Poly(ADP-ribosylation) protects maternally derived histones from proteolysis after fertilization

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Fertilization in sea urchins is followed by the replacement of sperm-specific histones by cleavage-stage histone variants recruited from maternal stores. Such remodelling of zygote chromatin involves a cysteine proteinase that degrades the sperm-specific histones in a selective manner, leaving the maternal cleavage-stage histone variants intact. The mechanism that determines the selectivity of the sperm-histone-selective proteinase (SpH-proteinase) was analysed by focusing on the post-translational modification status of both sets of histones. It has previously been reported that only native cleavage-stage histones are poly(ADP-ribosylated), whereas the sperm-specific histones are not modified. To determine whether the poly(ADP-ribose) moiety afforded protection from degradation, the ADP-ribose polymers were removed from the cleavage-stage histones *in vitro*;

these proteins were then assayed as potential substrates of the SpH-proteinase. Strikingly, the cleavage-stage histone variants were extensively degraded after the enzymic removal of their ADP-ribose moieties. In addition, the SpH cysteine proteinase was not inhibited by isolated poly(ADP-ribose) polymers. Consequently, only poly(ADP-ribosylated) cleavage-stage histone variants are protected from proteolysis. These results demonstrate a novel role for this type of post-translational modification, namely the protection of nuclear proteins against nuclear proteinases after fertilization.

Key words: chromatin, cysteine-proteinase, pronucleus, sea urchins, zygotes.

INTRODUCTION

Sperm nuclei are transformed after fertilization into male pronuclei, which fuse with female pronuclei, thus re-establishing the diploid genome of embryos. Biochemical evidence indicates that during the formation of the male pronucleus the whole set of sperm-specific histone variants is replaced by cleavage-stage (CS) histone variants derived from maternal stores. After amphimixis, the nuclei of zygotes are formed from nucleoprotein particles containing only CS histone variants [1–3]. It has been inferred from these results that male pronucleus remodelling involves the selective proteolysis of the sperm-specific histones. We have reported recently the presence of a cysteine proteinase that recognizes the sperm histones but leaves the maternally derived CS histone variants unaffected in sea urchin zygotes [4]. The role of this proteinase in sperm-specific histone degradation was substantiated by the effects of its inhibition *in vivo* in zygotes of sea urchins. Owing to the selective proteolytic behaviour of this enzyme it was designated the sperm-histone-selective proteinase (SpH-proteinase) [4].

We have previously reported that CS histone variants are extensively poly(ADP-ribosylated) in unfertilized sea urchin eggs, as well as during the initial two cleavage divisions [5]. This post-translational modification changes in a cell cycle-dependent manner and is related to the normal progress of DNA replication of sea urchin zygotes [6,7]. This information validates a role for the poly(ADP-ribosylation) of CS histone variants during the normal cleavage divisions, but does not explain its potential function in unfertilized eggs. The present study was undertaken to investigate whether this post-translational modification is responsible for the protection of the CS histone variants from proteolysis after fertilization.

The poly(ADP-ribosylation) of nuclear proteins is catalysed by poly(ADP-ribose) polymerase (EC 2.4.2.30). This enzyme

transfers the ADP-ribose moiety of NAD⁺ to chromosomal proteins to form either oligo-(ADP-ribosyl) or poly(ADP-ribosyl) homopolymers. This post-translational modification has been implicated in cellular responses to DNA damage (reviewed in [8]). More recently it has been postulated that poly(ADP-ribose) polymerase has a role downstream of DNA repair events and affects pathways of cell survival and cell death [9,10].

The possible protective effects of poly(ADP-ribose) moieties for the CS histone variants was investigated by assaying the SpH-proteinase with CS histone variants that had been treated with phosphodiesterase (PDE) bound to CNBr-activated Sepharose 4B (Sepharose–PDE) to remove the polymers of ADP-ribose. It was shown that the poly(ADP-ribosylated) forms of the CS histone variants were not degraded by the SpH-proteinase, whereas the naked proteins were readily degraded. This result indicates that the poly(ADP-ribosylation) of maternal histones has a fundamental role after fertilization in preserving the integrity of female histone variants while sperm histones are degraded.

EXPERIMENTAL

Isolation of sperm and CS histone variants

Sperm-specific and native CS histone variants were isolated from sperms, eggs and zygotes of sea urchins *Tetrapygus niger*, as described previously [2]. Sperm histone H1 was purified by exclusion chromatography on Bio-Gel P-60 by the method of von Holt et al. [11]. SDS/PAGE was performed as described by Laemmli [12].

SpH-proteinase purification

The SpH-proteinase was isolated from the chromatin of sea urchin zygotes harvested 5 min after fertilization. Purification

Abbreviations used: CS, cleavage-stage; PDE, phosphodiesterase; SpH-proteinase, sperm-histone-selective proteinase; TCA, trichloroacetic acid.

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was done on a sucrose gradient [10–40% (w/v)], followed by exclusion chromatography on Sephadex G-100 (120 mesh), as described previously [4]. The peak containing the activity of the proteinase was collected, concentrated and stored at -20°C . No significant loss of proteinase activity was observed after up to 2 months of storage. Sperm histone H1 labelled with ^{14}C was used as substrate to detect the enzyme in all steps of purification [4].

Proteinase assays

Proteinase activity was determined by measuring the hydrolysis of labelled protein substrates into trichloroacetic acid (TCA)-soluble peptides, essentially as described by De Martino [13]. The reaction mixture, in a total volume of $100\ \mu\text{l}$, contained 50 mM phosphate buffer, pH 7.5, with 1 mM dithiothreitol, 1 mM EDTA and 0.1% (v/v) Triton X-100, in accordance with the requirements of this proteinase as determined previously [4]. Radioactively labelled proteins used as substrates were obtained by incubating 1 mg of each protein in a solution containing 100 mM Hepes buffer, pH 7.5, 200 mM sodium cyanoborohydride and $56\ \mu\text{Ci}$ of [^{14}C]formaldehyde ($53\ \text{mCi/mmol}$) for 2 h at room temperature. The reaction mixture was dialysed and the labelled proteins were precipitated with 20% (w/v) TCA, washed once with acetone containing 0.07 M HCl and twice with acetone, as described previously [4]. By following this procedure the labelling of proteins achieved was as follows: [^{14}C]methyl-labelled nucleosomal whole sperm histone (specific radioactivity 80000 c.p.m./ μg of protein), [^{14}C]methyl-labelled sperm-specific histone H1 (specific radioactivity 28000 c.p.m./ μg protein), [^{14}C]methyl-labelled poly(ADP-ribosylated) CS histone variants (specific radioactivity 22000 c.p.m./ μg of protein) and [^{14}C]methyl-labelled CS histone variants recovered after hydrolysis of ADP-ribose moieties with PDE (specific radioactivity 13000 c.p.m./ μg of protein).

The proteinase activity is reported as the amount of radioactivity released as TCA-soluble peptides (c.p.m./min per μg of protein) at 37°C . The TCA-insoluble proteins/peptides remaining after digestion were subjected to SDS/PAGE; the radioactivity associated with each electrophoretic band was detected by fluorography [14]. No difference was observed in the electrophoretic pattern of the peptides obtained after proteolysis when unlabelled proteins were used as substrates. The unlabelled peptides derived from the proteinase digestion were stained with ammoniacal silver [15].

Hydrolysis of the ADP-ribose moiety of CS histone variants

The ADP-ribose polymers were removed from the native CS histone variants with Sepharose–PDE. The procedure for obtaining the Sepharose–PDE has been described previously [5]. In brief, snake venom PDE I (Worthington Biochemical Corp., Freehold, NJ, U.S.A.) in a solution containing 0.1 M NaHCO_3 , pH 8.3, and 0.5 M NaCl was incubated overnight at 4°C with CNBr-activated Sepharose 4B (Pharmacia AB, Uppsala, Sweden). The remaining reactive groups of the Sepharose–PDE were blocked with 0.2 M glycine, pH 8.0. Any uncoupled protein was removed from the Sepharose–PDE by being washed with 0.1 M NaHCO_3 , pH 8.3, then with 0.5 M NaCl and finally with a solution containing 1 M KCl and 0.2 M glycine, pH 8.0. The Sepharose–PDE obtained was stable for several months at 4°C [5].

The Sepharose–PDE was subsequently used to hydrolyse the ADP-ribose polymers bound to the native CS histone variants. The reaction mixture, in a total volume of $500\ \mu\text{l}$, contained 300 μg of native CS histone variants in 20 mM Tris/HCl, pH 8.0,

4 mM urea, 2 mM NaCN and a cocktail of proteinase inhibitors [2 mM PMSF, 2 $\mu\text{g/ml}$ antipain, 100 $\mu\text{g/ml}$ Tos-Phe- CH_2Cl ('TPCK') and 50 $\mu\text{g/ml}$ Tos-Lys- CH_2Cl ('TLCK')]. This solution was incubated for 120 min at 37°C with 500 μl of a suspension of Sepharose–PDE (1.8 units of PDE activity). The Sepharose–PDE was recovered by centrifugation and the supernatant containing the CS histone variants was extracted with 0.25 M HCl, precipitated with 20% (w/v) TCA, washed once with acetone containing 0.07 M HCl and twice with acetone, dried and stored at -20°C . As described previously, this procedure hydrolyses the vast majority of the ADP-ribose moiety of CS histone variants [5].

Preparation of polymers of ADP-ribose

Polymers of ADP-ribose were synthesized in a rat-liver cell-free system *in vitro* as described previously [6]. To purify these ADP-ribose polymers, the extract was deproteinized with proteinase K (Boehringer Mannheim) and then several times with chloroform/3-methylbutan-1-ol (24:1, v/v). The polymers obtained were dissolved in 0.3 M sodium acetate and precipitated with ethanol. The nucleic acids contaminating the polymers of ADP-ribose were eliminated with 100 $\mu\text{g/ml}$ DNase I and 100 $\mu\text{g/ml}$ pancreatic ribonuclease (Sigma) and finally with 300 units/ml micrococcal nuclease (Worthington). The ADP-ribose polymers were deproteinized again and precipitated with ethanol, as described previously [6]. The resulting polymers were further analysed by electrophoresis in a 20% (w/v) polyacrylamide gel performed in a 100 mM Tris/borate buffer, pH 8.3, as described by Malanga et al. [16]. The quantification of polymers of ADP-ribose was estimated spectrophotometrically with the molar absorption coefficient ($\epsilon\ 1.27 \times 10^4\ \text{M}^{-1}\cdot\text{cm}^{-1}$) [17].

RESULTS

Protection of CS histones by poly(ADP-ribosylation)

Native poly(ADP-ribosylated) CS histones were subjected to treatment with PDE coupled to Sepharose 4B and then labelled radioactively *in vitro*. These proteins were tested as potential substrates of the SpH-proteinase. As shown in Figure 1, the

Figure 1 Substrate selectivity of the SpH-proteinase

Time course of SpH-proteinase activity with the use of the following labelled substrates: $^{14}\text{C-SpH}$, total sperm histones; $^{14}\text{C-CS}$, native poly(ADP-ribosylated) CS histone variants; $^{14}\text{C-CS+PDE}$, CS histone variants treated with Sepharose–PDE to remove the poly(ADP-ribose) moiety. Results are means $\pm$ S.D. for four determinations.

Figure 2 SpH-proteinase does not degrade maternally derived CS histone variants

Time course of incubation of the SpH-proteinase with labelled substrates. The proteins/peptides obtained after each incubation were analysed by SDS/PAGE followed by fluorography. Abbreviations: ^{14}C -CS + PDE, CS histone variants treated with Sepharose-PDE to remove the poly(ADP-ribose) moiety; ^{14}C -poly(ADPR)-CS, native poly(ADP-ribosylated) CS histone variants; S, whole native ^{14}C -poly(ADP-ribosylated) CS histone variants from unfertilized eggs, included for comparison. The positions of molecular mass markers are indicated at the left.

Figure 3 Effect of poly(ADP-ribose) on SpH-proteinase activity

SpH-proteinase activity was determined after 60 min of incubation with 100 $\mu\text{g/ml}$ ^{14}C -labelled sperm histone H1 used as substrate in the presence of increasing amounts of poly(ADP-ribose). The amount of poly(ADP-ribose) was estimated spectrophotometrically. Results are means $\pm$ S.D. for four determinations.

SpH-proteinase caused extensive hydrolysis of CS histone variants after the removal of the ADP-ribose moieties. As expected, poly(ADP-ribosylated) CS histones remained unaltered, whereas sperm histones were almost totally degraded. These results are supported by fluorography of the proteins and peptides remaining after the incubation of CS histones with and without poly(ADP-ribosylation) with the SpH-proteinase (Figure 2). The non-poly(ADP-ribosylated) forms of the CS histones were almost completely degraded (left panel), whereas the modified forms remained intact (right panel). The electrophoretic migration pattern of the poly(ADP-ribosylated) forms of the CS histone variants reveals the presence of proteins containing polymers of

Figure 4 Poly(ADP-ribose) polymers do not inhibit the proteolytic degradation of sperm histone H1

SpH-proteinase was incubated for 60 min with ^{14}C -labelled sperm histone H1 as substrate; the proteins and peptides generated were analysed by SDS/PAGE followed by fluorography. Lane A, 0.5 μg of uncleaved ^{14}C -labelled sperm histone H1; lane B, 0.5 μg of ^{14}C -labelled sperm histone H1 digestion products; lane C, 0.5 μg of ^{14}C -labelled sperm histone H1 digestion products generated in the presence of 0.5 $\mu\text{g/ml}$ of isolated polymers of ADP-ribose. The migration of uncleaved sperm histone H1 is indicated by an arrow. The positions of molecular mass markers are indicated at the left.

ADP-ribose, evidenced as slowly migrating components, as well as those containing short oligomers that migrated in the lower region of the gel. As shown in Figure 2 (right panel), the proteins containing oligomers of ADP-ribose were protected from proteolysis in a similar manner to those containing polymers of ADP-ribose. From these results it can be inferred that the ADP-ribose moiety of CS variants should be extensively degraded to remove inhibition.

SpH-proteinase is not inhibited by polymers of ADP-ribose

ADP-ribose polymers were synthesized *in vitro* and purified as described in the Experimental section. The polymers obtained were analysed by electrophoresis; it was observed that most of the molecules remained in the upper part of the gel and that oligomers were barely detectable (results not shown). It can therefore be predicted that the average size of these polymers was more than 38 ADP-ribosyl units [18]. To investigate whether these ADP-ribose polymers might behave as inhibitors of the SpH-proteinase, the polymers were added in increasing amounts to a proteolytic assay in which ^{14}C -labelled sperm histone H1 was used as substrate. As shown in Figure 3, only a very slight decrease in the activity of the proteinase was observed in the presence of increasing amounts of ADP-ribose polymers. This result was confirmed by fluorography of the proteins and peptides remaining after incubation with the SpH-proteinase (Figure 4). We therefore concluded that isolated polymers of ADP-ribose are not inhibitors of the SpH-proteinase.

DISCUSSION

Our results demonstrate that the poly(ADP-ribose) moiety of maternal CS histone variants protects them from being degraded by the nuclear SpH-proteinase after fertilization in sea urchins. As reported previously, this enzyme selectively removes the sperm-specific histones while the maternally derived histones remain intact. This SpH-proteinase has been found to be

activated in sea urchin zygotes shortly after fertilization, although it is also present in an inactive form in unfertilized eggs [4]. Consequently, our results explain the previously puzzling proteolytic selectivity displayed by the SpH-proteinase that participates in male pronucleus formation in sea urchins.

Inagaki et al. [19] have reported that isolated ADP-ribose polymers can behave as inhibitors of proteinases. They have identified ADP-ribose molecules whose chain length ranged from four to seven ADP-ribosyl units as potential inhibitors of a chromatin-associated chymotrypsin-like proteinase from rat peritoneal macrophages. The inhibition described by Inagaki et al. affects only one-third of this enzymic activity. In contrast, we have demonstrated that isolated polymers of ADP-ribose are not inhibitors of the nuclear SpH-proteinase from sea urchin zygotes. In contrast with the results of Inagaki et al. [19], the size of the polymers tested in this report was greater than 38 ADP-ribosyl units. At present the information available supports the idea that large multibranched polymers are mainly involved in the potential functions of poly(ADP-ribose) *in vivo*. As reported previously by Alvarez-Gonzalez and Jacobson [18], polymers of over 67 residues of ADP-ribose per molecule can be isolated from intact cells even under conditions of rapid turnover [20]. The size of these polymers increases significantly if cells are treated with DNA-damaging agents [20]. It therefore seems that the role of the proteinase inhibitor tested by Inagaki et al. as a protector against nuclear proteolysis was less representative of the ADP-ribose polymers that can be found *in vivo* and was also less efficient and quite non-specific. In contrast, our results clearly demonstrate that the SpH-proteinase can be inhibited completely but only when the ADP-ribose polymers are covalently bound to maternal histones. These results therefore give strong evidence for the potential role of poly(ADP-ribosylation) of nuclear proteins as a mechanism to protect them from proteolysis in an efficient and specific manner.

Other acceptors for poly(ADP-ribosylation) in the nucleus include somatic and embryonic histone variants as well as a great number of non-histone chromosomal proteins. Furthermore, it is well established that the poly(ADP-ribosyl) synthetase itself behaves as the major potential target for poly(ADP-ribosylation) in chromatin [8]. Interestingly, the poly(ADP-ribosyl) synthetase seems to be one of the initial targets of the proteinase encoded by the gene *ced3* in *Caenorhabditis elegans*. This gene product is activated when cells are committed to starting apoptosis [21].

So far it is unclear whether the poly(ADP-ribosylation) of nuclear proteins represents a more general mechanism designed to protect nuclear proteins other than CS histones from degra-

dation. However, in the context of the results presented here, poly(ADP-ribosylation) seems to be a specific manner in which nuclear proteins are preserved from proteolysis. We consider that this is an interesting question that remains open and should be investigated further.

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