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Sperm Nucleosomes Disassembly is a Requirement for Histones Proteolysis During Male Pronucleus Formation

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Abstract We had previously reported that a cysteine-protease catalyzes the sperm histones (SpH) degradation associated to male chromatin remodeling in sea urchins. We found that this protease selectively degraded the SpH leaving maternal cleavage stage (CS) histone variants unaffected, therefore we named it SpH-protease. It is yet unknown if the SpH-protease catalyzes the SpH degradation while these histones are organized as nucleosomes or if alternatively these histones should be released from DNA before their proteolysis. To investigate this issue we had performed an in vitro assay in which polynucleosomes were exposed to the active purified protease. As shown in this report, we found that sperm histones organized as nucleosomes remains unaffected after their incubation with the protease. In contrast the SpH unbound and free from DNA were readily degraded. Interestingly, we also found that free DNA inhibits SpH proteolysis in a dose-dependent manner, further strengthening the requirement of SpH release from DNA before in order to be degraded by the SpH-protease. In this context, we have also investigated the presence of a sperm-nucleosome disassembly activity (SNDA) after fertilization. We found a SNDA associated to the nuclear extracts from zygotes that were harvested during the time of male chromatin remodeling. This SNDA was undetectable in the nuclear extracts from unfertilized eggs and in zygotes harvested after the fusion of both pronucleus. We postulate that this SNDA is responsible for the SpH release from DNA which is required for their degradation by the cysteine-protease associated to male chromatin remodeling after fertilization. J. Cell. Biochem. 103: 447–455, 2008.

Key words: chromatin remodeling; cysteine-protease; fertilization; histones; sea urchins

Chromatin remodeling during male pronucleus formation represent one of the most dramatic changes in chromatin structure and function in most species. In sea urchins, the complete set of sperm histones (SpH) is removed from chromatin and replaced by maternally inherited CS histone variants (reviewed by Imschenetzky et al. [2003]). We had also shown that the SpH released from male chromatin are degraded by a nuclear cysteine protease (SpH-protease) that catalyzes the SpH proteolysis, leaving the CS histone variants unaffected [Imschenetzky et al., 1997]. Such selective degradation was shown to be modulated by post-translational modification of substrates. We have previously shown that SpH1 and SpH2B were protected by phosphorylation from being degraded by this protease at intermediate steps of male chromatin remodeling and the CS variants are protected by poly(ADP-ribosylation) [Morin et al., 1999a,b]. Consistent with the independence of male chromatin remodeling from the proteins newly synthesized after fertilization [Poccia et al., 1981; Imschenetzky et al., 1991], this protease was found as an inactive precursor in the nucleus of unfertilized eggs and shown to be activated and mobilized into male pronucleus after fertilization [Concha et al., 2005a]. The role of this protease in SpH degradation was further supported by the persistence of the complete set of SpH at the end of the initial cell cycle in zygotes treated with E-64-d, an inhibitor of cysteine-proteases [Monardes et al., 2005]. Based on this evidence

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we proposed a model in which this SpH-protease exhibits a crucial role in male chromatin remodeling after fertilization [Imschenetzky et al., 2003]. However, it remains unknown if this protease degrades the SpH organized as nucleosomes or if these SpH should be released from DNA prior to their degradation. To investigate this issue we had developed an in vitro assay in which isolated sperm nucleosomes were incubated with the SpH-protease. As shown in this report the SpH organized as nucleosomes were not degraded, in contrast the SpH were totally degraded when they were released from DNA. Interestingly we also found that this protease was inhibited by free DNA, further supporting the idea that the SpH should be released from DNA before their degradation. Taken together our results indicate that sperm nucleosome disassembly from DNA by histone chaperones or other chromatin remodeling complexes is a prerequisite for their degradation.

Among the different nuclear chaperones, nucleoplasmin isolated initially in batracians, promotes sperm nucleosomes remodeling after fertilization in concert with proteins N1/N2. Nucleoplasmin participates as a chaperone in the assembly of nucleosome cores, by facilitating the addition of H2A–H2B dimers to the preformed H3–H4 tetramers organized by the chaperones N1/N2 (reviewed by Philpott et al. [2000]). Unfortunately, previous attempts to detect nucleoplasmin in sea urchins were unsuccessful [Stephens et al., 2002]. In this context we have further investigated if a sperm nucleosome disassembly activity (SNDA) was detectable in sea urchins after fertilization. As reported here, we have found a SNDA that promotes sperm nucleosomes disassembly in vitro. This activity exhibits a remarkable temporal correlation with male pronucleus formation. The SNDA was detected only in nuclear extracts of zygotes harvested during the time of male pronucleus formation and it was undetectable in unfertilized eggs and in zygotes harvested after the time of the fusion of both pronuclei. Based on these results we postulate that this SNDA compensates in sea urchins the role of batracians histone-chaperones nucleoplasmin and proteins N1/N2. Therefore this SNDA should induce the release of the SpH from DNA before their degradation by the cysteine-protease as proposed previously for sperm chromatin remodeling after fertilization in sea urchins [Imschenetzky et al., 2003].

MATERIALS AND METHODS

Gametes and Zygotes

Sea urchin *Tetrapygus niger*, were collected from the bay of Concepción, Chile and maintained in an aquarium containing natural sea water under constant aeration. Unfertilized eggs, sperm and zygotes were obtained as described previously [Imschenetzky et al., 1991].

Polynucleosomes Isolation

Nuclei were obtained from sperms by an aqueous ethanol/TritonX-100 procedure performed basically as described by Poccia et al. [1981]. Nucleosomes were obtained by incubating the isolated nuclei with 72 units/ml of micrococcal nuclease (MNase) (Worthington, New Jersey) in a buffer 0.01 M Tris–HCl at pH 7.6, 0.01 M NaCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$ at 37°C for 10 min. The nucleoprotein particles derived from MNase digestion were further purified on a sucrose density gradient 5–20% (w/v) in a buffer 10 mM Tris–HCl pH 7.2 containing 0.7 mM Na_2EDTA and analyzed by electrophoresis on horizontal 1% (w/v) agarose gels in 1 mM EDTA and 0.04 M Tris-acetate buffer pH 8.0, as described by Oliver et al. [2002]. The initial fractions (1–2) of the sucrose gradient containing polynucleosomes free of unbound DNA were used to determine the effect of the protease on the polynucleosomes in vitro or alternatively for the screening of a SNDA after fertilization.

Proteolytic Assay

The protease was purified from nuclear extracts of zygotes harvested 5 min post-insemination (p.i.) by sucrose gradient (10–40%, w/v) containing 10 mM sodium phosphate pH 6.0 followed by gel filtration through a column (2.0 cm × 80 cm) of Sephadex G-100 equilibrated with 10 mM sodium phosphate buffer pH 6.0. The products derived from the incubation of the (methyl-^{14}C) SpH with the purified protease were analyzed by SDS-PAGE and by fluorography as described previously [Imschenetzky et al., 1997].

To determine the effect of the protease on the SpH organized as nucleosomes, the purified protease was incubated with the polynucleosomes that were primarily isolated by sucrose gradients in 0.05 M phosphate buffer pH 7.5 containing 1 mM DTT, 1 mM EDTA and 0.1% Triton X-100 at 37°C. The maintenance of the

polynucleosomes organization after the incubation with the protease was analyzed by following its electrophoretic migration on 1% (w/v) agarose gels. The integrity of the SpH that were forming these polynucleosomes were further analyzed by SDS-PAGE followed by Western blots revealed with polyclonal antibodies against SpH that were performed as described previously [Oliver et al., 2002]. Western blots final detection was performed using a quimioluminiscence ECL Kit (Amersham Pharmacia Biotech, UK).

Screening of a Sperms Nucleosome Disassembly Activity (SNDA)

Polynucleosomes isolated from free DNA by sucrose gradients that were contained in fraction 1 or 2 of the gradient were used to detect the potential SNDA present either in nuclear or cytoplasmic extracts obtained from unfertilized eggs or from zygotes harvested at different times post-insemination. To obtain the nuclear and cytoplasmic extracts, the unfertilized eggs or the zygotes harvested at different times post-insemination, were homogenized in 10 mM phosphate buffer pH 6.0, 10 mM NaCl, 10 mM EDTA pH 6.0 and 0.5% Triton X-100 and centrifuged at 4450*g* for 10 min to separate the nuclear fraction that was obtained in the pellet from the cytoplasmic fraction that remains in the supernatant. To analyze the SNDA present in the different extracts, 0.5 μg of polynucleosomes were incubated at 37°C with 2 μg of nuclear extracts, or alternatively with 2 μg of cytoplasmic extracts in a solution containing 10 mM phosphate buffer pH 6.0, 10 mM NaCl, 10 mM EDTA pH 6.0. After incubation the maintenance of the polynucleosomes was evaluated by following their electrophoretic migration in 1% (w/v) agarose gels.

RESULTS

Sperms-Nucleosomes Disassembly is a Prerequisite for Histones Degradation

As reported before, the SpH-protease characterized in our laboratory was able to efficiently degrade the five SpH in vitro and in vivo [Imschenetzky et al., 1997; Monardes et al., 2005]. However, it was unknown if the SpH may be degraded while forming nucleosomes or if alternatively, the histones should be released from DNA prior to their degradation. To investigate this issue we have established an in vitro assay in which isolated sperm polynucleosomes were exposed to the active protease. Sperm polynucleosomes were obtained by micrococcal nuclease digestion, as shown in Figure 1, these polynucleosomes were migrating in 1% agarose gels in a position corresponding to the DNA fragments 1.26–1.78 kb, although free DNA fragments unbound from the SpH was also observed in this gel. When we analyzed the effect of increasing amounts of free DNA on the activity of the protease, we found that this enzyme was inhibited by DNA fragments in a dose-dependent manner (Fig. 2). Consequently, we had further purify sperm polynucleosomes from free DNA in order to avoid the potential inhibitory effect on the protease activity of the free DNA fragments present in the extracts derived from micrococcal nuclease digestion. As shown in Figure 3 the sucrose gradient described in methods allowed a successful separation of polynucleosomes (fractions 1–2) from free DNA (fractions 3–13). Subsequently we used these fractions (1 and 2) of the sucrose gradient that was free from unbound DNA to perform the in vitro assay in which these polynucleosomes were incubated for different times either alone or with the purified protease (Fig. 4). We observed that the migration of the polynucleosomes, as well as, the binding of the five SpH to DNA remained unaltered up till 120 min of incubation, either in

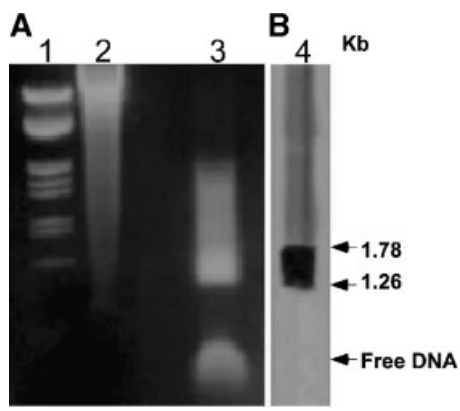

Fig. 1. Sperm nucleoprotein particles. Sperm nucleus obtained from sea urchins were digested for 10 min with micrococcal nuclease (72 U/ml), the nucleoprotein particles obtained by nuclease digestion were separated by electrophoresis in 1% (w/v) agarose gel as described in methods. **Lane 1**: Lambda DNA digested with *Hin*dIII and *Eco*RI. **Lane 2**: Free DNA digested with micrococcal nuclease. **Lane 3**: Nucleoprotein particles obtained by nuclease digestion stained with ethidium bromide, **Lane 4**: Nucleoprotein particles obtained by nuclease digestions stained with Coomassie blue.

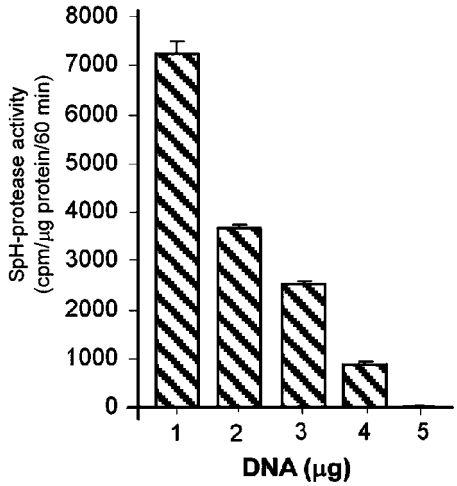

Fig. 2. DNA inhibits the SpH-protease. The proteolytic assay was performed in the presence of increasing amount of free DNA as described in methods. ^{14}C-SpH1 was used as substrate.

absence (Fig. 4A,B) or the presence of the active protease (Fig. 4C,D). These results confirm the stability of these polynucleosomes in the incubation media and further indicate that the polynucleosomal organization remains unaffected in the presence of the SpH-protease. It seems particularly important to note the integrity of the five SpH that were forming the polynucleosomes after the incubation with the protease (Fig. 4D). In contrast, the complete set of SpH are degraded when they are incubated with this protease in the absence of DNA, as shown in Figure 5. Taken together, these results indicate that the SpH-protease is unable to catalyze SpH degradation while these histones are organized as nucleosomes. Consequently, a step in which the SpH are released from DNA emerges as an absolute requirement for male histones degradation that is associated to chromatin remodeling after fertilization. Hence these results strongly suggest the presence of sperm nucleosomes disassembly activity (SNDA) after fertilization.

Sperms Nucleosome Disassembly Activity (SNDA) Correlates With Male Pronucleus Formation After Fertilization

The strategy used to analyze the potential presence of a SNDA involved in male chromatin

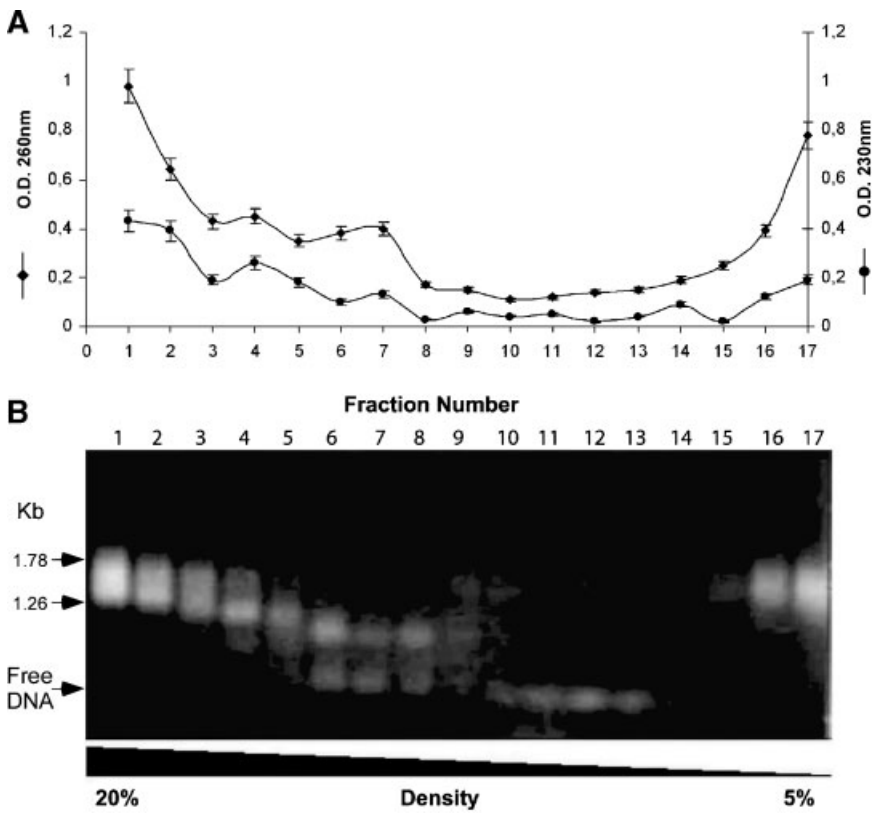

Fig. 3. Nucleosomes purification. To purify mono and polinucleosomes from free DNA, the nucleoprotein particles obtained by nuclease digestion were fractionated in a sucrose gradient (5–20%, w/v) (**A**) and each fraction obtained was further analyzed in an agarose gel (1%, w/v) (**B**).

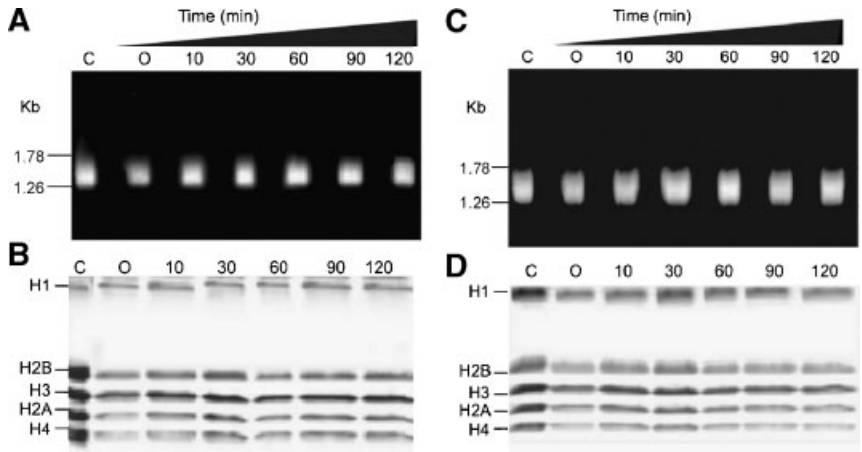

Fig. 4. Proteolytic assay using purified nucleosomes as substrates. Nucleosomes were isolated by sucrose gradients and incubated up to 120 min. in the conditions described in methods for the protease assay. Incubation was performed in the absence of the SpH-protease (**A**,**B**) or in the presence of 50 μg protease (**C**,**D**). The integrity of nucleosomes was monitored by following the electrophoretic mobility in agarose gels stained with ethidium bromide (A,C). The SpH that were forming the nucleosomes were further analyzed by Western blots revealed with anti-SpH antibodies (B,D).

remodeling after fertilization, relays on predictable changes of electrophoretic migration in 1% agarose gels displayed by the isolated sperm polynucleosomes. As we described above, sperm polynucleosomes migrate as discrete nucleoprotein particles in a position of these gels corresponding to the DNA fragments 1.26–1.78 kb (Fig. 1). Therefore we had investigated if the extracts isolated from unfertilized eggs or from zygotes harvested at different times post-insemination (p.i.) may alter the electrophoretic migration of the sperms polynucleosomes by promoting nucleosomes disassembly, thus generating a band corresponding to free DNA which is positioned at the bottom of this gel. Figure 6 summarizes the results obtained from the extracts derived from unfertilized eggs or from zygotes harvested during the time of male pronucleus formation (up to 30 min p.i.) and afterwards (up to 60 min p.i.). As previously reported, male chromatin remodeling occurs up till 30 min after fertilization, then the SpH are no longer detectable in zygotes, and the decondensed male pronuclei fuses with the female pronuclei re-establishing the dipolid condition of the zygote (reviewed by Imschenetzky et al. [2003]). The results obtained for the assays of the nuclear extracts are shown in Figure 6A and those obtained for the cytoplasmic extracts are shown in Figure 6B. As shown the migration of sperm nucleosomes remains unaltered when extracts from unfertilized eggs, either nuclear or cytoplasmic, were assayed. In contrast, sperm nucleosomes were disassembled, generating free DNA, when the nuclear extracts derived from zygotes harvested at 5, 10 or 15 min p.i. were used in these assays indicating that these extracts in harbor a SNDA. As shown in Figure 6, this SNDA was absent from the nuclear extracts from zygotes harvested 30 or 60 min p.i., as well as, when the cytoplasmic extracts from the different zygotes were assayed. It is noteworthy to consider that this SNDA correlates perfectly with the time of male chromatin remodeling, moreover, the SNDA was undetectable in the nuclear fraction from unfertilized eggs or from zygotes harvested after the fusion of male and female pronuclei. These results are consistent with the presence of SNDA in the nuclear fraction of zygotes harvested during the time of male pronuclei formation which may be responsible for sperm

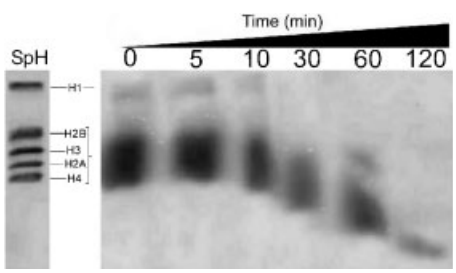

Fig. 5. Degradation of Sperm histones. Total ^{14}C-SpH were used as substrates for the assay that was performed as described in methods. The products remaining after the digestion with the SpH-protease were revealed by fluorography.

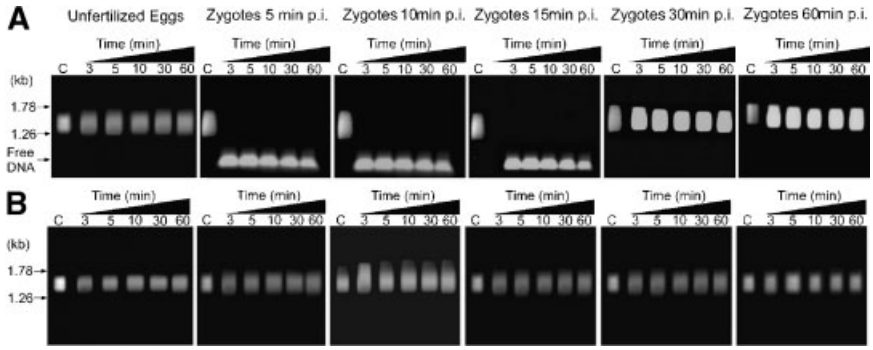

Fig. 6. Chromatin remodeling activity present in zygotes harvested at different times after fertilization. Nuclear and cytoplasmic extracts were obtained from unfertilized eggs or zygotes harvested at 5 min p.i., 10 min p.i., 15 min p.i., 30 min p.i. and 60 min p.i. Each extract was incubated with purified sperm nucleosomes and the integrity of nucleosomes after incubation was determined by following its migration in agarose gels, as described in methods. **A**: Nuclear extracts. **B**: Cytoplasmic extracts. It is to note nucleosomes disassembly which is evidenced by the presence of free DNA observed when the nuclear extracts obtained from zygotes harvested at 5 min p.i., 10 min p.i. and 15 min p.i. are used in the assay.

nucleosomes disassemble which is required in order to allow SpH degradation by the SpH-protease.

DISCUSSION

This report further precises the mechanism by which the SpH-protease catalyzes sperm histones degradation after fertilization by providing two additional lines of evidence [Imschenetzky et al., 2003]. First we demonstrated that the SpH-protease requires the disassembly of sperm histones from DNA in order to catalyze SpH degradation. This evidence is strengthened by the finding that free DNA inhibits the enzyme in vitro, further supporting the requirement of SpH release from DNA before their degradation. As previously reported the N-terminal sequence of this protease classifies the SpH-protease as a member of the cathepsin L family of proteases [Concha et al., 2005a,b]. Cathepsin L proteases are traditionally part of the endopeptidases responsible for terminal protein degradation in endo-lysosomal vesicles. Although its extracellular secretion has also been documented and related to the progression of neoplastic cells [reviewed by Mohamed and Sloane, 2006]. Despite the similarities in the N-terminal amino-acid sequence with the cathepsin L family of proteases, the substrate selectivity and modulation of its catalytic activity by post-translational modification of its substrates as reported previously [Morin et al., 1999a,b], or its binding to DNA as shown herein, are particular to the SpH-protease and have not being associated to other members of the cathepsin L family of proteases. In functional terms, this enzyme is involved in histones metabolism after fertilization [Imschenetzky et al., 1993] and also in cell cycle control. Its inhibition blocks SpH degradation, then provokes the arrest of the initial cell cycle and ultimately aborts early development [Concha et al., 2005b; Puchi et al., 2006]. Interestingly, a murine nuclear variant of cathepsin L was also postulated to be involved in cell cycle control. This variant catalyses the limited proteolysis of the CDP/cux transcription factor at the G1/S transition of the cell cycle, modulating its activity (reviewed by Goulet and Nepveu [2004]). More recently, it was reported that cathepsin L in mouse fibroblasts knockouts alters the distribution pattern of histone H3 (K9) methylation in centromeres and reverses the epigenetic landscape of the Y chromosome [Bulynko et al., 2006]. In this context, novel potential roles of the modulation of the activity of nuclear cathepsin L variants in histones metabolism and cell cycle progression appears as an emergent issue that we think merits further investigation.

In the second line of evidence reported herein, we describe the presence of a sperm nucleosome disassembly activity (SNDA) that induces the SpH release from DNA in in vitro assays. We found that this SNDA correlates remarkably the events that occur during male chromatin remodeling after fertilization. Hence, this SNDA establishes the predictable link between the events concomitant to male chromatin remodeling and SpH degradation as catalyzed by the SpH-protease [Imschenetzky et al., 2003]. Three major functional types of nuclear chaperones that belong to the nucleoplasmin/nucleophosmin family has been well documen-

ted: NPM1, NPM2 and NMP3. NPM1 and NPM3 are ubiquitously expressed in many tissues [Shackleford et al., 2001] and are functionally related mainly to ribosome biogenesis [Huang et al., 2005]. Although NPM1 appears to be a multifunctional protein, it binds to pRb thus stimulating DNA polymerase α [Takemura et al., 1999], participates in centrosomes duplication [Okuda et al., 2000], is involved in transcription through its histone chaperone activity and its association to specific transcription factors [Kondo et al., 1997; Okuwaki et al., 2001; Swaminathan et al., 2005; Weng and Yung, 2005]. In addition, it has being shown to participate in apoptosis [Wu et al., 2002; Maiguel et al., 2004; Lu et al., 2005].

NMP2 was primarily described in *Xenopus laevis*, is only expressed in oocytes and eggs and is involved in male chromatin remodeling after fertilization. It is a thermostable acid protein that in-harbors polyglutamic acid clusters providing binding tracts for the dimers of histones H2A and H2B. Hence, it was postulated that the unpacking of *Xenopus* male chromatin results from the binding of nucleoplasmin to sperm specific basic proteins (SSBP) with a higher affinity than that displayed by nucleoplasmin to histones H2A-H2B. During sperm chromatin remodeling, nucleoplasmin facilitates the removal of SSBP and the deposit of H2A-H2B dimers in a concerted manner. These events are followed by histone phosphorylation and the H1-like linker histone B4 assembly at the final stages of male chromatin. Analysis of the crystal structure of the N terminal domain of NMP2 reveals that the core of this protein is an eight-stranded beta barrel that fits within a stable pentamer. In the crystal structure, two pentamers are associated forming a decamer and it was shown that this decamer may assemble large protein complexes that contain the four nucleosome core histones [Dutta et al., 2001]. Intriguingly, in NMP2-null mices major events after fertilization, as meiosis, sperm DNA decondensation and the first embryonic S phase remain unaffected. These results suggest that NMP1 and/or NMP3 may compensate the functions of NPM 2 in male chromatin remodeling after fertilization [Burns et al., 2003].

In invertebrates, a highly hydrophilic protein of 18.5 kDa was isolated from embryonic chromatin of Drosophila and designated as Df31. It was postulated that Df31 functioned in the removal of sperm basic chromosomal proteins and interacted with histones facilitating their loading into the chromatin during male chromatin remodeling after fertilization. This protein was found to be associated to poly-nucleosomes and suggested to participate in higher order folding of chromatin [Crevel et al., 2001].

In sea urchins, previous attempts to detect a NMP2-like activity in vitro were unsuccessful and it was suggested that this cells lack nucleoplasmin [Stephens et al., 2002]. In contrast, NMP-like molecules were found to be encoded in the genomes of the sea urchins *Lyctechinus pictus* and *Strogylocentrutus purpuratus*. This NMP-like sequences shares the closest common ancestor with the NMP1 group of proteins. Similarly to NMP from other phyla the NMP-like sequences of sea urchins in-harbor potential phosphorylation sites required for their activation [Eirin-Lopez et al., 2006]. Hence it is plausible that the SNDA described in this report corresponds to the product of NMP-like proteins from the sea urchin *T. niger*. We think that the apparent contradiction of the detection of the SNDA in this report with the lack of detection of a NMP-like activity by Stephens et al. may be explained by the fact that the previous detection system was based on the information concerning the activity of NMP2 from *Xenopus*. Stephens et al. [2002] tested the ability of sea urchin thermo-heated nuclear extract to promote sperm nuclear swelling in vitro. In contrast our assay was performed measuring the ability of the nuclear extracts obtained at low temperatures to disorganize isolated poly-nucleosomes. We think that our assay is more representative of the in vivo condition and is more in agreement with the closest similarity to NMP1 of the histone NMP-like chaperones encoded in the genome of sea urchins. Consequently, this in vitro assay should be useful to further purify and characterize in molecular and functional terms this SNDA. Purification of the SNDA should allow the obtainment of antibodies that may be useful to perform SNDA immune-depletion experiments in order to establish in the future a hierarchical relationship between SpH release from male chromatin and its degradation by the SpH-protease.

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