C. Next, bacteria were pelleted (4000 rpm at 4 °C for 20 min) and resuspended in the lysis buffer (50 mM Tris-HCl pH = 7.9; 300 mM KCl; 1% Triton X-100; 2 mM DTT and protease inhibitor cocktail set III (Calbiochem)) followed by the sonication (3 times for 20 s). After sonication bacteria were spun at 20000 rpm at 4 °C for 1 h followed by the addition of equilibrated Gluthatione sepharose beads (GE Healthcare) and incubation for 2 h at 4 °C. Next, beads were subsequently washed 3 times (15 min each) with: buffer I (PBS; 1% Triton X-100; 2 mM DTT and protease inhibitor cocktail set III (Calbiochem)), buffer II (300 mM KCl; 50 mM Tris-HCl pH = 7.9; 2 mM DTT and protease inhibitor cocktail set III (Calbiochem)) and buffer III (50 mM Tris-HCl pH = 7.9; 100 mM KCl; 10% glycerol; 2 mM DTT and protease inhibitor cocktail set III (Calbiochem)). At the end beads were resuspended in the buffer III (1:1), aliquoted and stored at −80 °C until the use.

2.6. In vitro DNA-PKcs kinase assay

In vitro DNA-PKcs kinase assay kit was obtained from Promega and used according to the manufacturer's protocol. Briefly, 20U of recombinant DNA-PK holoenzyme (DNA-PKcs; KU70/80) was preincubated with the recombinant N1IC-Flag (1–6 ng) or NU7441 (1 μM) for 20 min on ice followed by the addition of ATP (150 μM) with liner dsDNA (Promega) and subsequent incubation at 24 °C for 40 min (20 μl reaction volume). 5 μg of the substrate peptide was used per single reaction (Glu − Pro − Pro − Leu − **Ser** − Gln − Glu − Ala − Phe − Ala − Asp − Leu − Trp − Lys − Lys)(Promega))[14]. ATP consumption by DNA-PKcs kinase was measured with the ADP-Glo assay (Promega).

2.7. NHEJ GFP reporter system (EJ5 U2OS)

U2OS EJ5-GFP cells were transfected with the use of Lipofectamine 2000 (Invitrogen) with: empty vector (EV) or N1ΔE-Flag constructs and: EV or SceI-HA constructs. 5 μM of NU7441 was added immediately after transfection. 24 h and 72 h post transfection cells were fixed in 4% PFA and subjected to fluorescence analysis with the use of wide field microscope (Olympus Biosystems Microscope). Images were analysed with the use of Cell profiler software (2.1.1)[12]

2.8. Colony assay

HEK293T cells were transfected with N1IC-GFP or EV-GFP, collected 16 h after and subjected to fluorescence-activated cell sorting (FACS). After FACS sorting cells were plated (3000 cells per 1 well of a 6 well pate − in a technical triplicate) and allowed to recover for 8 h. Next, cells were subjected to IR with indicated doses. 11 days post IR treatment cells were stained with the use of crystal violet (Millipore). Stained colonies were scanned and images were analysed with the use of Cell profiler software (2.1.1)[12].

2.9. Plasmids

EGFP and N1IC-GFP (1759–2556aa) plasmids were a kind gift of A. Sarin (NCBS, India)[15]. pcDNA3 N1ΔE-Flag (1715-2556aa) was a kind gift of G. Del Sal (University of Trieste, Italy)[16]. pUC19 SceI-HA was a kind gift of E. Soutoglou (IGBMC, France)[17]. GST DNA-PKcs fragments were a kind gift of B. Chen (UT Southwestern, U.S.A.)[13].

2.10. Statistical analysis

Presented data are represented as mean ± SEM. Statistical analysis of all the experiments was performed with the use of a two-tailed Student's *t*-test.