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Cross-talk between sumoylation and phosphorylation in mouse spermatocytes

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ABSTRACT

The meiotic G2/M1 transition is mostly regulated by posttranslational modifications, however, the cross-talk between different posttranslational modifications is not well-understood, especially in spermatocytes. Sumoylation has emerged as a critical regulatory event in several developmental processes, including reproduction. In mouse oocytes, inhibition of sumoylation caused various meiotic defects and led to aneuploidy. However, the role of sumoylation in male reproduction has only begun to be elucidated. Given the important role of several SUMO targets (including kinases) in meiosis, in this study, the role of sumoylation was addressed by monitoring the G2/M1 transition in pachytene spermatocytes *in vitro* upon inhibition of sumoylation. Furthermore, to better understand the cross-talk between sumoylation and phosphorylation, the activity of several kinases implicated in meiotic progression was also assessed upon down-regulation of sumoylation. The results of the analysis demonstrate that inhibition of sumoylation with ginkgolic acid (GA) arrests the G2/M1 transition in mouse spermatocytes preventing chromosome condensation and disassembling of the synaptonemal complex. Our results revealed that the activity of PLK1 and the Aurora kinases increased during the G2/M1 meiotic transition, but was negatively regulated by the inhibition of sumoylation. In the same experiment, the activity of c-Abl, the ERKs, and AKT were not affected or increased after GA treatment. Both the AURKs and PLK1 appear to be "at the right place, at the right time" to at least, in part, explain the meiotic arrest obtained in the spermatocyte culture.

1. Introduction

Successful progression through the G2/M1 transition in spermatocytes is a prerequisite for the formation of normal, genetically balanced gametes. Errors in meiotic recombination and chromosome segregation are the major causes of meiotic nondisjunction and aneuploidy [1]. However, the molecular mechanisms responsible for these meiotic errors are not well-understood, especially in males. Knockout mouse models arrested at the G2/M1 transition, in addition to the development of an *in vitro* system where pachytene spermatocytes have been induced to undergo G2/M1 transition upon treatment with okadaic acid (OA, an inhibitor of phosphatases PP1 and PP2A), have identified several important regulators of the process. For example, mice with a deletion of Cyclin A1 (CCNA1) or spermatocyte-specific deletion of cyclin dependent kinase 1 (CDK1) exhibit meiotic arrest during the exit from the meiotic prophase [2,3]. In a similar manner, mice with meiotic expression of an inactive isoform of AURKB display abnormalities during the exit from meiosis I [4]. Events in spermatocytes during the OA-induced G2/M1 transition *in vitro* closely mimic the ones observed *in vivo*. The Initiation of desynapsis is hallmarked by the removal of the central element protein (SYCP1) of the synaptonemal complex (SC) and precedes phosphorylation of histone H3 on Serine 10 (H3SerPh). These events are followed by the re-localization of the lateral element protein of the SC (SYCP3) to the centrosomes, and the formation of condensed bivalents. Although the translation of specific proteins during the pachytene stage is a prerequisite for the successful completion of meiosis, inhibition of protein synthesis in spermatocytes at the time of G2/M1 transition does not affect the exit from meiotic prophase *in vitro* [5]. Therefore, the G2/M1 transition is mostly regulated by posttranslational modifications. In

Abbreviations: GA, ginkgolic acid; OA, okadaic acid; SUMO, Small Ubiquitin-like Modifiers.

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support of this finding, the inhibition of global tyrosine phosphorylation or the activity of specific kinases causes meiotic arrests at different stages during the OA-induced G2/M1 progression [6,7]. For example, CDK inhibitor Butyrolactone (BLI) does not affect desynapsis or H3SerPh but does inhibit the OA-induced re-localization of SYCP3 and condensation of bivalents [8]; Interestingly, CDK inhibition also inhibits the activity of mitogen-activated protein kinases (MAPKs) through an unknown mechanism [9]. ZM447439 (ZM), an inhibitor of AURKs, did not affect initiation of desynapsis but inhibited H3SerPh (which is a direct target of AURKB) [7]. Inhibition of PLK1 with dihydropteridinone BI 2536 fully inhibited the first step of desynapsis (the removal of SYC1) and affected H3 phosphorylation to a certain degree [6]. Notably, general tyrosine kinase inhibitor completely abolishes desynapsis and chromosome condensation [5]. In addition to kinase activity, the activity of topoisomerases (TOPs, enzymes unwinding DNA) is also required for G2/M1 progression. Teniposide and ICRF-193, inhibitors of TOP2, dramatically affect the condensation of chromosomes [8].

Sumoylation is yet another type of posttranslational modification by Small Ubiquitin-like Modifiers or SUMO proteins that has been identified as an important regulatory event in several cellular processes including cell cycle progression [10–14]. Covalent conjugation of SUMO to the target protein happens through the action of SUMO activating enzyme (E1, a heterodimer of SAE1-SAE2), SUMO-conjugating enzyme UBC9 (E2), and SUMO ligases (E3). Sumoylation is reversed through the action of SENPs, which cleave the isopeptide bond between SUMO and its substrate. Four SUMO paralogs have been identified: SUMO1, 2, 3 (often termed SUMO2/3 because of their 95% sequence identity), and 4. While SUMO1, 2, and 3 are abundantly expressed in different tissues, SUMO4 is restricted to the kidneys and lymphatic tissues [15–17]. SUMO1-knockout mice show no phenotypic consequences because the protein's function is compensated for by SUMO2 and SUMO3 [18]. SUMO2 is apparently the major SUMO isoform present during embryonic development, and as a result, SUMO2-knockout mice show early embryonic lethality [19]. In a similar manner, UBC9 (the only SUMO-conjugating enzyme)-knockout mice show early embryonic lethality with severe disruptions in mitosis; a finding that supports the indispensable role of sumoylation in cell cycle progression [20]. In mouse oocytes, sumoylation plays a crucial role in spindle organization in addition to chromosome congression and segregation. Inhibition of sumoylation by SUMO1 or UBC9 with a specific antibody or their depletion by specific si-RNA microinjection in oocytes caused defective spindle organization, misaligned chromosomes, and led to aneuploidy. In a similar manner, overexpression of SENP-2, a SUMO-specific isopeptidase, led to defects in MII spindle organization in mature eggs [21,22]. However, the role of sumoylation in male reproduction has only begun to be elucidated. We and others have localized SUMO proteins in germ and somatic testicular cells and have obtained evidence implicating sumoylation in different aspects of normal and impaired spermatogenesis. We have also identified sumoylated targets in purified mouse spermatocytes and spermatids and in human sperm. In mouse spermatocytes, numerous proteins have been identified as SUMO targets including those regulating transcription, chromatin dynamics, and other processes. Sumoylation of several targets with potentially important roles during meiosis (such as CDK1, TOP2, RNAP II, MILI, DDX4, MDC1, KAP1, and TDP-43) were further supported by co-immunoprecipitation, co-localization, and *in vitro* sumoylation studies [23–29]. SYCP1 and SYCP2 have also been co-immunoprecipitated with SUMO from testicular lysate, as shown by another group [23]. Interestingly, some kinases have been identified by our screen as SUMO targets [29]. This finding is consistent with growing evidence that phosphorylation and sumoylation interact at multiple levels. A phosphorylation-dependent motif has been identified [30] and inhibition of sumoylation in somatic cells by a sumoylation inhibitor (ginkgolic acid, GA) significantly affected tyrosine phosphorylation (PhosphoTyr) of multiple proteins [31].

Overall, a cross-talk between different post-translation modifications during meiotic prophase and the G2/M transition is not well-understood, especially in spermatocytes. Given the important role of several SUMO targets in meiosis, in this study, the role of sumoylation was addressed by monitoring the G2/M1 transition in pachytene spermatocytes *in vitro* upon inhibition of sumoylation. Furthermore, to better understand a cross-talk between sumoylation and phosphorylation, the activity of several kinases implicated in meiotic progression was also assessed upon down-regulated sumoylation.

2. Materials and methods

2.1. Short-term culture of mouse spermatocytes; okadaic and ginkgolic acid treatment

C57BL/6NCrl mice were purchased from Charles River (Kingston, NY). The Animal Committee of Albert Einstein College of Medicine, Yeshiva University approved all animal protocols. A spermatocyte-enriched fraction was prepared as described in our recent publication. A flow-cytometry and microscopic analysis to confirm fraction purity was also performed as previously described by our group [29]. Spermatocytes were cultured according to [32]. Spermatocyte-enriched fractions were pooled together, washed three times by centrifugation at 500g for 7 min, and resuspended in minimal essential medium (MEM) (Sigma-Aldrich, M0894) supplemented with 0.29% (v/v) DL-lactic acid, 5% (v/v) FBS, 5.9 mg/ml HEPES, 0.05 mg/ml streptomycin sulfate (Life Technologies, 11860-038), and 0.075 mg/ml penicillin G (Sigma-Aldrich, P3032) to the final concentration of 2.5×10^6 cells/ml. Next, 986.7 μl of cell suspension was added to each well of a 4-well dish (Thermo Scientific, Rockford, IL), and the cells were incubated at 32 °C with 5% CO_2 for 10 h (after STA-PUT separation) or were used the same day after differential plating. The results of the experiments were similar with the both types of spermatocyte isolation, but the differential plating procedure shortened the time of the experiment by one day. Following the incubation, 1 μl of freshly prepared ginkgolic acid (GA, a specific inhibitor of sumoylation that blocks formation of the E1-SUMO intermediate [33]) or 1 μl of 100% DMSO was added to the experimental or control wells, respectively. The final concentration of GA was 10–50 μM. After one hour, G2/M transition was induced by the addition of 13.3 μl of 300 μM okadaic acid (OA, a phosphatase inhibitor [7,34]) for an additional 4 h. Following treatment, 100 μl of cell suspension from each well was centrifuged at 5000 rpm (Eppendorf, 5415 C, 5 min, 4 °C) and resuspended in 20 μl of a 2% PFA + 0.03% SDS solution. To this, 20 μl of 0.4% Photoflo solution (Kodak Professional, #74257, Hatfield, PA) was added. For chromosome spreads, 2 μl of well-mixed cell suspension was pipetted onto each well of a Shandon™ multi-spot slide (Thermo Scientific, 9991090) and gently spread in a circular motion over the well using a tip. After a brief air drying of the cells (5–10 min), the slides were washed in a series of solutions in Coplin jars: a. 2% PFA +0.03% SDS for 3 min; b. 2% PFA for 3 min; c. 0.4% Photoflo for 3 × 1 min. The slides were then air dried for 1 h before being subjected to IF or storage at −20 °C. In some experiments, the remaining 900 μl of cell suspension from each well was used for preparation of cell lysate. Whole cell protein lysates were prepared as previously described, using the whole cell extraction kit and protease inhibitor from Millipore (2910, Billerica MA) complemented with 2.5 mg/ml of N-ethylmaleimide (NEM (a de-

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