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Regulation of inositol 1,4,5-trisphosphate receptor type 1 function during oocyte maturation by MPM-2 phosphorylation

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ABSTRACT

Egg activation and further embryo development require a sperm-induced intracellular Ca²⁺ signal at the time of fertilization. Prior to fertilization, the egg's Ca²⁺ machinery is therefore optimized. To this end, during oocyte maturation, the sensitivity, i.e. the Ca²⁺ releasing ability, of the inositol 1,4,5-trisphosphate receptor type 1 (IP₃R1), which is responsible for most of this Ca²⁺ release, markedly increases. In this study, the recently discovered specific Polo-like kinase (Plk) inhibitor BI2536 was used to investigate the role of Plk1 in this process. BI2536 inactivates Plk1 in oocytes at the early stages of maturation and significantly decreases IP₃R1 phosphorylation at an MPM-2 epitope at this stage. Moreover, this decrease in Plk1-dependent MPM-2 phosphorylation significantly lowers IP₃R1 sensitivity. Finally, using *in vitro* phosphorylation techniques we identified T²⁶⁵⁶ as a major Plk1 site on IP₃R1. We therefore propose that the initial increase in IP₃R1 sensitivity during oocyte maturation is underpinned by IP₃R1 phosphorylation at an MPM-2 epitope(s).

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1. Introduction

Prior to fertilization, the mammalian egg is arrested at metaphase of meiosis II (MII), until the sperm induces a dramatic increase in the egg's intracellular Ca²⁺ concentration ([Ca²⁺]_i). This increase in [Ca²⁺]_i will induce all the subsequent events of egg activation, including cortical granule exocytosis, resumption of meiosis, extrusion of the second polar body (2PB), pronucleus (PN) formation and entry into first mitosis [1,2].

In mammals, the sperm-induced [Ca²⁺]_i signal consists of [Ca²⁺]_i oscillations that last for several hours [3]. In order to give rise to this specific spatio-temporal [Ca²⁺]_i oscillation pattern at fertilization, the Ca²⁺-release machinery of the mammalian oocyte is optimized during maturation. For instance, when immature germinal vesicle (GV) oocytes are fertilized *in vitro*, the sperm-induced [Ca²⁺]_i oscillations are fewer in number and each [Ca²⁺]_i rise shows a smaller amplitude and duration than those observed in MII eggs [4,5]. The Ca²⁺ channel responsible for most of this Ca²⁺ release

is the inositol 1,4,5-trisphosphate (IP₃) receptor type 1 (IP₃R1) [3], which is localized in the endoplasmic reticulum (ER). During oocyte maturation, the IP₃R1 undergoes important changes, including an increase in its concentration [6,7] and a change in its cellular distribution [6–8], which may bring about the enhanced IP₃R1 sensitivity, i.e. increased IP₃R1-mediated Ca²⁺ release, that is observed at the conclusion of maturation [4]. It is thought that these changes underlie the greater ability of eggs, compared to GV oocytes, to mount persistent [Ca²⁺]_i oscillations. Nevertheless, other cytoplasmic parameters are also optimized during oocyte maturation, although how they impact the egg's ability to mount oscillations is not well understood. For example, a significant increase in the [Ca²⁺]_{ER} [5] is observed during this period, and the ER itself undergoes a cellular redistribution [9–11] spreading from its area of concentration around the spindle at the MI stage to a more spread out distribution in MII eggs with conspicuous cortical clusters. Finally, changes in the activity of Ca²⁺ entry pathways may also be involved [12,13], although their identity and function remain unexplored.

Until now, only the effect of IP₃R1 concentrations has been thoroughly investigated and results show that this parameter has a role in optimizing Ca²⁺ release during oocyte maturation [14]. However, given the modest increase in IP₃R1 mass during maturation, this parameter alone can not completely explain the increased ability of eggs to release Ca²⁺ at the MII stage [14]. Hence, taking into account that [Ca²⁺]_i oscillations, IP₃R1 sensitivity, and ER cortical cluster for-

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mation are all maximal at the MII stage, and minimal at interphase [15], it is logical to propose that the same M-phase kinases that control the initiation and progression of meiosis [16,17] might also be regulating IP₃R1 function and Ca²⁺ release in eggs. Towards this end, we have previously reported that IP₃R1 is phosphorylated in a cell-cycle dependent manner at an MPM-2 epitope [18,19], which is commonly phosphorylated by M-phase kinases. This modification seemed to affect IP₃R1-mediated [Ca²⁺]_i oscillations [19]. Importantly, IP₃R1 becomes first phosphorylated at an MPM-2 epitope(s) at the beginning of maturation and it is almost maximal after 3 h [19,20], which coincides with the initial increase in IP₃R1 sensitivity observed during oocyte maturation [4,5].

Phosphorylation of different IP₃R isoforms by various kinases in somatic cells generally increases IP₃-induced Ca²⁺ release (IICR) [21,22]. However, most of the kinases known to phosphorylate IP₃Rs are not involved in cell cycle regulation and/or inhibition of their activity does not affect IP₃R1 function in eggs [23–25]. Recently, cyclin-dependent kinase 1 (CDK1), also known as maturation promoting factor (MPF), and extracellular signal-regulated kinase (ERK1/2), also known as mitogen activated protein kinase (MAPK), two M-phase kinases known to be involved in the regulation of oocyte maturation, have been shown to phosphorylate IP₃Rs [26–29]. Importantly, both kinases can phosphorylate MPM-2 epitopes on their substrates but whether they are responsible for the MPM-2 phosphorylation of IP₃R1 has not yet been elucidated. Interestingly, we found that inhibition of the MAPK pathway affects both IP₃R1 MPM-2 phosphorylation and the [Ca²⁺]_i-oscillating capability of the egg, which first suggested that MPM-2 phosphorylation of IP₃R1 might indeed increase IP₃R1 sensitivity during maturation [19]. Although MAPK is important to maintain IP₃R1 MPM-2 phosphorylation, its effects however become more evident towards the late stages of oocyte maturation and are possibly not associated with the initial MPM-2 phosphorylation of the receptor [20].

Another M-phase kinase that is known to phosphorylate MPM-2 epitopes is Polo-like kinase 1 (Plk1) [30]. Importantly for this study, Plk1 is also involved in the G2 to M phase transition in oocytes and is required for the completion of meiosis after fertilization [31,32]. In a previous study, we showed that Plk1 appeared directly responsible for the majority of IP₃R1 MPM-2 phosphorylation at the beginning of oocyte maturation [20] while MAPK regulated MPM-2 phosphorylation between MI and MII [19,20]. Therefore, our goals in this study were to investigate the functional effects of Plk1-mediated MPM-2 phosphorylation of IP₃R1 during oocyte maturation and to identify possible Plk1 phosphorylation site(s) on IP₃R1. Using the Plk1-specific inhibitor BI2536 we found that inhibition of Plk1 activity at the beginning of oocyte maturation diminished MPM-2 phosphorylation of IP₃R1. Moreover, BI2536-treated oocytes showed decreased IICR compared to control oocytes. Finally, we identified T²⁶⁵⁶ as a Plk1 phosphorylation site on IP₃R1.

2. Materials and methods

2.1. Reagents

All chemicals were purchased from Sigma, unless otherwise mentioned. Active His-Plk1 was purchased from Invitrogen Ltd. and radioactive ATP ([γ-³²P]ATP) from PerkinElmer. All peptides were synthesized in their amidated form and purified (>95%) by HPLC unless otherwise mentioned (Thermo Fisher Scientific GmbH). Peptides were diluted in DMSO.

2.2. Collection of oocytes and culture conditions

GV oocytes were collected from the ovaries of CD-1 female mice. Females were injected with 5 IU pregnant mare serum

gonadotropin (PMSG) and GV oocytes were recovered 42–46 h post-PMSG into HEPES-buffered Tyrode-Lactate solution (TL-HEPES) supplemented with 5% heat-treated fetal calf serum (FCS; Gibco) and 100 μM 3-isobutyl-1-methylxanthine (IBMX). GV oocytes were matured in Chatot, Ziomek, and Bavister (CZB) medium [33] containing 0.1% polyvinyl alcohol (PVA) at 36.5 °C under an atmosphere of 6% CO₂.

2.3. Antibodies

Immunological detection of IP₃R1 was carried out using the Rbt03 polyclonal antibody raised against C-terminal amino acids 2735–2749 of mouse IP₃R1 [34]. For simultaneous detection of IP₃R1 and IP₃R3, the panspecific antibody Rbt475 was used, the epitope of which (amino acids 127–141 of mouse IP₃R1) is conserved between isoforms and across species [35]. The anti-GST antibody (Zymed Laboratories) was used to detect the different GST-IP₃R1 domains. The MPM-2 monoclonal antibody (Upstate biotechnology) was used to ascertain IP₃R1 phosphorylation as previously reported [19]. Total Plk1 was detected by the use of a rabbit polyclonal antibody against human Plk1 (Cell Signaling Technology).

2.4. Preparation of pharmacological inhibitors

BI2536 (Axon Medchem), a recently discovered specific Plk inhibitor [36], was dissolved in DMSO (5 mM stock) and further diluted in CZB-PVA medium or phosphorylation buffer to its final concentration.

2.5. IP₃R1 GST constructs and mutagenesis

For domain analysis we expressed GST-fusion proteins corresponding to the various IP₃R1 domains that can be obtained by limited proteolysis [37] as described previously [19]. T²⁶⁵⁶ was mutated into an A by using pGEX6p2-IP₃R1 domain 6 as a template. Site-directed mutagenesis was performed using the Quick-Change point-mutation kit (Stratagene). Forward primers were designed according to the manufacturer's recommendation and reverse primers were the complementary sequence of the forward primers. This construct was sequenced to confirm mutation and frame. All GST-fusion proteins were purified as previously described [38].

2.6. In vitro phosphorylation of IP₃R1 and 3 by Plk1

Full-length mouse IP₃R1 and full-length rat IP₃R3 were expressed in Sf9 insect cells and microsomes were prepared as described previously [39]. Sf9 microsomes (2 μg) or GST-IP₃R1 domains (0.5 μg) were diluted in phosphorylation buffer (50 mM Hepes pH 7.5; 10 mM MgCl₂; 2.5 mM DTT; 0.01% Triton X-100; 200 μM ATP) supplemented with 20 μCi [γ-³²P]ATP. Reactions were performed at 30 °C for 1 h and initiated by the addition of 250 ng (for GST-IP₃R1 fragments) or 400 ng (for Sf9-microsomes) of the active His-Plk1 kinase (Invitrogen). Reactions were stopped by heating the samples for 10 min at 70 °C in 1× Laemmli sample buffer (LSB).

2.7. Immunoblotting

Cell lysates from 25 to 35 mouse oocytes were prepared by adding 5 μl 2× LSB and then boiling these for 10 min at 70 °C. Samples were loaded onto NuPAGE Novex 3–8% Tris-acetate gel (Invitrogen). After electrophoresis, proteins were transferred onto nitrocellulose membranes and probed with MPM-2 antibody (1/500). After stripping, the membranes were probed with Rbt03 antibody (1/1000). Membranes were developed, digitally captured

and quantified as described [19]. The intensity of the MPM-2 reactive band from GVBD oocytes was arbitrarily given the value of 1 and values in other lanes were expressed relative to this band.

After Plk1-mediated *in vitro* phosphorylation of GST-IP₃R1 domains or of Sf9-microsomes expressing IP₃Rs, proteins were loaded onto NuPAGE Novex 4–12% Bis-tris or 3–8% Tris-acetate (Invitrogen) gels, respectively. After electrophoresis, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes and incorporation of γ -³²P was determined using the Storm 840 PhosphorImager (Molecular Dynamics) as previously described [40]. Subsequently the blot was probed with anti-GST antibody (1/2000) or Rbt475 antibody (1/1000), respectively, to verify identical loading. After extensive washing, alkaline phosphatase-labeled anti-rabbit antibody was used as secondary antibody. The immunoreactivity was visualized by conversion of the Vistra™ ECF substrate into a fluorescent probe (Amersham) and scanned with the Storm 840 FluorImager, equipped with the Imagequant NT4.2 software (Molecular Dynamics). Quantification of the phosphorylation level was performed by comparing the radioactive signal of the GST-domain or full-size IP₃R to the total amount of, respectively, GST-domain or full-size IP₃R as estimated by Western blotting.

2.8. Fluorescence recordings and [Ca²⁺]_i determinations

To analyze the Ca²⁺-store content of the oocyte, oocytes were first loaded with Fura-2AM as previously described [41]. Oocytes were placed in Ca²⁺-free TL-Hepes with 1 mM EGTA for 30 min, after which they were treated with 10 μ M thapsigargin. Fluorescence recordings and [Ca²⁺]_i determinations were performed as described [19].

To analyze the IICR of the oocyte, caged IP₃ (0.25 mM) was microinjected into oocytes using the previously reported technique [19]. IP₃-injected oocytes were loaded with 1 mM Fluo-4AM (Molecular Probes) supplemented with 0.02% pluronic acid (Molecular Probes) for 20 min at room temperature. Fluo-4 (excitation at 480 nm and emission at 510 nm) was chosen since its excitation wavelength (480 nm) does not interfere with the uncaging of IP₃ (360 nm). [Ca²⁺]_i monitoring was performed in drops of Ca²⁺-free TL-HEPES using a Nikon Diaphot microscope fitted for fluorescence measurements. UV light to photolyze caged IP₃ was provided by a 75 W Xenon arc lamp and passed through a filter cube equipped with a 360/480 excitation filter. The emitted light above 510 nm was collected by a cooled Photometrics SenSys CCD camera (Roper Scientific). Fluo-4 fluorescence was obtained after 50 ms of UV exposure and the intensity was modulated using neutral density filters. [Ca²⁺]_i values were monitored using the software SimplePCI

(C-Imaging System), which controls the frequency and duration of photolysis.

2.9. Statistical analysis

Values from three or more experiments performed on different batches of oocytes were analyzed by one-way ANOVA followed by Fisher's protected least significant difference test using the STATVIEW (Abacus Concepts, Inc.) program. Differences were considered significant at $p < 0.05$. Values are given as means $\pm$ S.E.M.

3. Results

3.1. Plk1-mediated MPM-2 phosphorylation of IP₃R1 increases IICR in mouse oocytes

The effects of Plk1-mediated MPM-2 phosphorylation on IP₃R1 sensitivity were until now difficult to ascertain, at least in part, due to the lack of a specific Plk1 inhibitor. Thus, the recent finding that the small molecule BI2536 specifically inhibits Plks [36] has provided a new tool to probe the role of Plk1 on IICR. To first determine whether BI2536 was capable of inhibiting Plk1 activity in oocytes, we evaluated two commonly known consequences of Plk1 inhibition. Firstly, because inhibition of Plk1 activity delays GVBD [42,43], we examined the rate of GVBD in oocytes matured in medium supplemented with BI2536. Secondly, given that Plk1 plays an important role in the formation of the spindle poles, inhibition of Plk1 activity should compromise meiotic progression [44] and therefore BI2536-treated oocytes should also fail to extrude the 1st polar body (1PB) [42,43]. Accordingly, GV oocytes were matured in the presence of increasing concentrations of BI2536 and the time to GVBD and presence of 1PB were examined. Our results show that 100 nM or higher concentrations of BI2536 delayed GVBD for about 1 h, while lower concentrations were without effect on this parameter (Fig. 1A). Moreover, extrusion of the 1PB was abrogated by 100 nM BI2536 (Fig. 1B), while significant inhibition was already observed using 10 nM BI2536 (Fig. 1B). These results provide evidence that BI2536 is effectively inhibiting Plk1 activity in oocytes.

Subsequently, we tested whether inhibition of Plk1 activity by BI2536 reduced IP₃R1 MPM-2 phosphorylation at the GVBD stage. As expected, BI2536 decreased MPM-2 reactivity of IP₃R1 in a dose-dependent manner (Fig. 2), although 1 and 10 μ M BI2536 produced nearly comparable suppression of MPM-2 phosphorylation ($p < 0.015$). Importantly, even at the highest BI2536 concentration, ~40% of MPM-2 reactivity remained present (Fig. 2), suggesting that

Fig. 1. Inactivation of Plk1 by BI2536 delays germinal vesicle breakdown (GVBD) and inhibits extrusion of the first polar body (1PB) in mouse oocytes. (A) Percentage (%) of oocytes, which are matured in medium supplemented without or with the indicated concentrations of BI2536, that underwent germinal vesicle breakdown (GVBD) at different times of *in vitro* maturation. (B) Percentage (%) of oocytes that extruded their first polar body (1PB) after 12–15 h of *in vitro* maturation in medium containing 0 nM (control), 1 nM, 10 nM or 100 nM BI2536.

Fig. 2. Inhibition of Plk1 activity by BI2536 decreases IP₃R1 MPM-2 reactivity in GVBD oocytes. (A) Immunoblotting of oocyte lysates collected at 0 h (GV) or 2 h (GVBD) and treated with the indicated concentrations of BI2536 (BI). The blot was probed with an MPM-2 antibody and after stripping re-probed with an anti-IP₃R1 (Rbt03) antibody. (B) Quantification of IP₃R1 MPM-2 reactivity. The intensity of the MPM-2 reactive band from GVBD oocytes was arbitrarily given the value of 1 and values in the other lanes were expressed relative to this band (** *p* < 0.015).

another kinase may also be phosphorylating IP₃R1 at an MPM-2 epitope at this stage.

As the addition of BI2536 effectively suppressed Plk1 activity and decreased IP₃R1 MPM-2 reactivity in oocytes, we used this inhibitor to investigate how MPM-2 reactivity affected IP₃R1 sensitivity. We therefore compared IICR in GVBD oocytes matured for 2 h in medium supplemented with and without 10 μM BI2536. If Plk1 phosphorylation of IP₃R1 plays a role in receptor sensitivity, we would expect Ca²⁺ release in BI2536-treated GVBD oocytes to be smaller than in control GVBD oocytes. For this, GV and GVBD

oocytes were injected with caged IP₃ and IICR was measured by recording the Fluo-4 fluorescence signal obtained after uncaging IP₃ with a UV flash (Fig. 3A–D). In agreement with our hypothesis, BI2536 greatly reduced the number of GVBD oocytes (from 78% without treatment to 30% after treatment with BI2536) capable of responding to IP₃ release with a Ca²⁺ rise (Fig. 3D; *p* < 0.03). In the BI2536-treated GVBD oocytes still capable of releasing Ca²⁺, the duration of this release was 3 times shorter in BI2536-treated GVBD oocytes than in control GVBD oocytes (*p* < 0.03) while the amplitude was not significantly altered (Fig. 3A–C). For comparison the data

Fig. 3. BI2536 reduces the increased IP₃-induced Ca²⁺ release (IICR) in GVBD oocytes but does not affect the Ca²⁺-store content. The duration (A) and amplitude (B) of the Ca²⁺ rise was measured with Fluo-4 after releasing inositol 1,4,5-trisphosphate (IP₃) by a UV-flash in GV and GVBD oocytes ± 10 μM BI2536. (C) A typical trace of IICR in GV, GVBD and GVBD oocytes treated with 10 μM BI2536. (D) The number of GV, GVBD and GVBD oocytes treated with 10 μM BI2536 that respond to IP₃ with a Ca²⁺ rise. (E) The content of the Ca²⁺ stores in GV and GVBD oocytes ± 10 μM BI2536 was estimated by measuring the area under the curve of the Ca²⁺ response induced by the addition of thapsigargin (10 μM) in Ca²⁺-free media (* *p* < 0.03; ** *p* < 0.015).

Table 1
Plk1 phosphorylation consensus sequences in IP₃Rs.

	T ¹⁰⁴⁸	S ¹⁴⁹²	S ¹⁷⁹⁰	T ²⁶⁵⁶
IP ₃ R1 (mouse)	SEENTP-LDL	TFFSSPFSDQ	RGEMSLAEVQ	STEYTGPESEY
IP ₃ R1 (human)	SEENTP-LDL	TFFSSPFSDQ	RGEMSLAEVQ	STEYTGPESEY
IP ₃ R1 (rat)	SEENTP-LDL	TFFSSPFSDQ	RGEMSLAEVQ	STEYTGPESEY
IP ₃ R1 (chicken)	RKENTP-LDL	TFFSSPFSDQ	RGEMSLADVQ	STEYTGPESEY
IP ₃ R1 (<i>Xenopus</i>)	SEENTP-LDL	TFFSSPFSDQ	RSEMSLSDVQ	STEYTGPESEY
IP ₃ R (<i>Drosophila</i>)	ERSDAANLDL	SFFASPFSDQ	RAGKTLHEMQ	PTEFTGPESEY
IP ₃ R (<i>C. elegans</i>)	YRSSGHELHL	KFFEAPYSAL	SQERDLIAIQ	ETEFTGPESEY
IP ₃ R2 (rat)	GKTSSM-LEV	GFFNSPFSDN	PDWSAIAATQ	PTEYTGPESEY
IP ₃ R2 (human)	GKTSSM-LEV	GFFNSPFSDN	QDWSAIAATQ	PTEYTGPESEY
IP ₃ R3 (rat)	RKEKTP-VQL	AFSSPFSEN	TG-ISMSDIQ	KTDYTGPESEY
IP ₃ R3 (human)	RKEKNP-VQL	AFSSPFSEN	MG-ISMSDIQ	KTDYTGPESEY

The sequences of IP₃R1, IP₃R2 and IP₃R3 of various species were examined for the presence of Plk1 consensus sites. Four highly conserved Plk1 consensus sites were found in IP₃R1, centered around the phosphorylatable amino acids T¹⁰⁴⁸, S¹⁴⁹², S¹⁷⁹⁰ and T²⁶⁵⁶ of mouse IP₃R1. Only the sequences around S¹⁴⁹² and T²⁶⁵⁶ were conserved in IP₃R2 and IP₃R3. See text for more information. The amino acids indicated in bold are the amino acids checked for agreement and the underlined amino acids are those in agreement with the published Plk1 consensus sequences.

for GV oocytes are also shown (Fig. 3A, B, C). Given that there is a marked increase in the Ca²⁺ content of the ER during the transition from GV to GVBD (Fig. 3E, $p < 0.015$), which in turn could affect the sensitivity of IP₃R1, it was important to determine that BI2536 was not affecting this parameter and thereby inhibiting IICR via an indirect mechanism. Thus, we assessed whether the [Ca²⁺]_i content of the ER, as evaluated by the [Ca²⁺]_i response induced by thapsigargin (TG), was influenced by the BI2536 treatment. Our results show that BI2536 does not significantly affect Ca²⁺ release by TG (Fig. 3E). Moreover, there was no difference in the duration of the TG-induced Ca²⁺ transient between control and BI2536-treated GVBD oocytes (data not shown) suggesting that no major changes occur in Ca²⁺ extrusion or Ca²⁺ buffering mechanisms after BI2536 treatment. We can conclude from these data that BI2536 reduces IICR through inhibition of Plk1-mediated MPM-2 phosphorylation of IP₃R1. These results demonstrate that BI2536 effectively inhibits Plk1 activity in oocytes and by doing so decreases MPM-2 phosphorylation of IP₃R1. Importantly, Plk1-mediated MPM-2 phosphorylation increases the sensitivity of IP₃R1 at the beginning of oocyte maturation. This is the first study demonstrating that phosphorylation of IP₃R1 in oocytes plays an important role in the optimization of the egg's Ca²⁺-releasing capability.

3.2. In silico prediction of possible Plk1 consensus sites in IP₃R1

In a previous study [20] we identified Plk1 as a major MPM-2 generating kinase of IP₃R1 during the early stages of maturation, although the exact Plk1 phosphorylation site(s) in the receptor was not established. Therefore, we first searched *in silico* for possible Plk1 phosphorylation consensus sequences in IP₃R1. Based on the most recently published Plk1 consensus sequence consisting of X-φ/E-E/D-E/D/S-S/T⁺-φ-S/φ-S/E/D-E residues [45], we found only one potential site centered on S¹⁴⁹² of IP₃R1 (Table 1). However, when using the earlier, simpler version of Plk1 consensus sequence that consists of E/D-X-S/T⁺-φ-X-D/E [46], three additional sites centered on amino acids T¹⁰⁴⁸, S¹⁷⁹⁰ and T²⁶⁵⁶ were found in IP₃R1 (Table 1). Importantly, only the consensus sequences around S¹⁴⁹² and around T²⁶⁵⁶ are conserved in all IP₃R isoforms and resemble possible MPM-2 consensus sites [47].

3.3. In vitro phosphorylation of IP₃R1 and IP₃R3

In vitro phosphorylation techniques were next used to identify Plk1-dependent phosphorylation sites on IP₃R1. To check whether Plk1-mediated phosphorylation is conserved over the various isoforms, we subjected both IP₃R1 and IP₃R3 to *in vitro* phosphorylation. Microsomes of Sf9 insect cells overexpressing IP₃R1 or IP₃R3 were diluted in phosphorylation buffer containing

[γ-³²P]ATP (20 μCi) and incubated in the presence of active Plk1 for 1 h at 30 °C. Both IP₃R1 and IP₃R3 were *in vitro* phosphorylated by Plk1 (Fig. 4A, upper panel), and phosphorylation was quantified and normalized by comparing phosphorylation levels relative to the total amount of IP₃R as detected with the polyclonal antibody Rbt475 (Fig. 4A, lower panel) that recognizes both isoforms equally well [35]. IP₃R3 showed a 2.47 ± 0.07 ($n = 3$) stronger phosphorylation than IP₃R1, confirming that Plk1 can phosphorylate different IP₃R isoforms and suggesting that isoform-dependent differences can occur. To further confirm that the phosphorylation was indeed due to the activity of Plk1, the specific Plk inhibitor BI2536 was added to the *in vitro* reactions. The addition of BI2536 (10 μM) decreased Plk1-mediated phosphorylation of both IP₃R isoforms by ~90%, confirming that γ-³²P incorporation was due to Plk1 activity (Fig. 4B).

To narrow down the possible Plk1 phosphorylation sites on the full-size IP₃R1, we investigated the phosphorylation properties of IP₃R1 fragments. To preserve the most natural conformation that those fragments adopt in the full-size IP₃R1, domains corresponding to IP₃R1 fragments obtained after limited proteolysis were used

Fig. 4. Plk1 phosphorylates full-size IP₃R1 and IP₃R3. (A) Sf9 microsomes expressing full-length IP₃R1 and IP₃R3 were incubated with or without (negative control) Plk1 in the presence of [γ-³²P]ATP for 1 h at 30 °C. (B) Plk1-mediated phosphorylation of Sf9 microsomes ± 10 μM BI2536 (BI). Phosphorylated IP₃Rs were detected using phosphorimaging ([γ-³²P]ATP) and loading of IP₃Rs was determined with an anti-IP₃R antibody (Rbt475) that recognizes both isoforms equally well. All results are typical of at least three experiments.

Fig. 5. Plk1-mediated phosphorylation of domains 1–6 of IP₃R1. Phosphorylation of the IP₃R1 domains expressed as GST-fusion proteins was performed as in Fig. 4. Phosphorylated IP₃R1 domains were detected using phosphorimaging ([γ -³²P]ATP) (left panel) and loading of the IP₃R1 domains was determined with an anti-GST antibody (right panel). The arrow indicates the position of Plk1 (autophosphorylated) and arrowheads indicate the position of the various IP₃R1-domains: domain 1, a.a. 1–345, domain 2, a.a. 346–922, domain 3, a.a. 923–1581, domain 4, a.a. 1582–1931, domain 5, a.a. 1932–2216, domain 6, a.a. 2590–2749. Result typical of at least three experiments.

[19,37,48]. After the expression and purification of those domains as GST-fusion proteins, each IP₃R1 domain was subjected to *in vitro* phosphorylation by Plk1 in the presence of [γ -³²P]ATP (20 μ Ci). The results show that domain 1 (a.a. 1–345) and domain 6 (a.a. 2590–2749) were most strongly phosphorylated by Plk1 (Fig. 5, left panel). The low level of phosphorylation observed in some of the other domains (2, 3 and 5) did not differ much from the γ -³²P incorporation observed for GST alone (negative control). Equal loading of the different GST-domains was confirmed by an anti-GST antibody (Fig. 5, right panel).

Together, those results suggest that Plk1 phosphorylation sites are most probably located in domain 1 and/or domain 6 of IP₃R1, and that they may be conserved in IP₃R3. Taking into account the *in silico* predictions, T²⁶⁵⁶ is the best candidate for phosphorylation by Plk1, as domain 6 is strongly phosphorylated and this site is conserved in IP₃R3. It is noteworthy that a recognizable consensus site for Plk1 could not be found in domain 1 of IP₃R1.

3.4. Identification of T²⁶⁵⁶ in IP₃R1 as a Plk1 phosphorylation site

To further investigate whether T²⁶⁵⁶ is indeed the main site on IP₃R1 phosphorylated *in vitro* by Plk1, we first synthesized a peptide of 20 a.a. mimicking the sequence surrounding T²⁶⁵⁶ of IP₃R1 (referred to as wild type (wt) peptide; NH₂-FIVLVKVKDSTEYTT²⁶⁵⁶GPESYV-CONH₂). Different concentrations of wt peptide were incubated for 1 h at 30 °C with GST-domain 6 in phosphorylation buffer containing [γ -³²P]ATP and active Plk1. If T²⁶⁵⁶ is the main phosphorylation site in domain 6, the addition of increasing amounts of wt peptide into the phosphorylation reaction should compete in a dose-dependent manner with the phosphorylation site in the domain. In agreement herewith, 333 μ M wt peptide inhibited the phosphorylation of domain 6 by 74 $\pm$ 3% (mean $\pm$ S.E.M., *n* = 4) as measured by the incorporation of [γ -³²P]ATP (Fig. 6A). Moreover, the wt peptide itself appeared to become phosphorylated by Plk1. This result suggests that T²⁶⁵⁶ is,

Fig. 6. wt peptide decreases Plk1-mediated phosphorylation of domain 6, and of full-size IP₃R1 *in vitro*. Plk1-mediated *in vitro* phosphorylation of (A) domain 6 + wild type (wt) peptide (left panel), domain 6 + wt peptide or control (ctr) peptide (right panel) at the indicated concentrations. After separation by SDS-polyacrylamide electrophoresis on a 4–12% Bis-Tris gel, phosphorylated domain 6 (upper panels) and wt peptide (lower panels) were detected using a phosphorimager ([γ -³²P]ATP) and loading of domain 6 (middle panels) was determined with an anti-GST antibody. (B) Plk1-mediated *in vitro* phosphorylation of Sf9-microsomes expressing IP₃R1 in the presence of the indicated concentrations of wt or ctr peptide. Phosphorylated IP₃R1s were detected using phosphorimaging (upper panel) and loading of IP₃R1s and Plk1 were determined with an anti-IP₃R1 antibody (Rbt475) and anti-Plk1 antibody, respectively. All results are typical of at least three experiments.

Fig. 7. Identification of T²⁶⁵⁶ as an *in vitro* Plk1 phosphorylation site in domain 6 of IP₃R1. Plk1-mediated *in vitro* phosphorylation of wild type (wt) domain 6 and T^{2656A} domain 6. Phosphorylated domain 6 was detected using phosphorimaging ([γ -³²P]ATP) and loading of domain 6 was determined with an anti-GST antibody. The result is typical of at least three experiments.

at least *in vitro*, a strong target for Plk1 on domain 6. A control peptide (referred to as ctr peptide; NH₂-SIVQTSFPMFLSVDSTSTF-CONH₂) at the same concentration as the wt peptide was however completely ineffective in lowering the phosphorylation of domain 6, demonstrating the specificity of the effect of the wt peptide (Fig. 6A, right panel). Equal loading of the different GST-domains was confirmed by an anti-GST antibody (Fig. 6A).

To extend this finding to the full-size IP₃R1, the wt peptide (333 μ M) was incubated with Sf9 microsomes overexpressing IP₃R1, in the presence of [γ -³²P]ATP and active Plk1. The addition of wt peptide, unlike ctr peptide, here also strongly (>90%) inhibited the incorporation of γ -³²P in the full-size IP₃R1 (Fig. 6B), strongly suggesting that T²⁶⁵⁶ is an effective Plk1 phosphorylation site. Equal loading of the IP₃Rs and Plk1 was confirmed by an anti-IP₃R (Rbt475) and anti-Plk1 antibody, respectively (Fig. 6B).

The previous data indicate that the wt peptide is a good substrate for Plk1. However, besides T²⁶⁵⁶, the wt peptide contains two other serines and threonines that also might form targets for Plk1 phosphorylation and/or binding. Therefore, in a subsequent experiment, we mutated T²⁶⁵⁶ in GST-domain 6 into an alanine, and investigated whether Plk1 could still phosphorylate it. After incubation under conditions that allow for Plk1-dependent phosphorylation, much less [γ -³²P] was incorporated in GST-domain 6 T^{2656A} (35 $\pm$ 4.3%, mean $\pm$ S.E.M., *n* = 4) as compared to wild type GST-domain 6 (Fig. 7). This result implies that T²⁶⁵⁶ is indeed the major Plk1 phosphorylation site in domain 6 of IP₃R1.

4. Discussion

Mammalian egg activation is achieved following sperm entry and the subsequent initiation of [Ca²⁺]_i oscillations in the egg [1,49]. To be able to induce this signal, the Ca²⁺ machinery of the egg in general, and IP₃R1 in particular, have to be optimized during oocyte maturation [4,5,50]. In somatic cells, one possible mechanism to increase IP₃R1 sensitivity is through phosphorylation [21], and protein phosphorylation plays a key role in regulating oocyte maturation and meiosis resumption [16,43,51]. In line with this, we have shown that during oocyte maturation, IP₃R1 becomes phosphorylated at an MPM-2 epitope [18–20], which is commonly the target of M-phase kinases [52]. Importantly, while we have shown that Plk1 is directly involved in this MPM-2 phosphorylation of IP₃R1 [20], it was not yet known whether this modification affects IP₃R1 sensitivity. Accordingly, in this study we examined whether the Plk1-mediated MPM-2 phosphorylation of IP₃R1 underlies the increased sensitivity of IP₃R1 observed during oocyte maturation. Our results show that the specific Plk inhibitor BI2536 suppresses Plk1 activity in oocytes and consequently decreases IP₃R1 MPM-2 reactivity. We also found that inhibition of MPM-2 reactivity coincided with decreased IICR in GVBD oocytes, indicating that Plk1-mediated MPM-2 phosphorylation of IP₃R1 might be one of the mechanisms increasing IP₃R1 sensitivity at the beginning of oocyte maturation.

Using *in vitro* assays, we suggest that T²⁶⁵⁶ is the preferred phosphorylation site for Plk1 in IP₃R1.

4.1. BI2536, a specific Plk inhibitor, inhibits Plk1 activity and decreases the MPM-2 reactivity of the IP₃R1 at the early stages of oocyte maturation

Until recently, specific Plk1 inhibitors were not available and, instead, compounds such as wortmannin and doxorubicin, which have a broad range of targets, were routinely used to block Plk1. Therefore, to confirm our previous findings that Plk1 is responsible for the MPM-2 reactivity of the IP₃R1 observed during the beginning of oocyte maturation, the Plk-specific inhibitor BI2536 [36] was used. Since Plk1 is the only Plk isoform active at the G2/M transition [53], which corresponds with the beginning of oocyte maturation, this inhibitor is ideally suited to study the effects of Plk1 in oocytes. Exposure of somatic cells to BI2536 produces the same effects on mitotic progression than Plk1 RNAi: a delay into prophase and a spindle-assembly-checkpoint-induced arrest in prometaphase [44]. Other studies using mammalian oocytes showed that inhibition of Plk1 activity resulted in delayed GVBD (corresponding with delayed prophase) and absence of 1PB extrusion (indicating a spindle-assembly-checkpoint arrest) [42,43]. Here we found that 100 nM BI2536 was sufficient for delaying GVBD and blocking 1PB extrusion, suggesting that Plk1 was effectively inhibited during oocyte maturation.

After demonstrating the effectiveness of BI2536 in oocytes, we used this compound to inhibit the Plk1-mediated MPM-2 reactivity of IP₃R1 in oocytes undergoing GVBD. BI2536 decreased MPM-2 reactivity in a dose-dependent manner and 10 μ M BI2536 inhibited it maximally, confirming our previous finding that Plk1 is the major MPM-2 generating kinase at the beginning of oocyte maturation [20]. Although 10 μ M BI2536 completely inhibited Plk1-mediated phosphorylation of IP₃Rs *in vitro*, it did not completely inhibit MPM-2 reactivity *in vivo*, suggesting the presence of another MPM-2 generating kinase, such as CDK1 or MAPK. We have already demonstrated that the latter kinase is not responsible for MPM-2 reactivity of IP₃R1 at the beginning of oocyte maturation [20], and we therefore can safely exclude it as a candidate kinase at this stage. Importantly, in our previous study, we found that initiation of maturation by a brief pulse of okadaic acid in the presence of IBMX, which largely precludes the activation of CDK1, reduces MPM-2 reactivity of IP₃R1 in MI oocytes by 30% compared to controls [20], raising the possibility that CDK1 may also function as an MPM-2 generating kinase of IP₃R1 in oocytes.

4.2. Plk1-mediated MPM-2 phosphorylation of IP₃R1 increases the sensitivity of IP₃R1 at the GVBD stage

During the early stages of oocyte maturation, which encompasses the first 4 h, there is an increase in the sensitivity of IP₃R1 that is manifested by an increase in Ca²⁺ release [4]; this increase correlates with the appearance of Plk1-mediated MPM-2 phosphorylation of IP₃R1 [20]. Our results show that 10 μ M of BI2536 drastically decreased the MPM-2 reactivity of IP₃R1. Therefore, under the same conditions the sensitivity of IP₃R1 was examined by uncaging IP₃ in the oocyte. Releasing the same amount of IP₃ into BI2536-treated GVBD oocytes resulted in fewer showing a [Ca²⁺]_i rise and those that did, exhibited [Ca²⁺]_i responses with reduced duration compared to control GVBD oocytes. In our study, the Ca²⁺ store content was not affected by the use of BI2536, suggesting that the decrease in MPM-2 phosphorylation was responsible for the observed decrease in IP₃R1 sensitivity.

4.3. T²⁶⁵⁶ is *in vitro* a major Plk1 site of IP₃R1

To elucidate the mechanism by which Plk1-mediated IP₃R1 MPM-2 phosphorylation affects IP₃R1 sensitivity, it is important to identify the possible Plk1 phosphorylation site(s) in the receptor. Four possible consensus sites (T¹⁰⁴⁸, S¹⁴⁹², S¹⁷⁹⁰ and T²⁶⁵⁶) are present in full-size IP₃R1 but only T²⁶⁵⁶, localized in the C-terminal part of IP₃R1, was unequivocally identified *in vitro* as a Plk1 phosphorylation site. Mutation of T^{2656A} in domain 6 significantly decreased *in vitro* phosphorylation of this domain while adding a wt peptide encompassing this site almost abolished the phosphorylation of full-size IP₃R1. These results however do not rule out the possibility that other Plk1 sites might exist in IP₃R1. In this regard, domain 1 of IP₃R1 was strongly phosphorylated *in vitro* by Plk1, although it did not contain a recognized Plk1 consensus motif. A tempting hypothesis is that this site represents a Plk1 docking site. Plk1 docking sites consist of S-S/T*-P/X where the S/T* has to be phosphorylated before Plk1 can bind [54]. In line with this, domain 1 has one site resembling such a docking site (S-S²⁷⁸-K). Moreover, it is known that Plk1 can phosphorylate its own docking sites [55], and our previous study suggested that in oocytes Plk1 could indeed be capable of self priming to phosphorylate IP₃R1 [20]. Additional experiments will be needed to check this hypothesis.

It is not yet known how Plk1-mediated phosphorylation regulates IP₃R1 sensitivity. It is possible that T²⁶⁵⁶ phosphorylation affects the gating mechanism of the receptor [56]. This hypothesis is particularly interesting because the C-terminus, also called the gate-keeper, plays an important role in determining the activity of the receptor [57,58]. Moreover, several proteins have already been identified that bind the C-terminus of the receptor and influence the conductivity of the receptor [59–61]. Alternatively, it was reported that phosphorylation by some kinases can increase IP₃R affinity for IP₃ or, in some cases, determine the exact subcellular localization of IP₃R1 [21]. Phosphorylation at T²⁶⁵⁶ potentially can affect IP₃R sensitivity by each of these three pathways, alone or in combination.

Based on the above results, we therefore suggest that Plk1 is involved in the initial MPM-2 IP₃R1 phosphorylation and as a result it increases the sensitivity of IP₃R1 during early oocyte maturation. Moreover, our results suggest that Plk1 acts by phosphorylating T²⁶⁵⁶ in the C-terminal region of IP₃R1. Importantly, we cannot discount that phosphorylation of other sites in the receptor, and possibly by other kinases, may contribute to MPM-2 reactivity and further enhance IP₃R1 sensitivity under *in vivo* conditions. This study is the first to reveal that phosphorylation of IP₃R1 is involved in increasing the sensitivity of IP₃R1 during oocyte maturation thereby contributing to prepare the oocyte for fertilization.

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