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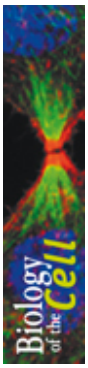


Cellular origin of the *Bufo arenarum* sperm receptor gp75, a ZP2 family member: its proteolysis after fertilization

Sonia L. Scarpeci, Mercedes L. Sanchez and Marcelo O. Cabada[1]

División Biología del Desarrollo, IBR (CONICET-UNR) and Area Biología, Facultad de Ciencias Bioquímicas y Farmacéuticas, UNR (Universidad Nacional de Rosario), Suipacha 531, Rosario S2002RLK, Republic of Argentina

Background information. The egg envelope is an extracellular matrix that surrounds oocytes. In frogs and mammals, a prominent feature of envelope modification following fertilization is the N-terminal proteolysis of the envelope glycoproteins, ZPA [ZP (zona pellucida) A]. It was proposed that ZPA N-terminal proteolysis leads to a conformational change in egg envelope glycoproteins, resulting in the prevention of polyspermy. *Bufo arenarum* VE (vitelline envelope) is made up of at least four glycoproteins: gp120 (glycoprotein 120), gp75, gp41 and gp38. The aim of the present study was to identify and characterize the baZPA (*B. arenarum* ZPA homologue). Also, our aim was to evaluate its integrity and functional significance during fertilization.

Results. VE components were labelled with FITC in order to study their sperm-binding capacity. The assay showed that gp75, gp41 and gp38 possess sperm-binding activity. We obtained a full-length cDNA of 2062 bp containing one ORF (open reading frame) with a sequence for 687 amino acids. The predicted amino acid sequence had close similarity to that of mammalian ZPA. This result indicates that gp75 is the baZPA. Antibodies raised against an N-terminal sequence recognized baZPA and inhibited sperm–baZPA extracted from VE binding. This protein does not induce the acrosome reaction in homologue sperm. Northern-blot studies indicated that the transcript is exclusively expressed in the ovary. *In situ* hybridization studies confirmed this and pointed to previtellogenic oocytes and follicle cells surrounding the oocyte as the source of the transcript. baZPA was cleaved during fertilization and the N-terminal peptide fragment remained disulfide bonded to the glycoprotein moiety following proteolysis.

Conclusion. From the sequence analysis, it was possible to consider that gp75 is the baZPA. It is expressed by previtellogenic oocytes and follicle cells. Also, it can be considered as a sperm receptor that undergoes N-terminal proteolysis during fertilization. The N-terminal peptide could be necessary for sperm binding.

Introduction

Amphibian oocytes are surrounded by an extracellular glycoprotein coat that participates in the interaction with the sperm and protects the embryo from physical injuries. *Bufo arenarum* VE (vitelline envelope) is formed by at least four glycoproteins: gp120, gp75, gp41 and gp38 (Barisone et al., 2002). This coat participates in the specificity of sperm binding, the induction of the acrosome reaction and the block to polyspermy. In mice, it was shown that the sperm binds to ZP3, undergoes the acrosome reaction and penetrates the ZP (zona pellucida), remaining tethered to the envelope by a weak secondary binding to ZP2 (Wassarman, 1999). A recent model has proposed that ZP2 would regulate the supramolecular structure of the ZP required to support sperm binding (Rankin et al., 2003).

[1]To whom correspondence should be addressed (email cabada@ibr.gov.ar).
Key words: amphibian, vitelline envelope, fertilization, gamete recognition, glycoprotein 75 (gp75), zona pellucida.
Abbreviations used: CGP, cortical granule product; DIG, digoxigenin; EHP, external hydrophobic patch; FE, fertilization envelope; gp, glycoprotein; IHP, internal hydrophobic patch; ORF, open reading frame; 5′-RACE, 5′-rapid amplification of cDNA ends; VE, vitelline envelope; ZP, zona pellucida; baZPA, *B. arenarum* ZPA homologue.

In amphibians, conflicting results have been published on the roles of the VE glycoproteins in *Xenopus laevis* and *Bufo japonicus* (Omata and Katagiri, 1996; Tian et al., 1997). More recently, other researchers provided evidence that in *Xenopus*, ZPA, ZPB and ZPC interact in a synergistic manner to promote sperm–envelope binding (Vo and Hedrick, 2000). It has been reported that ZPA homologues in different species participate in the interaction with sperm but also contribute to the ZP hardening when fertilization takes place, leading to the inhibition of polyspermy (Lindsay and Hedrick, 2004). The mechanisms involved in this process are not completely understood, but several proteases and glycosidases seem to take part in the elimination of sperm-binding sites and the modification of the extracellular matrix of the egg, making it impenetrable to additional spermatozoa. It was proposed that the proteolysis of the N-terminal region of the ZPA produced by the action of the cortical granules contents released in the process of fertilization leads to a conformational change in the protein. As a result, ZPA no longer binds to sperm, probably due to a modification in the three-dimensional structure of the VE (Kiefer and Saling, 2002; Jovine et al., 2004).

In the present study, we report that sperm can bind *in vitro* to gp75, gp41 and gp38. We have cloned gp75 and found that it is a homologue of sperm receptor ZP2 in mammals. The mRNA is expressed in early-stage oocytes and in the follicle cells, and the protein is synthesized in these cells as well. After the fertilization process, the N-terminal domain of the baZPA (*B. arenarum* ZPA homologue) undergoes proteolysis by a metalloprotease but remains bounded to the glycoprotein moiety via a disulfide bond.

Results

Sperm binding to the components of the VE

An important aspect in the fertilization process is the recognition and binding of spermatozoa to the VE. We used a sperm binding assay to evaluate sperm binding to isolated glycoprotein labelled with FITC. After extensive dialysis and filtration, the labelling reaction was checked by SDS/PAGE, all four components of the envelope proved to be labelled, and no free dye was observed (results not shown). Sperm suspensions were fixed with formaldehyde before incubation with each of the labelled glycoproteins, to avoid an acrosome reaction during the incubation periods.

Incubation of sperm with gp75–FITC (baZPA–FITC), gp41–FITC and gp38–FITC resulted in staining of the sperm head (Figures 1C–1H). BSA–FITC was used as a control (Figures 1A and 1B). Incubation of sperm with gp120–FITC or without FITC-labelled protein (results not shown) resulted in weak staining of whole sperm. These results indicate that gp75 (baZPA), gp41 and gp38 can be considered as candidates for the sperm binding to the VE.

Inhibition of sperm binding to baZPA with anti-baZPA N-terminal peptide

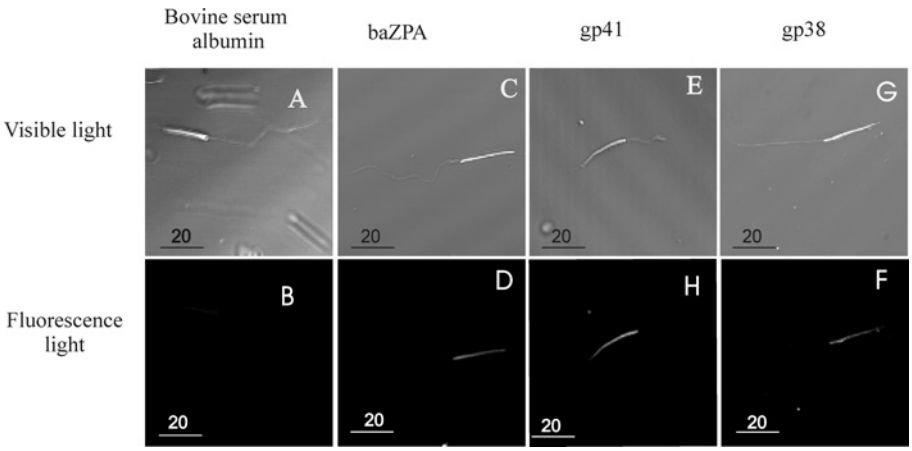
In the present study, we focused on baZPA, to find out specifically the role of N-terminal peptide of this molecule in sperm binding. We developed an antibody specific for the N-terminal domain of baZPA. To test whether antibodies against baZPA N-terminal peptide could inhibit sperm binding to baZPA, FITC-labelled baZPA were isolated from both VE and FE (fertilization envelope). Both proteins were incubated, separately, with the antibody against N-terminal baZPA. After incubations, spermatozoa were treated with these preparations. In the case of baZPA isolated from VE, a significant decrease in binding (evaluated as the number of stained sperm heads) was observed. We also found that in this experiment, the binding decreased as a function of the antibody concentration used during the incubations.

On the other hand, binding of the sperm to the baZPA obtained from FE was significantly lower than to the baZPA isolated from VE. In the competitive inhibition assay using baZPA from FE, incubation with the specific antibodies showed no significant differences in sperm binding levels when compared with the results obtained without the antibodies. No significant decrease in stained sperm heads was observed in control samples incubated with normal rabbit serum, even at high concentrations (Figure 2).

Induction of the acrosome reaction

As was previously shown, the solubilized VE was able to induce acrosome breakdown in homologous sperm in the conditions assays (Barisone et al., 2002). We tested whether the solubilized baZPA from VE and FE induced acrosome breakdown in homologous

Figure 1 | Binding of VE glycoproteins to sperm evidenced by immunofluorescence
B. arenarum spermatozoa were treated with BSA–FITC (**A**, **B**), gp75–FITC (**C**, **D**), gp41–FITC (**E**, **F**) or with gp38–FITC (**G**, **H**), as indicated in the Materials and methods section. (**A**, **C**, **E**, **G**) Fluorescence images. (**B**, **D**, **F**, **H**) Visible light micrographs (×1000). Scale bars, μm.



sperm. Acrosomal status was evaluated using a technique developed in our laboratory (Martinez and Cabada, 1996). For each experiment, controls were run in parallel, incubating sperm in saline medium without baZPA for the same times (Figure 3). In a series of three experiments, no significant differences were observed between baZPA from VE-treated, baZPA from FE-treated and control suspensions, indicating that baZPA does not induce the acrosome reaction in *B. arenarum* sperm. For each experiment, sperm were incubated with solubilized VE as well.

gp75 is a homologue of mammalian ZP2

The full-length *B. arenarum* gp75 cDNA consists of a sequence of 2062 nt with a single ORF (open reading frame) (see Supplementary Figure S1 at http://www.biolcell.org/boc/100/boc1000219add.htm). Both the 5′- and 3′-untranslated regions flanking the coding sequence were rather short, with 54 nt from the start methionine residue and 72 nt from the stop codon to the poly A tail respectively. The AUG initiation codon was identified by the −3, +4 base motif (CNNAUGG) associated with vertebrate initiator codons (Kozak, 1991). The polyadenylation site, AATAA, is located 11 nt upstream of the poly A tail and 28 nt downstream of the stop codon. The ORF encodes a polypeptide of 687 amino acids, with a calculated molecular mass of 76 789 Da. It contains the chemically determined peptide sequence at the N-terminus of mature gp75 protein (see Supplementary Figure S1, underlined residues). Using the algorithm of Bendtsen et al. (2004), the signal peptide cleavage site was predicted to occur between Ala^{34} and Leu^{35}, yielding a signal peptide of 21 amino acids.

The hydropathy plot of the translated protein showed strong hydrophobicity in the N-terminal (signal peptide) and C-terminal regions (see Supplementary Figure S2A at http://www.biolcell.org/boc/100/boc1000219add.htm). The C-terminal transmembrane domain (residues 659–681) probably reflects the secretory pathway of this glycoprotein and its targeting to the extracellular matrix of *B. arenarum* eggs. A dibasic amino acid motif (Lys^{617}-Arg^{618}) 41 residues upstream from the transmembrane domain may serve as a convertase or furin-like cleavage site; such sites are characteristic of glycoproteins biosynthesized via the constitutive secretory pathway. Cleavage at the furin-like site releases the glycoproteins from its membrane anchor for the subsequent assembly into the egg envelope.

In mouse, the assembly of the ZP proteins is believed to be dependent on two regions, the EHP (external hydrophobic patch) and the IHP (internal hydrophobic patch) (Jovine et al., 2004). Alignment of the amino acid regions constituting the IHP and EHP of mouse, human and *X. laevis* with the amino acid sequence of gp75 and *Discoglossus pictus* ZP2

Figure 2 | Effect of baZPA antibody on the binding of baZPA (from VE or FE) to spermatozoa

Bars indicate sperm binding levels for sperm binding to: 1, VE (100 μg/ml); 2, baZPA from VE (100 μg/ml); 3–5, baZPA from VE (100 μg/ml) pre-incubated in 1:10, 1:100 or 1:1000 dilution of antibodies for 1 h respectively; 6, baZPA from FE (100 μg/ml); 7–9, baZPA from FE (100 μg/ml) pre-incubated in 1:10, 1:100 or 1:1000 dilution of antibodies for 1 h respectively; 10, baZPA from VE pre-incubated in 1:10 dilution of pre-immune rabbit serum for 1 h; 11, BSA (100 μg/ml). Errors bars represent S.E.M. for three experiments.

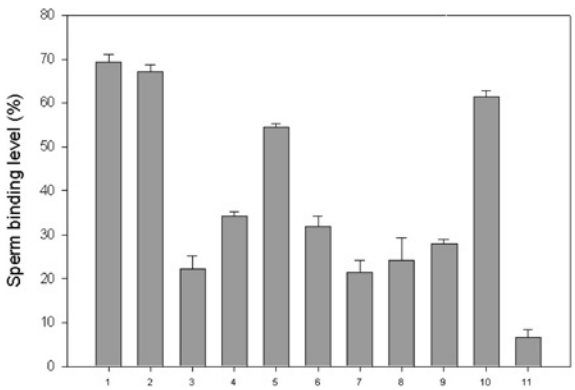
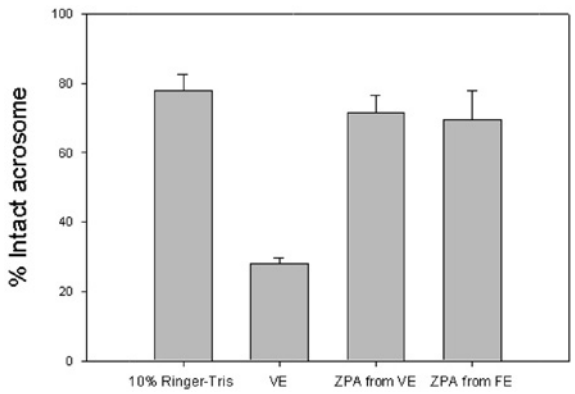


Figure 3 | Effects of purified baZPA from VE and FE on sperm acrosome reaction

Sperm were incubated either with solubilized baZPA from VE or baZPA from FE in a hypo-osmotic medium (10% Ringer/Tris) or 10% Ringer/Tris (control) or with solubilized VEs in 10% Ringer/Tris. Errors bars represent S.E.M. for three experiments. Values for incubation of sperm with baZPA from VE and baZPA from FE were not significantly different from that for incubation of sperm with 10% Ringer/Tris ($P < 0.05$).



shows the presence of two high-homology regions (see Supplementary Figure S2B).

In addition, we found a conserved ZP domain present in most of the sequences informed for envelope glycoproteins in many species. In our sequence, this domain is 266 residues long (Lys^{350}-Ser^{616}). It contains the invariant ten cysteine residues and two glycine residues immediately after and before the third and sixth cysteine residues respectively found in other ZPA/ZPB-like proteins (see Supplementary Figure S2B).

Four potential N-glycosylation sites were found in the proposed mature form of the protein. Numerous potential O-linked glycosylation sites (serine or threonine) were also found in the sequence.

A GenBank® Nucleotide Sequence Database search revealed that the primary sequence of *B. arenarum* gp75 is homologous with the mammalian ZP glycoprotein ZP2 family and *X. laevis* ZPA. The level of sequence identity was found to be highly significant, with the probability of a random match between the *B. arenarum* gp75 and various mammalian and non-mammalian ZPA family members between 4×10^{-169} and 3×10^{-87}. Pairwise sequence alignments revealed 44–46% overall sequence identity between the *B. arenarum* gp75 and the other amphibians ZPA proteins and 34–37% between *B. arenarum* gp75 and the human, mouse and pig ZP2 proteins.

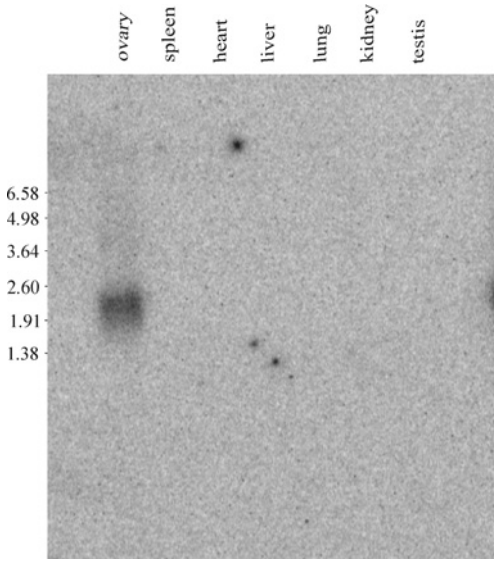
A dendrogram of the phylogenetic relationship of envelope components confirmed that the sequence represented a ZPA family component (see Supplementary Figure S2C).

Tissue and cellular distribution of baZPA mRNA

The distribution of baZPA mRNA was analysed by Northern-blot hybridization. Total RNA from ovary, spleen, heart, liver, lung, kidney and testis were electrophoresed on a formaldehyde-denaturing agarose gel and blotted on to a nylon membrane. The transcript was detected only in the ovary with a length of 2.2 kb (Figure 4), which is consistent with the length of the baZPA cDNA (2062 bp) (Supplementary Figure S1 of the Supplementary data).

The distribution of baZPA mRNA in the ovary was further investigated by *in situ* hybridization (Figure 5). The transcript was localized in the cytoplasm of previtellogenic oocytes and in the follicle cells. The oocytes bearing a strongly positive signal are stages

Figure 4 | Northern-blot analysis of baZPA
Total RNA from different organs were electrophoresed on a formaldehyde-denaturing agarose gel and blotted on to a nylon membrane. They were hybridized with a radiolabelled probe prepared with a 496 bp DNA fragment (406–901) as a template.



I–II, as judged by the morphology and the diameter of the oocytes (<450 μm) according to Dumont (1972) (Figures 5A and 5B). In mature oocytes, the signal was absent from the cytoplasm (Figure 5A). As it is difficult to observe the exact position of the follicle cells under light microscopy, nuclear staining with the dye Hoechst 258 was carried out using the same slices.

Further demonstration of the origin of the glycoprotein baZPA was provided by immunostaining with polyclonal antibodies against the N-terminal region of baZPA. Slices of pieces of ovaries containing follicles with oocytes of different sizes were treated as indicated in the 'Materials and methods' section. Follicles of different sizes from different animals were studied. We present the results in previtellogenic oocytes (50–200 μm oocytes) and full-grown ones (1400 μm). In the first case (Figure 6A), a dense layer of coloured reaction (indicating the presence of antigens from baZPA) is observed in the follicle cells. Positive reaction in the oocytes is also observed.

In order to visualize the exact position of the follicle cells, the same slices were stained with Hoechst 258 (Figure 6B). This result confirms that the antigens of the glycoprotein baZPA are indeed in the follicle cells.

The same procedure was carried out with follicles containing fully-grown oocytes showing the presence of baZPA in the follicle cells, the oocyte cytoplasm and the VE (Figures 6D and 6E). Rabbit pre-immune serum was used in specificity controls (Figures 6G and 6H).

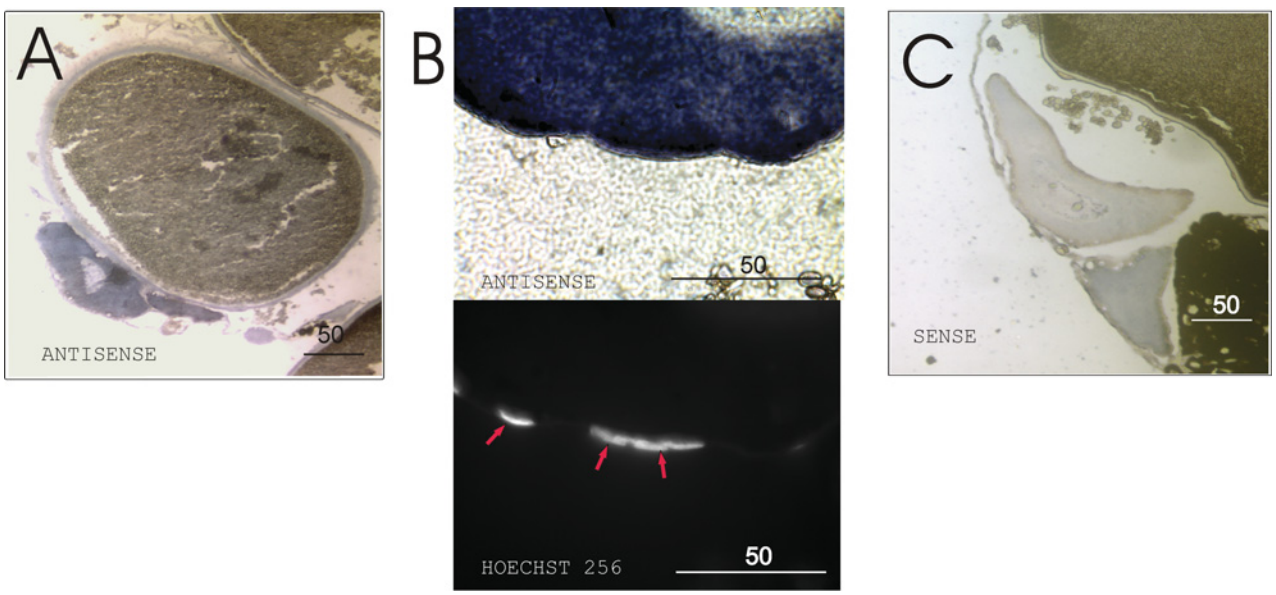
baZPA cleavage during the conversion of VE into FE

The N-terminal region of the ZPA presents a cleavage site for proteases Asp-Asp/Glu that is conserved in vertebrates (Lindsay and Hedrick, 2004). In order to determine whether the peptide remains bound to the rest of the protein after fertilization, we developed an antibody against this 28-amino-acids polypeptide. This antibody proved to be specific for gp75, supporting our hypothesis that gp75 is the homologue of ZPA in *B. arenarum.* This antibody was used in a Western-blot assay of the envelope egg samples treated under reducing and non-reducing conditions (Figure 7). Under reducing conditions, the envelope from unactivated eggs (VE) showed strong reactivity to the antibody, but the envelope from embryos (FE) and the envelope from unactivated eggs treated with CGPs (cortical granule products) (CGP-treated VE) showed much lower signal, indicating the loss of the ZPA N-terminal peptide. This low reactivity could be explained considering that not all the baZPA proteins in these samples were proteolytically cleaved in the fertilization process. However, under non-reducing conditions, the envelopes from both the VE and FE showed the same level of antibody binding, indicating that the ZPA N-terminal fragment remained linked to the ZPA glycoprotein fragment by disulfide bonds. Examination of the baZPA amino acid sequence (see Supplementary Figure S1) confirmed the existence of a cysteine residue in the N-terminal domain peptide that could be involved in such a disulfide bridge.

Immunostaining procedures were carried out using the antibodies with embryo sections. A positive reaction was observed, supporting the idea that the proteolytic process that takes place after fertilization does not completely release the N-terminal peptide from embryos (Figures 6G and 6H).

Figure 5 | Distribution of baZPA mRNA in the ovary by *in situ* hybridization

Paraffin sections of *B. arenarum* ovary were treated as indicated in the 'Materials and methods' section and hybridized with DIG-labelled antisense [(**A**) and upper panel of (**B**)] or sense (**C**). (**B**) Lower panel: the same paraffin section stained with Hoechst 258. Arrows indicate the nuclei of follicle cells. The specifically hybridized probe was visualized with an alkaline phosphatase-coupled anti-DIG antibody. The signal was detected in the cytoplasm of previtellogenic oocytes (stained dark blue) and in the somatic cells. Magnification: (**A**, **C**) ×400 and (**B**) ×1000. Scale bars, μm.



Discussion

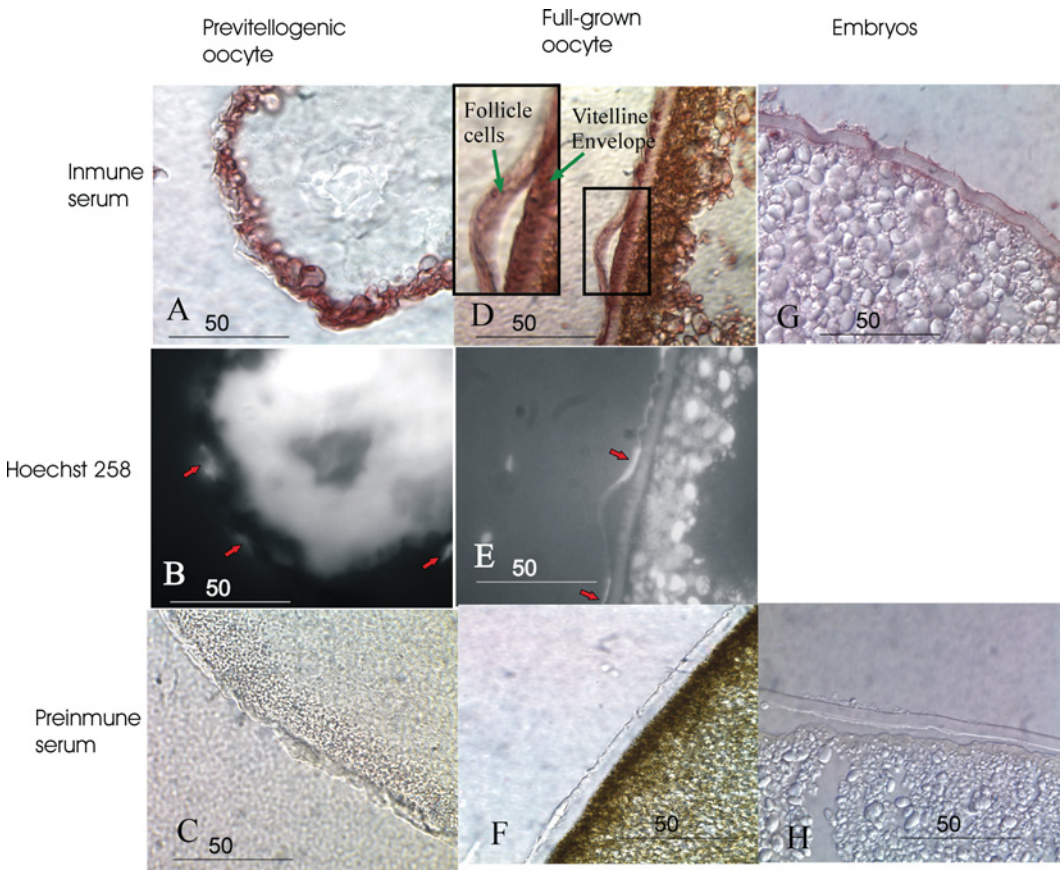
The egg envelope in vertebrates has been reported to be composed of ZP glycoproteins (Wassarman, 1988; Takeuchi et al., 2001; Primakoff and Myles, 2002). Many of these ZP glycoproteins have been isolated and cloned to date and form a large ZP glycoprotein family. The composition of the ZP glycoproteins expressed in one species shows obvious variations among vertebrates, especially non-mammalian ones. For example, while three kinds of glycoproteins (ZPA/ZP2, ZPB/ZP1 and ZPC/ZP3) are expressed in mice (Wassarman, 1988), eight and five genes encoding ZP glycoproteins have been found in *Oryzias latipes* (Kanamori et al., 2003) and *X. laevis* (Spargo and Hope, 2003) respectively. The biological significance of these variations is not well understood.

In *B. arenarum*, not all the VE proteins were characterized molecularly. As it was previously observed (Barisone et al., 2003; Spargo and Hope, 2003), the VE of the oocyte of *B. arenarum* is composed of at least four glycoproteins, originally named as gp120, gp75, gp41 and gp38. The homologues of ZPB (baZPB; G.A. Barisone and M.O. Cabada, unpublished data) and ZPC (Barisone et al., 2007) have been cloned, but the correlation between the obtained sequences and the isolated glycoproteins has not been determined to date.

We have cloned the gp75 component and clearly establish that it is the homologue of ZP2/ZPA in mammals. One of the conserved sequence features is the ZP domain, the putative cleavage site for furin-like protease (RXK/RR) and the putative C-terminal transmembrane domain. The hydropathy plots of these proteins are also similar. The ZP domain consists of ~260 residues common to proteins of apparently diverse functions and it can be considered as a 'polymerization module' in mammals (Jovine et al., 2002). It was possible to localize an IHP inside the ZP domain and an EHP between the putative furin cleavage site and the transmembrane domain. These regions were first described in mouse (Jovine et al., 2004) and it was found that in *B. arenarum*, there are many conserved amino acids. In the proposed model, it has been suggested that there is an interaction between EHP and IHP of ZP precursor proteins and, in this way, the ZP protein is prevented from

Figure 6 | *In situ* detection of baZPA antigens

Light micrograph of an ovarian follicle containing previtellogenic oocyte (**A**, **B**, **C**), an ovarian follicle containing full-grown oocyte (**D**, **E**, **F**) and embryo sections (**G**, **H**). (**A**, **D**, **G**) Slices were submitted to the procedure outlined in the 'Materials and methods' section for detection of baZPA. (**B**, **E**) The sibling slip of (**A**) and (**D**) respectively stained with Hoechst 258. Red arrows indicate the nuclei of follicle cells. Green arrows indicate the follicle cells and VE. In (**D**) the whole oocyte's cytoplasm is fluorescent. (**C**, **F**, **H**) Control sections showing no coloured staining. Magnification: (**A**, **B**) ×400 and (**C**, **D**, **E**, **F**, **G**) ×1000. Scale bars, μm.



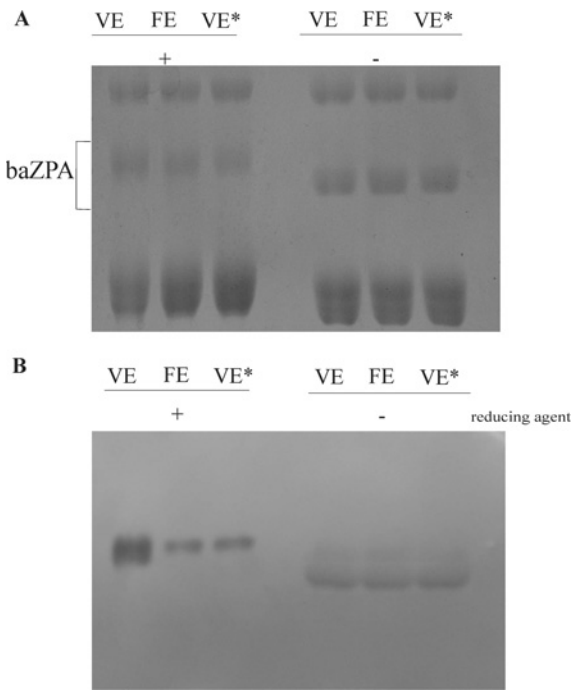
participating in assembly within the cell. The conservation of these interacting regions provides further evidence that the process forming the vertebrate eggshell is similar in amphibians and mammals.

The sequence of the first 28 amino acids in the N-terminal region shares 50% identity with *X. laevis*. Particularly, the eight amino acids surrounding the cleavage site Asp^{156}-Asp^{157} are identical. SDS/PAGE analysis under non-reducing and reducing conditions showed the presence of intramolecular bonds. The incubation after the oocyte activation with CGP produces a proteolytic cleavage of baZPA (Barisone et al., 2003). Nevertheless, this peptide remains bound to the molecule through the disulfide bonds as was shown by Western blot and immunohistochemical observation of embryo sections. Further studies should be performed in order to establish whether this process contributes to a conformational change of the protein, which facilitates the hardening of the envelope contributing to the block to polyspermy. The cortical granule enzyme responsible for the digestion of the baZPA remains to be purified and cloned, but previous studies showed that it has the characteristics of a pH-dependent metalloprotease (Barisone et al., 2003).

Notwithstanding the highly conserved nature of the glycoproteins that constitute the ZP or VE, important species-specific differences exist in the cellular mechanisms that underpin the creation of this pivotal structure.

Figure 7 | Immunodetection of the N-terminal peptide of baZPA before and after proteolysis

Solubilized VEs from oocytes, CGP-treated VEs ('VE*') and FEs from embryos ('FE') were run on an SDS/8% PAGE gel under reducing (+) and non-reducing (–) conditions. In each lane, 20 μg of protein was loaded. (**A**) Coomassie Brilliant Blue-stained gel; (**B**) an identical gel blotted on to nitrocellulose and probed with antibodies to the baZPA N-terminal fragment.



Mammalian and amphibian egg envelope glycoproteins are biosynthesized via the constitutive secretory pathways (Hosaka et al., 1991). In most of the informed glycoproteins, this process takes place in the ovary. However, in fish and birds, VE proteins are synthesized in either the liver or ovary, or in both (Chang et al., 1997; Bausek et al., 2000; Vaccaro, 2001; Kanamori et al., 2003; Modig et al., 2006). We detected baZPA mRNA exclusively in the ovary. In other organs such as spleen, heart, liver, lung, kidney and testis, baZPA mRNA was not present. Some disagreement exists as to the role of cumulus cell in this regard (Lee and Dunbar, 1993; Epifano et al., 1995). In mice, monkeys, human and *X. laevis*, zona proteins are expressed and assembled exclusively by the oocyte and not by the granulose cells (Kubo et al., 1997, 2000; Eberspaecher et al., 2001; Zhao and Dean, 2002; Rankin et al., 2003). In bull, rabbit, dog and *D. pictus*, the zona proteins are differentially expressed by the oocyte and the granulose cells (Lee and Dunbar, 1993; Sinowatz et al., 2001; Vaccaro, 2001; Blackmore et al., 2004).

By *in situ* hybridization, we found that baZPA mRNA is expressed in previtellogenic oocytes and in the follicle cells. This is in good agreement with immunohistochemical observation that baZPA protein is localized both in follicle cells and in oocytes.

The *B. arenarum* VE is a thick extracellular matrix. When it is completely formed, it is between 8 and 15 μm wide, whereas in *Xenopus*, where the VE components are expressed exclusively in previtellogenic oocyte, it is approx. 1 μm wide. The production of the substantial *B. arenarum* VE represents a considerable burden to the oocyte and could represent a serious challenge to the protein synthetic machinery of this cell if not for the participation of the follicle cells.

In amphibians, the VE glycoproteins are involved in the recognition of the sperm and the protection of the oocyte outside the body, since these animals present external fertilization. The classic paradigm sperm–egg envelope binding is believed to involve a single egg envelope glycoprotein ligand interacting with a receptor on the sperm surface. However, discrepancies with this model have been reported in studies with porcine (Yurewicz et al., 1998) and bovine models (Yonezawa et al., 2001). Moreover, experiments performed with specific antibodies against ZP components have suggested that all human ZP glycoproteins participate in the interaction with spermatozoa (Koyama et al., 1991; Tsubamoto et al., 1999; Rath et al., 2002; Sivapurapu et al., 2002). In agreement with these findings, an alternative model for mice has been recently proposed, in which sperm interaction with ZP glycoproteins would take place with a supramolecular ZP structure involving all components (Rankin et al., 2003).

Localization of sperm receptor for the VE ligands was studied using fluorescence microscopy techniques. Formaldehyde-fixed sperm were used in this assay to avoid acrosome breakdown during the incubation for long times. Therefore our results apply for non-reacted sperm; gp75 (baZPA), gp41 and gp38 seem to be sperm receptors. These results are in good agreement with previous studies carried out in our laboratory (Barisone et al., 2007). Competition experiment using specific antibodies to the baZPA

N-terminal peptide shows that sperm binding to baZPA from VE can be inhibited when this protein is pre-incubated with the antibodies. It has been shown that sperm are unable to bind to the FE (Barisone et al., 2002). When binding experiments were performed with baZPA from FE, a dramatic decrease in sperm binding occurs, but pre-incubation with the antibodies does not have any effect on sperm binding. This fact may indicate that N-terminal peptide is necessary for sperm binding. Although it was previously shown that VE is able to induce acrosome breakdown in homologous sperm (Barisone et al., 2002), our results suggest that baZPA by itself does not trigger the acrosome reaction.

In conclusion, our results clearly demonstrate that the gp75 component of *B. arenarum* VE is the ZPA homologue. We have obtained its cDNA and the N-terminal sequence. *In situ* experiments prove that ZPA is expressed in the ovary by oocytes and follicle cells. Specific antibodies raised against ZPA allowed us to show that this protein undergoes proteolytic processing during fertilization and that this N-terminal peptide is necessary for sperm binding.

Further experiments are in progress to describe the functional consequences of this processing.

Materials and methods

Animals and gametes

Sexually mature *B. arenarum* specimens were collected in the neighbourhood of Rosario city and kept in a moist chamber at 12°C until used. Experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* of Facultad de Ciencias Bioquímicas y Farmacéuticas, UNR (Rosario, Republic of Argentina).

Testes and spermatozoa were obtained as described elsewhere (Barisone et al., 2002).

Female specimens were kept in a moist chamber at 20–22°C for 1 day before stimulation with one homologous hypophysis homogenate injected intracoelomically. After 10–12 h, oocyte strings were collected from ovisacs (Houssay et al., 1929).

Envelope isolation and solubilization

VE and FE isolation was performed essentially as described by Barisone et al. (2002). VEs were solubilized in distilled water by heating at 100°C for 5–10 min or in 8 M urea. FEs were solubilized in 8 M urea.

Solubilized VEs and FEs were centrifuged at 10 000 *g* for 20 min at 4°C, and the supernatant solutions were stored at −20°C for up to 2 months. Envelope glycoprotein concentration was measured by the Folin phenol method (Lowry et al., 1951).

Cortical granule collection

CGP was obtained as described previously (Barisone et al., 2003).

Proteolytic activity

Proteolytic activity of CGP was assayed on VE preparations by incubating intact VE suspended in distilled water (5 µl) with 5 µg of total CGP protein and 30 µl of Ringer/Tris for 60 min at 25°C as previously described (Barisone et al., 2003). The VEs were then rinsed in Ringer/Tris solution and centrifuged to eliminate excess CGP proteins. The CGP-treated VE were solubilized in sample buffer (2% SDS, 10% glycerol, 0.125 M Tris/HCl, pH 6.8, and 0.005% Bromophenol Blue) and analysed by SDS/PAGE.

Protein labelling: sperm binding to isolated components of VE

Solubilized VEs and BSA were labelled with FITC as described elsewhere (Barisone et al., 2002). FITC-labelled VE samples were submitted to SDS/10% PAGE under non-denaturing conditions in preparative gels. The bands corresponding to each VE glycoproteins were excised from the gel and electroeluted by using an Electroeluter System (Bio-Rad, Hercules, CA, U.S.A.). SDS was eliminated by several washes with 4 vol. of cold acetone (80%) by centrifugation at 13 000 *g* for 15 min. The pellet was allowed to dry and resuspended in water for 30 min at 4°C. The purity and integrity of labelled protein were analysed by SDS/PAGE.

Sperm suspensions were prepared as described previously (Barisone et al., 2002). Fixed sperm were washed twice with PBS (2-fold volume), and finally resuspended in the same buffer (starting volume). Then, 100 µl samples were pelleted at 1200 *g* for 5 min, and resuspended in 100 µl of each labelled VE glycoprotein (1 mg/ml) or BSA–FITC (controls). After incubating for 45 min at 20–22°C, sperm were washed twice with PBS, pelleted as mentioned above and mounted in 0.1% phenylendiamine/50% (v/v) glycerol/PBS for fluorescence microscopy. Image acquisition was performed with an Axioplan 2 Imaging, LSM 5 Pascal confocal microscope kindly provided by Carl Zeiss.

Polyclonal antibodies

A 28-amino-acid peptide corresponding to the N-terminal region of the characterized protein was used to obtain antibodies that do not cross-react with the rest of the VE glycoproteins. Specific oligonucleotides that cover the N-terminal region and carry restriction sites BamHI and EcoRI were used in PCR reactions by using as a template the 5′-RACE (5′-rapid amplification of cDNA ends) clone corresponding to the N-terminal region of ZPA and the product was cloned into pGEM T Easy vector (Promega, Madison, WI, U.S.A.) and subcloned into an expression vector pGEX-2T glutathione transferase and then submitted to the conditions suggested by the manufacturer (Amersham Biosciences, Uppsala, Sweden). Cultures of the transformed cells were induced with IPTG (isopropyl β-D-thiogalactoside) to produce the recombinant protein. The purified protein was used in a standard immunization protocol in rabbits. No cross-reactivity with other VE components when assayed on Western blot of VE glycoproteins was observed.

Inhibition of sperm binding to baZPA with anti-baZPA N-terminal peptide

To test the effects of anti-baZPA antibodies on sperm–baZPA binding, 1 µg of FITC-labelled baZPA purified from either VE or FE was pre-incubated with 1:10, 1:100 or 1:1000 antibodies or pre-immune rabbit serum (1:10) for 1 h. The

antibodies-treated protein, as well as proteins not treated with antibodies, was used in the sperm binding assay as described above. Approx. 100 cells were evaluated in each of the three experiments for each condition assayed. Preparations were counted under an Olympus BH-2 epifluorescence microscope (Olympus Optical Co., Tokyo, Japan).

Acrosome reaction assays

Sperm were incubated either with solubilized baZPA from VE or baZPA from FE in a hypo-osmotic medium (10% Ringer/Tris) or 10% Ringer/Tris (control) or with solubilized VEs in 10% Ringer/Tris. In the conditions assays, solubilized VEs are able to induce acrosome breakdown in *B. arenarum* sperm (Barisone et al., 2002). Sperm (6.4×10^5) were incubated in 100 μl of medium containing baZPA from VE (1 μg), baZPA from FE, solubilized VEs and 10% Ringer/Tris for 10 min at 18°C. Ringer/Tris (1.4 ml) was immediately added to each aliquot to turn the medium back to iso-osmotic. Sperm were pelleted at 550 *g* for 5 min at 18°C and finally resuspended in 20 μl of Ringer/Tris.

Assessment of acrosome status

Acrosome status was assessed using an indirect immunofluorescence technique (Martinez and Cabada, 1996). Briefly, sperm were immobilized on polylysine-coated coverslips, fixed with 3% formaldehyde in PBS and permeabilized with 0.2% Triton X-100. Acrosomal content was detected using an antigen-specific primary antibody and a Cy3-coupled secondary antibody. In this way, fluorescence in the anterior end of the sperm head indicated presence of the acrosomal content and, therefore, non-reacted sperm. Approx. 100 cells were evaluated in each of the three experiments for each condition assayed. Preparations were counted under an Olympus BH-2 epifluorescence microscope (Olympus Optical Co.).

Statistical analysis

In the sperm binding inhibition experiments, results were analysed with non-parametric ANOVA techniques and multiple comparisons based on the Kruskal–Wallis method (range addition).

In the acrosome reaction assays, results were analysed by ANOVA to establish the significance of the percentage of acrosome-reacted sperm in baZPA from VE, baZPA from FE, VE and control media.

In all cases, comparisons were considered to be significantly different when P values were <0.05.

Northern blot

RNAs from different tissues (ovaries, spleen, heart, liver, lung, kidney and testis) were obtained using TRIzol® reagent (Gibco BRL) and 10 μg of total RNA was separated on 1% agarose gels with formaldehyde. The RNA was blotted on to a nylon membrane (Amersham Biosciences, Little Chalfont, Bucks., U.K.), followed by UV cross-linking. A 496 bp PCR product specific for the N-terminal region of the ZPA was used as a probe. Labelling reaction was done with Prime-a-gene Labeling System (Promega) and ^{32}P-labelled dATP (Amersham Biosciences, Little Chalfont, Bucks., U.K.). The blot was prehybridized for 4 h at 65°C in 6 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate), 2 × Denhardt's reagent (1 × Denhardt's = 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone and 0.02% BSA) and 0.5% SDS. Hybridization was performed overnight at 65°C in the prehybridization solution with 50 ng of probe. The membrane was washed three times for 25 min at 65°C in 2 × SSC and 0.5% SDS, exposed to a Storage Phosphor Screen and visualized using a Storm Scanner and Software (Amersham Biosciences).

Probe synthesis for *in situ* hybridization

A PCR product of approx. 500 bp corresponding to the N-terminal region of baZPA was obtained using the 5′-RACE clone as a template and the following oligonucleotides: 5eu: 5′-AAAggATCCACCATggACTCTgAATACATg-3′ with a BamHI site; and IN71: 5′-AAAAAgCTTgTCCAgCATTATA-3′ with a HindIII site. The product was cloned into a pCR2.1TOPO vector and subcloned into a pBluescriptII SK$^-$ vector (Stratagene, La Jolla, CA, U.S.A.) by using the restriction sites mentioned above. The plasmid was linearized by digestion with BamHI or HindIII, and the antisense and sense RNA probes were synthesized using T3 or T7 polymerase (Boehringer Mannheim). They were then labelled with DIG (digoxigenin) using DIG RNA-labelling mix (Roche Diagnostics, Indianapolis, IN, U.S.A.) according to the manufacturer's instructions. The cRNA probes were precipitated and resuspended in hybridization buffer.

In situ hybridization

Pieces of *B. arenarum* ovary were fixed with 4% (w/v) paraformaldehyde in PBS overnight at 4°C. Paraffin sections were cut to 1 μm, spread on gelatinized slips and dried. The sections were dewaxed with xylene, rehydrated with a series of ethanol concentrations, and post-fixed with paraformaldehyde, followed by digestion with proteinase K and H_2O_2 treatment in order to diminish the colour background of the oocyte's pigments (Sanchez Riera et al., 1988). Hybridization was performed with the antisense or sense probe, which was diluted with hybridization buffer at a concentration of 500 ng/ml at 50°C for 16 h in a moist chamber as described by Parhar et al. (1996). An alkaline phosphatase-conjugated anti-DIG antibody was added according to the manufacturer's recommendations (Boehringer Mannheim). Colour development was performed using NBT/BCIP (Nitro Blue Tetrazolium/5-bromo-4-chloroindol-3-yl phosphate) (Promega). To localize follicle cells, sections were stained with Hoechst 258 (Cross and Overstreet, 1987). After a 15 min incubation, the sections were washed with PBS, covered with mounting solution (0.1% diphenylamine and 50% glycerol in PBS) and coverslips were examined under light microscopy and fluorescence microscopy with a Nikon Eclipse E800.

Western blot

Electrophoresis was carried out as previously described (Barisone et al., 2007). Briefly, 20 μg of protein from dissolved VE, from VE treated with CGP as mentioned above or from FE were subjected to SDS/PAGE using 8% gels under denaturing, reducing and non-reducing conditions. Proteins were electrotransferred on to nitrocellulose membranes overnight at 30 mA/gel (MiniProtean II Gel System; Bio-Rad). Gels were processed for Coomassie Brilliant Blue staining or electrotransferred on to nitrocellulose membranes (Towbin et al., 1979). Membranes were washed twice with PBS and then blocked with PBS buffer supplemented with 5% non-fat dried milk.

The N-terminal baZPA was detected with the rabbit polyclonal antibody generated against the fusion protein diluted 1:2500 in PBS/milk. Membranes were incubated for 2 h with agitation. After being washed three times with PBS, the membranes were incubated (at 22°C for 1.5 h) with an anti-goat secondary antibody coupled with horseradish peroxidase (1:5000 dilution in PBS; Amersham Biosciences, Piscataway, NJ, U.S.A.). Antibody binding was detected using a peroxidase substrate solution: 1.25 ml of 0.8% AEC (3-amine-9-ethylcarbazole) in dimethylformamide, 12.5 µl of 30% H_2O_2 in 25 ml final volume of 50 mM acetate buffer (pH 5.0). When colour developed, the excess of developing solution was washed away with PBS.

Immunolocalization

Paraffin-embedded sections of small ovary pieces containing follicles of different sizes, and embryos were treated as described by Cabada et al. (1996). The first antibody was added at a 1:250 dilution in PBS and was left for 1 h (Harlow, 1988). Controls were treated with pre-immune serum under the same conditions. To localize follicle cells, sections were stained with Hoechst 258 (Cross and Overstreet, 1987). After a 15 min incubation, the sections were washed with PBS and covered with mounting solution (0.1% diphenylamine and 50% glycerol in PBS) and coverslips were examined under light microscopy and fluorescence microscopy with a Nikon Eclipse E800.

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