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During Male Pronuclei Formation Chromatin Remodeling Is Uncoupled From Nucleus Decondensation

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Abstract Male pronucleus formation involves sperm nucleus decondensation and sperm chromatin remodeling. In sea urchins, male pronucleus decondensation was shown to be modulated by protein kinase C and a cdc2-like kinase sensitive to olomoucine in vitro assays. It was further demonstrated that olomoucine blocks SpH2B and SpH1 phosphorylation. These phosphorylations were postulated to participate in the initial steps of male chromatin remodeling during male pronucleus formation. At final steps of male chromatin remodeling, all sperm histones (SpH) disappear from male chromatin and are subsequently degraded by a cysteine protease. As a result of this remodeling, the SpH are replaced by maternal histone variants (CS). To define if sperm nucleus decondensation is coupled with sperm chromatin remodeling, we have followed the loss of SpH in zygotes treated with olomoucine. SpH degradation was followed with anti-SpH antibodies that had no cross-reactivity with CS histone variants. We found that olomoucine blocks SpH1 and SpH2B phosphorylation and inhibits male pronucleus decondensation in vivo. Interestingly, the normal schedule of SpH degradation remains unaltered in the presence of olomoucine. Taken together these results, it was concluded that male nucleus decondensation is uncoupled from the degradation of SpH associated to male chromatin remodeling. From these results, it also emerges that the phosphorylation of SpH2B and SpH1 is not required for the degradation of the SpH that is concurrent to male chromatin remodeling. J. Cell. Biochem. 96: 235–241, 2005.

Key words: fertilization; chromatin remodeling; sperm-histones degradation; sperm-histones phosphorylation; sea urchin

In most vertebrates, eggs are fertilized in a non-haploid stage, so male pronucleus development occurs together with the resumption of meiosis. Since sea urchins eggs reach their haploid state before insemination this specie is especially suitable to investigate the events leading to male pronucleus formation without the potential interference of those related to the resumption of meiosis. In sea urchins, male pronucleus decondensation and sperm chromatin remodeling are contemporaneous and both events are required for a successful re-establishment of the diploid condition of the embryo.

In vivo studies using polyspermic fertilized eggs have shown that phosphorylation in multiple SPKK sites located in the extended N terminal regions of SpH2B and SpH1 occurs shortly after fertilization [Poccia and Green, 1992; reviewed by Green, 2001]. It was further postulated that the phosphorylation of these sperm histones (SpH) is catalyzed by a cdc-2 like protein kinase [Stephens et al., 2002]. More recently, these phosphorylation events associated to male pronucleus formation, visualized as changes in male pronucleus shape, has been elucidated by in vitro assays. It was shown that two protein kinases, a cdc2-like kinase and protein kinase C, act synergistically to decondense sperm nuclei after fertilization [Stephens et al., 2002]. In these assays, the cdc2-like kinase activity was blocked by olomoucine, an inhibitor of protein kinases exhibiting higher preference for Cdk1 (cdc2), Cdk2, and Cdk5

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than for Cdk4 and Cdk6 [reviewed by Meijer, 1996]. Histone H1 phosphorylation at SPKK sites by Cdk-like kinases has also been reported [Swank et al., 1997].

Contemporaneously to sperm nucleus decondensation, a complete remodeling of sperm chromatin occurs. During this remodeling, SpH are lost from chromatin and replaced by cleavage stage histone variants (CS) that are recruited from maternal stores [Imschenetzky et al., 1991; Poccia and Green, 1992]. By analyzing the composition of chromatin from zygotes harvested at different times after fertilization, we have determined that there is a specific sequence of histone exchange. Initially, SpH3-SpH4 tetramer is removed, then SpH2A-SpH2B dimers and SpH1 are lost from male chromatin at final stages of chromatin remodeling that occurs at the time of the fusion of both pronucleus [Imschenetzky et al., 1991]. Consistent with this schedule of events, we have isolated hybrid nucleosome cores at intermediate steps of male chromatin remodeling that are organized by phosphorylayted SpH2A-SpH2B dimers and a subset of poly(ADP-ribosylated) CS histone variants [Oliver et al., 2002]. After the fusion of both pronucleus, the chromatin was found to be formed by poly(ADP-ribosylated) CS histone variants [Imschenetzky et al., 1996]. Furthermore, we have identified a nuclear cysteine-protease that degrades the SpH leaving the maternal CS histones unaffected [Imschenetzky et al., 1997]. The activity of this enzyme is modulated by post-translational modifications of its substrates, phosphorylation protects SpH1 and SpH2B from degradation [Morin et al., 1999a], and poly(ADP-ribosylation) blocks the proteolysis of CS histone variants [Morin et al., 1999b]. It is unknown yet if this protease may catalyze the degradation of the SpH while these histones are organized as nucleosome cores, or if the SpH should be removed before degradation. In this context, the disappearance of the SpH3-SpH4 tetramer before the SpH2A-SpH2B dimers from male chromatin argues in favor of the potential participation of histone chaperons or of a yet undefined chromatin-remodeling complex in the removal of the SpH prior to their proteolysis. This protease was found as an inactive precursor in unfertilized eggs and shown to be activated and mobilized into male pronucleus after fertilization. The localization of this protease into male pronucleus further supports its role in the SpH degradation concomitant to male chromatin remodeling [reviewed by Imschenetzky et al., 2003; Concha et al., 2005].

It is unclear if the protein-phosphorylation events participating in male pronucleus decondensation are required for the degradation of SpH associated to male chromatin remodeling after fertilization. To investigate this issue, we perturbed the normal male pronucleus decondensation with olomoucine and followed SpH disappearance from male chromatin. We have also analyzed in vivo the effect of this protein-kinase inhibitor on the phosphorylation of SpH1 and SpH2B.

MATERIALS AND METHODS

Chemicals

Olomoucine and E-64d were purchased from Sigma Chemical Company (St. Louis, MO). In DMSO, 100 mM olomoucine and 30 mM E-64d stock solutions were prepared. These solutions were stored at -20°C.

Gametes and Embryos

Gametes were collected from the sea urchin *Tetrapygus niger*, insemination was performed and development took place in sea water at room temperature under constant aeration as described previously [Imschenetzky et al., 1991]. Zygotes were harvested at different times post insemination (p.i.) and processed as indicated in each experiment. In those experiments performed with the protein kinases inhibitor, unfertilized eggs were pre-incubated for 30 min at room temperature in sea water containing olomoucine (0.5 mM). To demonstrate that the lack of detection of SpH represents the disappearance of these proteins from the zygotes, we have included a control assay in which the SpH degradation was blocked by adding E-64d (150 μM) to the zygotes culture 30 min pre-insemination. As shown previously, this inhibitor targets a cysteine-protease that degrades the SpH after fertilization [Imschenetzky et al., 1997].

Microscopy

To visualize that the male and female pronuclei zygotes were harvested at different times after insemination and stained with 1 μg/ml DAPI dye (4′,6′ diamidino-2-phenylindole). Fluorescent signals were observed by epifluorescence microscope Nikon Eclipse E 600.

Phosphorylation of SpH

SpH phosphorylation after fertilization was determined as follows: unfertilized eggs were pre-incubated for 60 min at room temperature in sea water containing 20 μCi/ml of [^{32}P]-ortophosphate neutralized with NaOH 0.1 M. At 30 min before fertilization, 0.5 mM Olomoucine was added to the unfertilized eggs and then the zygotes were harvested 10 min after fertilization. Total histones were isolated and analyzed by PAGE/SDS electrophoresis as described below. After electrophoresis, the gels were stained with Coomassie blue, dried and the radioactivity associated to each protein band was determined by autoradiography on a Imaging Screen K analyzed in a Molecular Imager FX (Bio Rad).

Western Blots of SpH

Chromatin was purified from sea urchin zygotes, and histones were obtained as described previously [Imschenetzky et al., 1991]. Histones were separated by electrophoresis in one-dimensional 18% (w/v) polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS–PAGE), transferred to nitrocellulose membranes, and immuno-detected by Western blots revealed with polyclonal antibodies obtained against total histones isolated from sperms as described previously. As reported, these antibodies exhibited a slight cross-reactivity with maternally inherited CS histone variants that migrate in the region of the PAGE/SDS corresponding to SpH2A [Imschenetzky et al., 1991]. To avoid this problem, the IgG fraction was further purified on an affinity column performed with total CS histone variants isolated from unfertilized eggs. To perform the affinity column, the CS histone variants were covalently bound to Sepharose 4B as described by Tijssen [1988]. This column was used to retain the IgG fraction that was cross-reactive with the CS variants. The detection was performed using a chemiluminescent ECL system (Amersham Pharmacia Biotech, UK).

RESULTS

Male Pronucleus Decondensation and SpH Phosphorylation

Male pronucleus decondensation was visualized as a change of shape of the sperm nucleus in zygotes harvested at different times post-insemination (p.i.). Initially, the conical and condensed sperm nucleus changes to an ovoid shape that defines the intermediate stages of decondensation and may be observed at 10 min p.i. Finally, an almost spherical and fully decondensed sperm nucleus becomes visible that fuses with the female pronucleus at 30 min p.i. To visualize in vivo male pronucleus decondensation, the zygotes were harvested at an intermediate stage of male pronucleus formation (10 min p.i.) and at the time of the fusion of both pronuclei (30 min p.i.) and stained with DAPI as described in Materials and Methods. We have determined the dose of the inhibitor that affects male pronucleus decondensation in vivo. As shown in Figure 1, the changes of nuclear shape that defines male pronucleus decondensation observed in normal zygotes (Fig. 1A) remained almost unaffected in the presence of 0.1 mM of olomoucine (Fig. 1B). Among the different concentrations tested, we found that the normal swelling and spherical shape of male pronucleus were not attained when the zygotes were incubated in 0.5 mM of olomoucine (Fig. 1C). From these observations, it emerges that olomoucine inhibits male pronucleus decondensation in vivo, although the migration of male pronucleus towards the female pronucleus remains unaffected (Fig. 1C, plate 30 min).

We have also analyzed the effect in vivo of 0.5 mM olomoucine on the phosphorylation of SpH1 and SpH2B. As described above, this concentration of inhibitor was able to prevent male pronucleus decondensation. Zygotes were cultured in sea water containing [^{32}P]-orthophosphate. At 10 min p.i., the zygotes were harvested, and whole histones were isolated from the chromatin and analyzed by autoradiography as described in Materials and Methods. As shown, the phosphorylation of SpH SpH1 and SpH2B shown in control zygotes (Fig. 2, lane 1) was abolished when the zygotes were treated with olomoucine (Fig. 2, lane 2). These results are consistent with previous reports indicating that SpH1 and SpH2B become phosphorylated after fertilization [Green and Poccia, 1985] and further support in vivo, the role of an olomoucine sensitive protein-kinase in these post-translational modifications. This role was postulated previously by Stephens et al. [2002], based on in vitro assays.

SpH Degradation After Fertilization

The loss of SpH after fertilization was followed by analyzing the histones present in the

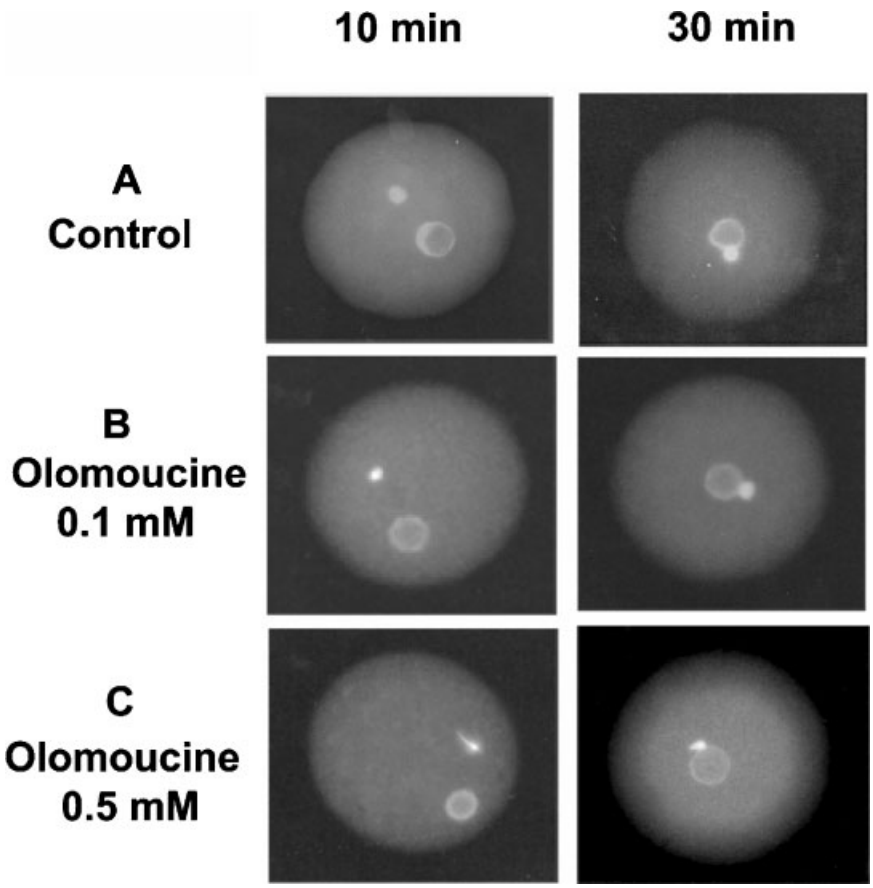

Fig. 1. Male pronucleus decondensation. To follow male pronuclei decondensation, zygotes were harvested at 10 and 30 min post insemination (p.i.) and stained with DAPI dye (1 μg/ml). **A**: Control. Zygotes incubated in sea water containing, (**B**) 0.1 mM olomoucine, and (**C**) 0.5 mM olomoucine.

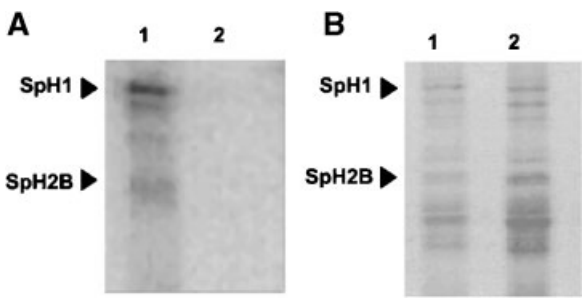

Fig. 2. Sperm histones phosphorylation in vivo. To determine sperm histones (SpH) phosphorylation unfertilized eggs were pre-incubated for 60 min in sea water containing 20 μCi/ml of [^{32}P]-ortophosphate. Olomoucine (0.5 mM) was added 30 min before fertilization. Zygotes were harvested 10 min after fertilization and used to isolate whole histones as described in Materials and Methods. Thirty micrograms of histones were loaded on each lane of the PAGE/SDS gels, and the radioactivity associated to each protein band was monitored by autoradiography. **Panel A**: Autoradiography of SpH isolated from control zygotes (**lane 1**) and from zygotes treated with olomoucine (**lane 2**). **Panel B**: Coomassie stained gel: Whole SpH from control zygotes (**lane 1**) and SpH from zygotes treated with olomoucine (**lane 2**).

zygotes harvested at an intermediate stage of male pronucleus formation (10 min p.i), and after the fusion of both pronuclei, at a time that corresponds to the end of the first embryonic cell cycle (90 min p.i.). The detection of SpH was performed by immunoblots, revealed with antibodies against SpH that were immunoadsorbed to a column of Sepharose 4B bound to the CS histone variants as described in Materials and Methods. By using these antibodies, the complete set of SpH were observed (Fig. 3, lane 1 in panels A–C) while no cross-reactivity with the CS variants was detected (Fig. 3, lane 4 in panels A–C). We found that the normal schedule of SpH disappearance concurrent to male chromatin remodeling (Fig. 3A) was not affected by the treatments of the zygotes with olomoucine (Fig. 3B). Only SpH2A-SpH2B dimers were found in the nucleosome cores at 10 min p.i. (Fig. 3, lane 2 in panels A and B). These SpH2A-SpH2B dimers were completely lost at 90 min p.i. (Fig. 3, lane 3 in panels A and

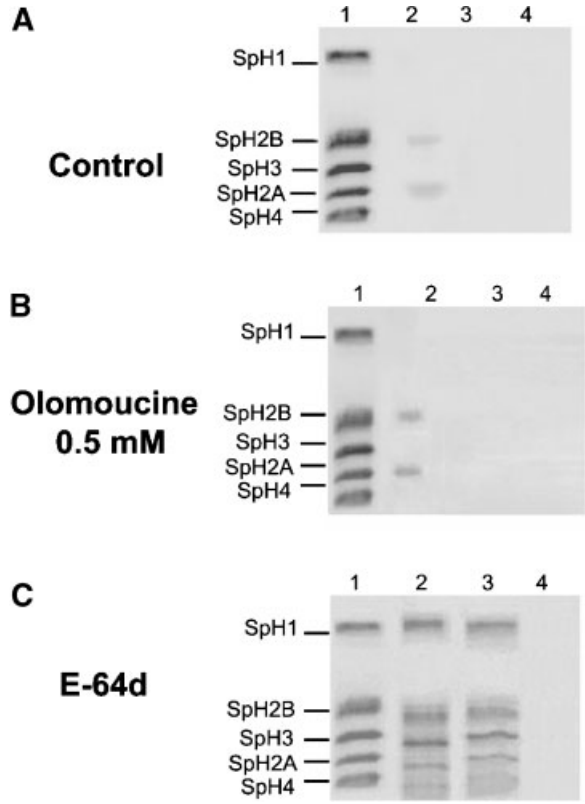

Fig. 3. Sperm histones degradation. To follow sperm histones (SpH) degradation, 30 μg of total histones isolated from zygotes were analyzed in SDS/PAGE and revealed with anti-SpH antibodies. Histones isolated from sperms (**lane 1**) and unfertilized eggs (**lane 4**) were included in each blot as positive and negative control, respectively. **A**: Histones from control zygotes harvested at 10 min p.i. (**lane 2**) and 90 min p.i. (**lane 3**). **B**: Histones from zygotes treated with 0.5 mM olomoucine, harvested at 10 min p.i. (**lane 2**) and 90 min p.i. (**lane 3**). **C**: Histones from zygotes treated with 150 μM E-64d, harvested at 10 min p.i. (**lane 2**), and 90 min p.i. (**lane 3**).

B). To demonstrate that the loss of SpH, followed by Western blots, is a reliable assay, we have analyzed the presence of SpH in the chromatin obtained from zygotes treated with E-64 d to inhibit the cysteine-protease that is involved in SpH degradation after fertilization [Imschenetzky et al., 1997; reviewed by Imschenetzky et al., 2003]. As shown, the complete set of SpH was visualized in the chromatin of zygotes harvested either at 10 min p.i or 90 min p.i. when the zygotes were incubated in E-64d (Fig. 3C, lanes 2 and 3, respectively). From this result, it is obvious that the complete set of SpH is clearly detectable when the cystein-protease involved in SpH degradation is inhibited by E-64d.

DISCUSSION

We have demonstrated that sperm nucleus decondensation may be uncoupled from male chromatin remodeling during male pronucleus formation. As reported, the loss of sperm specific histones concurrent to male chromatin remodeling remains unaltered when sperm nucleus decondensation has being perturbed in vivo by olomoucine. We have also observed that the migration of both pronucleus to each other and their subsequent fusion were insensitive to this protein-kinase inhibitor. Hence, the mechanisms involved in pronucleus migration seem to be independent of the phosphorylation/dephosphorylation cascades catalyzed by protein-kinases inhibited by olomoucine. As reported, the purine analog olomoucine exhibits a quite narrow selectivity; among the protein kinases tested, it inhibits the cyclin-dependent kinases Cdk1 (cdc2), Cdk2 and Cdk5, and erk1 to a lesser extent [Vesely et al., 1994]. It is very difficult to predict the real concentration of olomoucine attained into the male pronucleus when the inhibitor is added externally to alive zygotes, therefore it is not surprising that the dose of oloumucine that decreases male pronucleus decondensation in vivo, described in this report, is higher than the IC 50 values reported for the purified protein-kinases [reviewed by Meijer, 1996]. The inhibition of sperm nucleus decondensation in vivo by olomoucine is consistent and complements previous information indicating that protein kinase C together with an olomoucine sensitive protein kinase participates in male pronucleus decondensation in vitro [Stephens et al., 2002]. Concerning the potential role of these protein-kinases, it seems clear that the initial step of male pronucleus decondensation correlates with the disassembly of the nuclear lamina and is promoted by the protein kinase C-dependent phosphorylation of lamin B. The second step involves the formation of a new nuclear envelope and correlates with an ATP-dependent binding of nuclear membrane vesicles to chromatin and a GTP-dependent fusion of these vesicles with each other. When the male pronucleus is ready to fuse with the female pronucleus, an import of soluble lamin B occurs, leading to the final swelling of male pronucleus [reviewed by Collas et al., 2000]. The phosphorylation of an integral membrane protein (lamin B receptor) in arginine–serine repeat regions induces the targeting

of nuclear vesicles to chromatin [Takano et al., 2002]. Consequently, the role of protein kinase C in the dissasembly/reassembly of the nuclear membrane that follows fertilization seems to be well sustained. As reported by Stephens et al. [2002], protein kinase C alone was unable to induce a complete sperm nucleus decondensation in vitro without the participation of a protein kinase sensitive to olomoucine. In this context, the cyclin-dependent kinases sensitive to olomoucine emerge as potential candidates to complement PKC in male pronucleus decondensation. The cell cycle associated protein–kinases targeted by olomoucine, thus far, Cdk1 and Cdk2 has been immuno-localized into male pronucleus after fertilization [Moreau et al., 1998; Sumerel et al., 2001; Concha et al., 2005].

We have confirmed that olomoucine blocks in vivo the phosphorylation of SpH1 and SpH2B that occur shortly after fertilization, as reported previously for in vivo assays by Stephens et al. [2002]. It was postulated that these post-translational modifications decrease histones-binding capacity to linker DNA inducing chromatin relaxation [reviewed by Green, 2001]. However, the degradation of SpH in the presence of olomoucine, described in this report, indicates that the lack of SpH1 and SpH2B phosphorylation, induced by olomoucine, does not impede SpH removal from the male pronucleus and therefore the normal male chromatin remodeling. This result argues against the crucial role of histones phosphorylation in male chromatin remodeling that follows fertilization [reviewed by Green, 2001]. In contrast, it is consistent with our previous report, in which we have informed that the cysteine-protease that selectively degrades SpH after fertilization is negatively modulated by phosphorylation of SpH1 and SpH2B [Morin et al., 1999a]. Based on this result, we have postulated that a yet unknown phosphatase should remove the phosphate from these histones in order to be degraded [reviewed by Imschenetzky et al., 2003]. In this context, the inhibition of SpH1 and SpH2B phosphorylation by olomoucine may be expected to facilitate the protease-mediated SpH degradation concomitant to male chromatin remodeling. Taken together, these results indicate that the phosphorylation/dephosphorylation cascades that govern sperm nucleus decondensation after fertilization are not a requisite for the loss of SpH that are associated to male chromatin remodeling. However, there may be additional roles for the phosphorylation of SpH2B and SpH1 in male chromatin remodeling, which remain to be established.

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