Glucose-regulated protein 78 (Grp78/BiP) is secreted by human oviduct epithelial cells and the recombinant protein modulates sperm–zona pellucida binding

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Objective: To determine the secretion of Grp78 by human oviduct epithelial cells, its association to spermatozoa, and its involvement in gamete interaction.
Design: Prospective study.
Setting: Basic research laboratory.
Subject(s): Semen samples obtained from normozoospermic volunteers. Tubal tissue provided by patients undergoing hysterectomies. Oocytes collected from women undergoing IVF-ET.
Intervention(s): Analysis of Grp78 expression and secretion by oviductal tissue. Gamete incubation with recombinant Grp78 (rec-Grp78).
Main Outcome Measure(s): Assessment of protein expression and secretion by immunohistochemistry and Western immunoblotting, respectively. Evaluation of rec-Grp78 binding to human spermatozoa by immunocytochemistry, and analysis of its effect upon gamete interaction using the hemizona assay.
Result(s): Grp78 was found in the surface of oviduct epithelial cells. Soluble Grp78 was detected in oviductal fluids from women in the periovulatory period and in oviductal tissue conditioned medium. Rec-Grp78 was able to bind to the sperm acrosomal cap, and its presence during gamete interaction led to a decrease in the number of spermatozoa bound to the zona pellucida (ZP). When calcium ions from the incubation medium were replaced by strontium, rec-Grp78 enhanced sperm–ZP interaction.
Conclusion(s): Grp78 is expressed and secreted by oviduct epithelial cells. The protein would bind to the gametes and may modulate their interaction in a calcium-dependent manner. (Fertil Steril® 2010;93:1574–84. ©2010 by American Society for Reproductive Medicine.)

Key Words: Grp78, BiP, chaperone, oviduct, spermatozoa, zona pellucida, gamete interaction

Mammalian fertilization takes place in the ampulla region of the oviduct, as a result of successful interaction between the female and male gametes. Ejaculated spermatozoa are unable to fertilize an oocyte; full fertilization competence is acquired after their residence in the female reproductive tract, where sperm cells undergo several structural and functional changes collectively known as capacitation. Sperm capacitation has been mainly associated with modifications in the sperm plasma membrane composition and fluidity, changes in intracellular ion concentrations, generation of low and controlled levels of reactive oxygen species, and an increase in protein phosphorylation. Spermatozoa that have completed capacitation bind to the extracellular matrix that surrounds the oocyte (zona pellucida [ZP]) and undergo the release of the acrosomal content (acrosomal exocytosis [AE]). Acrosome-reacted spermatozoa penetrate the ZP, reach the perivitelline space, and finally bind and fuse to the oocyte plasma membrane [reviewed by (1, 2)].

Several lines of evidence indicate that the oviductal environment has an essential role in allowing the completion of gamete fertilization competence [reviewed by (3, 4)]. Upon arrival to the oviduct, spermatozoa are able to interact with oviduct epithelial cells (OEC), and are in contact with oviductal fluid (OF). The OF consists of a transudate from serum and of specific compounds secreted by OEC (5). In animal models, the existence of a sperm reservoir in the oviduct has been demonstrated, and this storage is required to preserve sperm fertility until ovulation [reviewed by (6)]. In humans, the relevance of physical contact between spermatozoa

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and OEC, as well as the effect of oviductal secretions upon sperm function, has been previously documented. Coculture of human spermatozoa with OEC or sperm incubation with OF or oviductal tissue conditioned medium (CM) have shown to maintain and even to improve sperm viability and motility (7–12). Regarding the effect of oviductal factors on the development of human sperm capacitation and AE, results are controversial; although some studies have demonstrated a significant stimulation of capacitation-related events and AE after sperm–OEC coculture (9, 10), other reports have shown delayed capacitation and low levels of induced AE when spermatozoa were subjected to the coculture or exposed to oviductal secretions (11, 13, 14).

Numerous studies have been conducted toward the identification of proteins present in OEC surface or secreted by the oviduct that would regulate mammalian gamete function and fertilization (11, 15–19). In the bovine model, it was demonstrated that two chaperone proteins, heat shock protein 60 (Hsp60) and Grp78 (glucose-regulated protein 78), also called immunoglobulin-binding protein (BiP), are localized in the apical membrane of OEC and associate to spermatozoa (20). Recently, both proteins were described in the human female reproductive tract; they were found to bind to human spermatozoa and to affect some capacitation-related events (21).

Grp78 is a well-characterized component of the endoplasmic reticulum (ER) that assists in the correct folding and assembly of newly synthesized proteins, and is responsible of targeting aberrant proteins for proteasomal degradation [reviewed by (22)]. Grp78 has also been localized on the plasma membrane of some cell types, where it may function as a surface signaling receptor (23–28). Moreover, there is evidence of Grp78 secretion by different human cells (23, 29, 30); however, the role of extracellular Grp78 has not been completely described.

The present study was aimed at investigating the expression and secretion of Grp78 by human tubal tissue, its association to spermatozoa and its participation in gamete interaction. Grp78 expression in ampulla and isthmus tissue sections was determined, and presence of the protein in native OF recovered from women in different stages of their menstrual cycle, as well as in oviductal tissue conditioned medium, was analyzed. Moreover, the ability of recombinant Grp78 to bind to human male gametes was evaluated by indirect immunocytochemistry, and its effect upon sperm–ZP interaction was analyzed using the hemizona assay.

MATERIALS AND METHODS

All human samples used in the present study were obtained under donor's written consent, and protocols were approved by the Ethics Committee from the Instituto de Biología y Medicina Experimental, from the Centro Médico Fertilab (Buenos Aires, Argentina), and from the Facultad de Ciencias Bioquímicas y Farmacéuticas, at the Universidad Nacional de Rosario (Rosario, Argentina).

Reagents, Antibodies and Culture Media

Unless specified, chemicals were of tissue culture grade and purchased from Sigma Chemical Co. (St. Louis, MO). Electrophoresis reagents were products of BioRad Laboratories (Hercules, CA).

Polyclonal rabbit antihuman Grp78 (sc-13968; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and monoclonal mouse antihuman Grp78 (G73320; BD Biosciences-Pharmigen, San Diego, CA) and antirat KDEL (KDEL: Lys/Asp/Glu/Leu; SPA-827; Stress Victoria, British Columbia, Canada) antibodies were used in the study. Other antibodies used were: rabbit immunoglobulin G (IgG), mouse IgG and horseradish peroxidase (HRP)-conjugated goat antirabbit IgG (Sigma Chemical Co.), HRP-conjugated horse antimouse IgG and goat antirabbit IgG labeled with fluorescein isothiocyanate (FITC) (Vector Laboratories, Inc., Burlingame, CA), antimouse IgG conjugated with FITC and antirabbit IgG conjugated with Cy3 (Chemicon-Millipore, Billerica, MA).

Recombinant human Grp78 (rec-Grp78) devoid of the KDEL sequence, which anchors the protein to the ER domain 2 receptor (erd2) (31), and containing an N-terminal histidine tag was produced in a prokaryotic system, purified, and resuspended in phosphate-buffered saline (PBS) as described (32). Recombinant β-galactosidase expressed in the same system (32) was used as a control recombinant protein in the hemizona assay.

The cell culture medium used throughout the study was human sperm medium (HSM Ca^{2+}). It is a modified Tyrode's medium that consists of 117.5 mM NaCl, 0.3 mM $NaH_2PO_4 \cdot H_2O$, 8.6 mM KCl, 2.5 mM $CaCl_2$, 0.49 mM $MgCl_2 \cdot 6H_2O$, 2 mM glucose, 19 mM sodium lactate, 25 mM $NaCO_3H$, 0.25 mM sodium pyruvate, 50 μg/mL penicillin, and 75 μg/mL streptomycin (33). Medium containing 2.5 mM $SrCl_2$ in replacement of 2.5 mM $CaCl_2$, called HSM Sr^{2+}, was used when indicated. DMEM/Ham F12 (Gibco BRL, Paisley, Scotland), Ham F10 (Hyclone, Road Logan, UT), and human tubal fluid (HTF; Irvine Scientific, Santa Ana, CA) media were also used, as specified.

Oviductal Tissues, Oviductal Tissue Conditioned Medium, Fluids, and Cells

Human oviductal tissues were obtained from premenopausal patients (n = 29; 29–51 years) with normal menstrual cycles and no clinical history of infection or neoplasic disease, who were scheduled for hysterectomies because of uterine fibromyomas or hypermenorrhea. In all cases, the cycle day was determined counting from the onset of the previous menses, and it was confirmed by ultrasonography. Fallopian tubes from two postpartum women were also obtained after tubal ligation for therapeutic purposes. All these procedures were done at the Hospital Provincial del Centenario (Rosario, Argentina). Tissues were immediately placed in a conical tube containing DMEM/Ham F12 medium, and kept on ice while transported to the laboratory. Within 1 hour after surgery, tubal tissues were placed in a culture plate (Nunclon Delta,

Nunc, Roskilde, Denmark), rests of connective and vascular tissues were removed with surgical scissors, and tubal pieces were sliced longitudinally and cut in 2–3 mm^3 sections.

Oviductal tissue CM was recovered after culture of tissue sections (containing both ampulla and isthmus) from women in the proliferative phase of their menstrual cycles as described elsewhere (11). Briefly, tissues were cultured at 37°C in 3-cm plastic culture plates (Costar, Cambridge, MA) containing 2 mL of DMEM/Ham F12 medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 10% fetal bovine serum (Bioser, Buenos Aires, Argentina), 2 mmol/L L-glutamine (Seromed; Biochrom KG, Berlin, Germany), and 15 mmol/L HEPES (Imperial, UK). After 24 hours of tissue culture in fresh serum-free medium, the CM was collected. Cellular viability of the cultured tissue was evaluated by electrophoretic analysis of DNA integrity, as previously reported (12). In brief, explants were manually disaggregated with buffer B (Tris 10 mmol/L, NaCl 0.0875 mmol/L, EDTA 9 mmol/L), supernatants were treated for 20 hours at 37°C with sodium dodecyl sulfate (SDS) (20% v/v) and proteinase K (10 mg/mL; Promega, Madison, WI), after which cellular DNA was sequentially extracted with phenol, phenol:chloroform:isoamyl alcohol (25:24:1) followed by chloroform:isoamyl alcohol (24:1). Total DNA was precipitated by adding NaCl (5 mol/L) and absolute ethanol and incubating for 2 hours at −20°C. After centrifugation at 7,000 × *g*, DNA was washed with 70% (v/v) ethanol, air dried, resuspended in sterile water and stored at −20°C until use. Electrophoresis of DNA samples was performed on 1% agarose gels in TBE buffer (Tris base 0.089 mol/L, pH 8.3, boric acid 0.089 mol/L, EDTA 0.02 mol/L) for 2 hours at 75 V. Conditioned media obtained from tissues with altered DNA integrity, as judged by the presence of low molecular weight DNA fragments, were excluded from the study. Conditioned media were centrifuged for 10 minutes at 700 × *g* to remove cellular debris and were dialyzed against distilled water for 24 hours at 4°C, lyophilized, redissolved in 200 μL of Ham F10 medium, and stored at −20°C until use. Aliquots of CM from different cultures were pooled and analyzed.

Oviductal fluids were obtained by injecting 1 mL of Ham F10 medium into the fimbria region of an intact Fallopian tube, and recovering the maximum volume of liquid drained from the lumen of the isthmus region. Diluted OF were centrifuged for 10 minutes at 700 × *g* to remove cells and debris, and supernatants were stored at −20°C until use. Presence of Grp78 was analyzed in individual fluids from different stages of the menstrual cycle, as well as in a pool of OF from women in the periovulatory period (days 12–18 of the menstrual cycle).

Human follicular fluids (FF) were recovered from patients undergoing an ovulation induction protocol coupled to an assisted fertilization procedure, treated as previously described (34). Fluids obtained from individual follicles of each woman were mixed, centrifuged for 10 minutes at 700 × *g* to remove cellular debris, and stored at −20°C until further use. Human sera were obtained from healthy women and processed similarly to FF. Aliquots from at least 10 samples were pooled and analyzed for the presence of Grp78.

Human oocytes were obtained from women undergoing ovarian stimulation protocols, as described (34). Maturation was achieved by oocyte incubation for 6 to 8 hours in HTF medium, cumulus oophorus cells were removed, and oocytes were stored at 4°C in 0.1 M Tris buffer (pH 7), 1.5 M $(NH_4)_2SO_4$, and 0.5% dextran, until used.

MCF-7 human breast tumor cells (American Type Culture Collection, Manassas, VA) were propagated in DMEM/Ham F12 culture medium supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA), and kept at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were grown until confluence was reached, harvested, washed twice at 4°C with PBS supplemented with 2 mM $CaCl_2$, and frozen at −20°C until preparation and processing of protein extracts.

Immunohistochemical Studies

Sections of isthmus and ampulla regions from oviducts recovered from women in the proliferative phase of their menstrual cycles were fixed in 10% formaldehyde and embedded in paraffin. Tissues were cut into 10-μm sections and were subjected to a standard deparaffination and rehydratation protocol. Antigens were exposed by tissue treatment with boiling sodium citrate solution (pH 6) when indicated. Slides were immersed for 15 minutes in a 150-mM glycine solution to neutralize free aldehyde sites and were incubated for 30 minutes with a solution of 2% normal goat serum, 1% bovine serum albumin (BSA), 0.1% gelatin, 0.1% Triton X-100, 0.05% Tween-20, and 0.05% azide in PBS to block nonspecific binding sites. Slides were then incubated overnight at 4°C with monoclonal or polyclonal anti-Grp78 antibodies (5 and 2 μg/mL, respectively, in PBS with 1% BSA, 0.1% gelatin, and 0.05% azide). Purified mouse or rabbit IgG were used as controls. Following three washes with 0.1% Triton X-100 in PBS, tissues were exposed for 1 hour to antimouse or rabbit IgG labeled with FITC (1:200 in PBS). Tissues were counterstained with propidium iodide, were mounted, and were observed with a laser confocal microscope (C1, Nikon, Tokyo, Japan). Images were acquired with an objective Plan Apo 40×/0.95 (excitation/emission: 488 nm/515–530 nm and 544 nm/570 LP), and analyzed using standard procedures for fluorescent imaging.

Electrophoresis and Western Immunoblotting

After thawing, CM, fluid, and serum samples were centrifuged (10,000 rpm at 4°C) for 1 hour, supernatants were recovered, and total protein concentration was determined using the Bradford method (BioRad Laboratories). Samples were processed, separated by 7% SDS-PAGE, and transferred to nitrocellulose membranes following standard protocols (35). Membranes were stained with 0.2% Ponceau to determine protein loading, they were blocked with PBS containing 0.1% Tween-20, 5% skimmed milk, and were incubated overnight with monoclonal or polyclonal anti-Grp78 (0.5 and 1

μg/mL, respectively), as well as with anti-KDEL (2.5 μg/mL) antibodies. Purified mouse or rabbit IgG added at the same concentrations as specific antibodies served as controls. Blots were washed three times for 5 minutes with PBS–0.1% Tween-20, and incubated with antimouse or antirabbit HRP-conjugated IgG (1:2,000 and 1:1,000, respectively). Reactive proteins were detected by enhanced chemiluminiscence using the ECL kit (Amersham Life Science Inc., Oakville, ON) according to the manufacturer's instructions. Each experiment was repeated at least three times.

Semen Samples and Sperm Processing

Semen samples were collected from normozoospermic donors, according to World Health Organization standards (36), after 3 to 5 days of sexual abstinence and by mechanical stimulation (masturbation). Only samples with >90% live spermatozoa, 75% progressive motile cells, and over 14% normal sperm forms by Kruger criteria (36) were included in the study.

After complete liquefaction, semen samples were subjected to sperm selection using the direct swim-up procedure using HSM Ca^{2+} containing 0.3% BSA (36); highly motile cells were resuspended in the same medium supplemented with 2.6% BSA, and sperm concentration was adjusted to 5×10^6 cells/mL. One-milliter aliquots were incubated for up to 8 hours at 37°C, 5% CO_2 in air to allow sperm capacitation. At the end of the incubation, sperm motility was determined by observation under light microscopy (400× magnification; Alphaphot-2 YS2, Nikon, Tokyo, Japan); only samples with >80% progressive motile cells (grade a + grade b) (36) were used in the study. To verify the occurrence of capacitation, spermatozoa incubated for 8 hours were exposed to FF, and AE was assessed by staining with 50 μg/mL FITC-labeled *Pisum sativum* agglutinin (FITC-PSA), as described (34).

Detection of Endogenous Grp78 in Human Spermatozoa

Presence of endogenous Grp78 in ejaculated and capacitated spermatozoa was determined by SDS-PAGE followed by Western immunoblotting, and by peptide sequencing of sperm protein extracts. In addition, immunodetection of Grp78 was done by fluorescence immunomicroscopy.

For Western immunoblot analyses, sperm cells were washed twice with PBS supplemented with protease inhibitors, and pellets were processed as previously described (35). Sperm proteins were resolved by SDS-PAGE, transferred onto nitrocellulose membranes, and subjected to immunodetection of Grp78.

For protein identification using mass spectrometry, motile spermatozoa were subjected to protein extraction by incubation with PBS containing 1% Triton X-100, 1% deoxycholate, and a cocktail of protease inhibitors; following sonication, the sperm extract was centrifuged at 10,000 rpm for 10 minutes (35). The supernatant was supplemented with Laemmli sample buffer with 2-β-mercaptoethanol and boiled for 5 minutes. After centrifugation, the supernatant was treated with 20 mM iodoacetamide for 20 minutes at room temperature and subjected to SDS-PAGE. Protein bands of interest were excised from the gel, digested with trypsin, and isolated peptides were analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, as described (37). The analysis was repeated twice using different sperm extracts, and protein assignment was done using the BLAST tool from the National Center for Biotechnology Information (NCBI) Web site (http://www.ncbi.nlm.nih.gov).

To immunolocalize endogenous Grp78, motile noncapacitated and capacitated spermatozoa were fixed in 2% formaldehyde for 4 minutes, washed two times with PBS, placed onto microscope slides, and air dried. Samples were blocked with 4% BSA in PBS and incubated overnight at 4°C with anti-Grp78 polyclonal antibody or rabbit IgG (4 μg/mL in blocking buffer). After three washes of 5 minutes each in PBS, antirabbit IgG labeled with Cy3 (1:750 in blocking solution) was added and slides were incubated for 1 hour at room temperature in darkness. Slides were extensively washed, mounted, and observed with a Nikon fluorescence microscope (100× magnification) coupled to an image analyzer (IPLab Scientific Imaging Software for Windows; BD Biosciences Bioimaging, Rockville, MD). At least 200 cells were evaluated in each slide. Presence of the endogenous protein was also analyzed in nonfixed, live spermatozoa. Motile cells were exposed to anti-Grp78 polyclonal antibody or rabbit IgG (8 μg/mL in blocking buffer), washed, fixed, and spotted onto slides; following overnight incubation with blocking buffer, the secondary antibody was added, and slides were processed as previously described.

Sperm Incubation with Recombinant Grp78

Spermatozoa incubated for 4 hours under capacitating conditions were exposed to 100 μg/mL rec-Grp78 or to the same volume of PBS, and incubated for additional 4 hours. Following two washes to remove the unbound protein, cells were processed for immunocytochemical studies using polyclonal anti-Grp78, and binding of the recombinant protein was assessed by fluorescence microscopy, as described earlier. Sperm motility (grade a + grade b) after exposure to rec-Grp78 or PBS was determined by subjective evaluation (36). The acrosomal status of these cells was analyzed by FITC-PSA staining, and protein tyrosine phosphorylation patterns were evaluated by Western immunoblotting, as reported (34).

Hemizona Assay

The hemizona assay (HZA) was performed as described elsewhere (34). Motile spermatozoa were incubated for 4 hours under capacitating conditions and an aliquot containing 1.5×10^4 motile cells was added to each HZ in the presence of rec-Grp78; the counterpart HZ was incubated with the

same volume of PBS, in the absence of the recombinant protein, as control. After a 4-hour incubation at 37°C, 5% CO_2 in air, the hemizonae were washed by repeated vigorous pipetting in drops of medium, and the number of spermatozoa tightly bound to the outer surface of each HZ was counted under a 400× magnification using Hoffman interference optics (Modulation Optics Inc., Greenvale, NY). Incubations in the presence of recombinant β-galactosidase were performed as a control. The assay was performed either in HSM Ca^{2+} or HSM Sr^{2+} media, as indicated. For assays done in the presence of HSM Sr^{2+}, motile sperm selection by the swim-up procedure was performed in this medium. Results from the HZA were reported as number of spermatozoa bound per HZ.

Statistical Analysis

Data were expressed as mean ± SEM. To assume normal distribution, percentages were converted to ratios and subjected to the arcsine square root transformation. All data were analyzed by paired Student *t* test. Statistical analyses were done using the GraphPad InStat program (GraphPad Software, San Diego, CA).

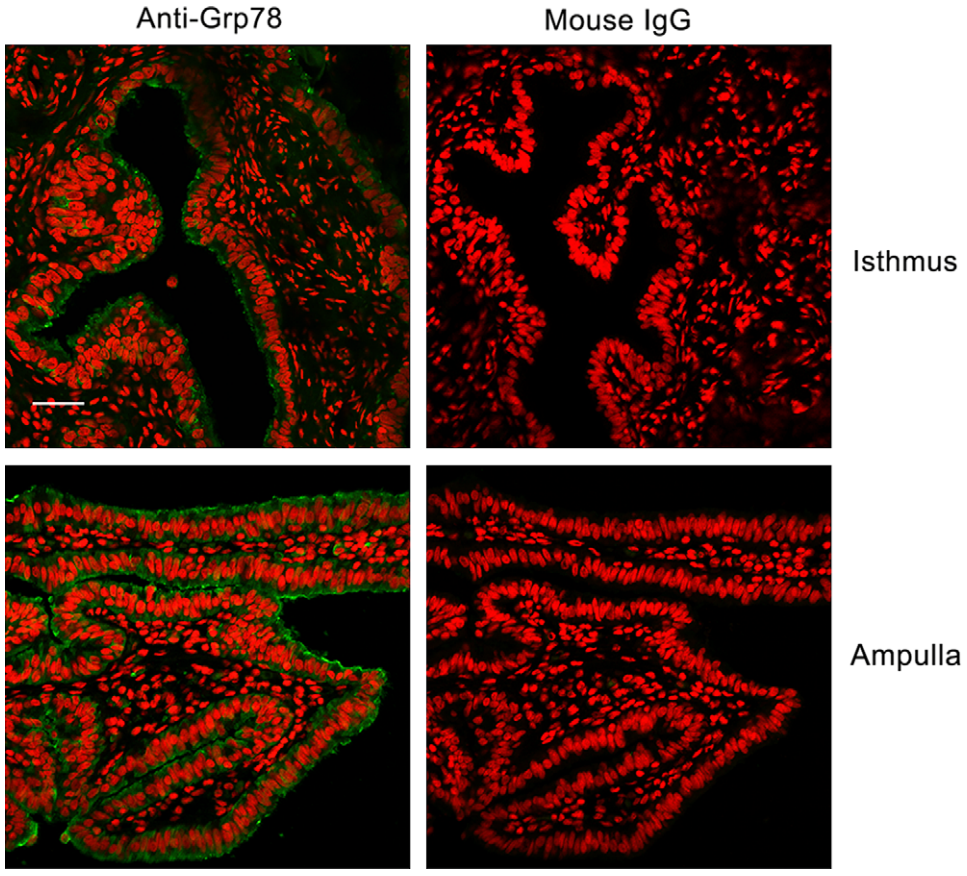
RESULTS

Expression and Secretion of Grp78 in Human Fallopian Tubes

The expression of Grp78 in human Fallopian tubes was determined by fluorescent immunohistochemistry using specific anti-Grp78 antibodies. In both isthmus and ampulla tissue sections Grp78 was detected in epithelial cells (Fig. 1). The protein was localized in the cytoplasm of ciliated and nonciliated cells, showing a more abundant signal on their luminal or apical surface. A weak immunoreactivity was also found in stromal cells of these oviductal regions. Higher expression levels of Grp78 were obtained in ampulla compared with isthmus sections; this result was observed using either different anti-Grp78 antibodies (monoclonal or polyclonal) or staining protocols (including or omitting antigen retrieval).

FIGURE 1

Expression of Grp78 in human Fallopian tubes. Sections of human isthmus and ampulla were subjected to immunohistochemical studies using monoclonal anti-Grp78 antibody and antimouse labeled with FITC. Incubations with mouse IgG instead of primary antibody served as negative controls. Cell nuclei were counterstained with propidium iodide. Bar: 40 μm. A typical result is shown.



Marín-Briggiler. Oviductal Grp78 and sperm-ZP binding. Fertil Steril 2010.

To evaluate Grp78 secretion by the human oviduct, presence of the protein in native OF and in CM from cultured oviductal cells was analyzed. Using a specific monoclonal antibody, a single Grp78 form of 72 to 74 kDa was detected (Fig. 2). A protein of the same molecular weight was found with rec-Grp78 and in MCF-7 protein extracts (positive controls), but no signal was observed in FF, in women sera or in culture medium not exposed to the cells. The immunodetection was specific because signal was absent when the membrane was developed with mouse IgG (data not shown).

To confirm the secretory nature of Grp78 detected in OF and CM, membranes were also probed with an anti-KDEL antibody. The chaperone Grp78 localized in the ER contains a KDEL sequence, which is absent in cell-free Grp78 found in synovial fluid (30). Our results showed a positive band with the anti-KDEL antibody only in MCF-7 cell extracts; no immunoreactivity was obtained in either OF or CM, indicating that Grp78 is a secretion product of OEC.

To determine whether Grp78 secretion by OEC changes throughout the menstrual cycle, its presence in individual OF from women in different days of the cycle was analyzed. Although Grp78 was detected in OF from women in cycle days 12 to 21, negligible protein levels were found in fluids from women in early follicular (OF from days 2–11) and in late luteal (OF from days 23 and 24) phases (Fig. 3). Moreover, no signal was observed in OF from postpartum women (Fig. 3). Equal protein loading was confirmed by Ponceau staining, and similar results were obtained using the monoclonal or polyclonal anti-Grp78 antibodies (data not shown).

FIGURE 2

Presence of Grp78 in human tubal tissue-conditioned medium and human fluids. Protein extracts from human oviductal tissue-conditioned medium (CM) and human fluids were subjected to SDS-PAGE, followed by Western immunoblotting using monoclonal anti-Grp78 and anti-KDEL antibodies. Lane 1: MCF-7 protein extract (40 μg); lane 2: rec-Grp78 (50 ng); lane 3: human oviductal tissue CM (40 μg); lane 4: control medium without cells (equal volume than that loaded on lane 3); lane 5: pool of oviductal fluid (OF, 40 μg); lane 6: pool of follicular fluid (FF, 100 μg); lane 7: pool of female serum (100 μg). A representative result is shown.

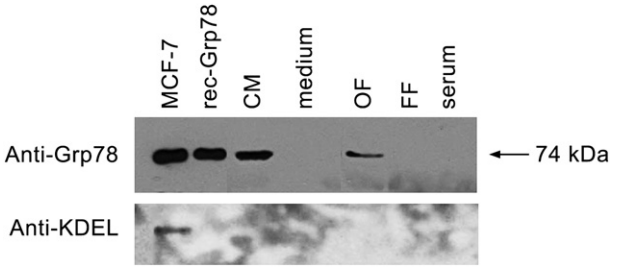


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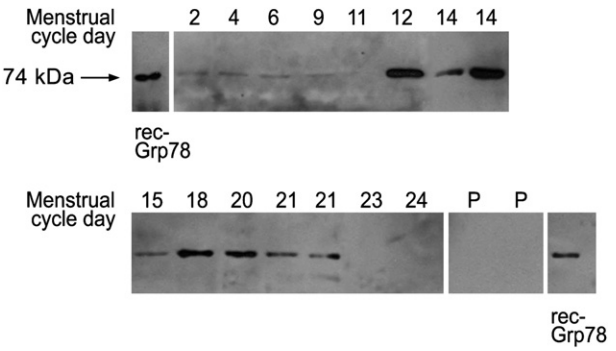
FIGURE 3

Presence of Grp78 in OFs from women in different stages of the menstrual cycle. Protein extracts from individual human OF (10 μg) were subjected to SDS-PAGE, followed by Western immunoblotting using a monoclonal anti-Grp78 antibody. The number at the top of each lane indicates the cycle day in which each OF was obtained; OF from two women in days 14 and 21 of their menstrual cycle were analyzed. P indicates OF from postpartum women. Rec-Grp78 (10 ng) was included as a control. A representative result is depicted. Equivalent protein loading in each lane was checked by Ponceau staining.



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Altogether, these results confirm the expression of Grp78 by OEC; Grp78 was present in CM from human tubal tissue and in native OF, finding higher protein levels in fluids from women in periovulatory period than in other stages of the menstrual cycle.

Association of Recombinant Grp78 to Human Spermatozoa

A recent report has described the association of Grp78 to the surface of human spermatozoa using Western blotting analysis (21). In the present study, the sperm region to which recombinant Grp78 (rec-Grp78) interacts was investigated, but previously we determined the presence and localization of the endogenous Grp78 sperm protein form. This evaluation revealed a form of an estimated molecular weight of 74 kDa in total sperm protein extracts using anti-Grp78 and anti-KDEL antibodies (Fig. 4A). Peptide mass fingerprinting by MALDI-TOF further confirmed the presence of Grp78 in human spermatozoa. The amino acid sequence of the endogenous sperm Grp78 matched with the Grp78 sequence already described in somatic cells (GenBank accession number NP_005338). Immunocytochemical studies showed that endogenous Grp78 is localized either to the whole flagellum or restricted to the midpiece or to the neck region (Fig. 4B a–h); Grp78 was also found in the cytoplasmic droplet of abnormal spermatozoa (Fig. 4B e and f). Similar protein localization was obtained in noncapacitated spermatozoa and in cells incubated for 4 and 8 hours under capacitating conditions

FIGURE 4

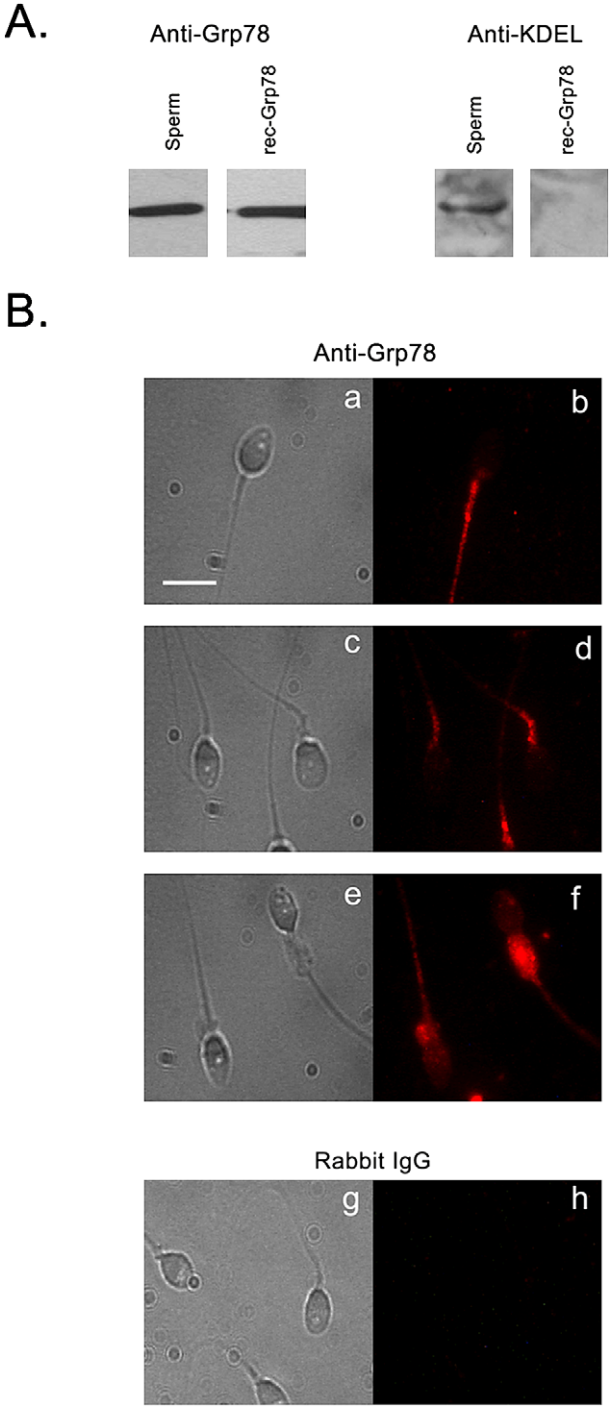

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FIGURE 4 Continued

Presence and localization of Grp78 in human spermatozoa. (**A**) Presence of Grp78 in human spermatozoa assessed by Western immunoblotting. Human sperm extracts (50 μg per lane) were subjected to SDS-PAGE and Western immunoblotting using monoclonal anti-Grp78 and anti-KDEL antibodies. Rec-Grp78 was used as a control (50 ng). The experiment was repeated three times, and a typical result is depicted. (**B**) Localization of Grp78 in human spermatozoa evaluated by indirect immunofluorescence. Motile sperm cells were processed for immunocytochemistry using a polyclonal anti-Grp78 antibody (b, d, and f) or rabbit IgG (h) and a secondary antibody labeled with Cy3; n = 9 semen samples. Examples of the staining patterns obtained in cells of different sperm donors, and the corresponding brightfield images are shown (a, c, e, and g). Bar: 5 μm.

(data not shown). Assays performed with live, nonfixed spermatozoa showed negative results, suggesting masked or intracellular localization of the sperm Grp78 epitope.

The association of Grp78 to human spermatozoa and localization of binding sites were determined in cells incubated with 100 μg/mL rec-Grp78 and analyzed by immunocytochemistry using the anti-Grp78 antibody. An intense labeling over the acrosomal cap was found in 18 $\pm$ 3% of the spermatozoa (Fig. 5). This signal was not observed in cells incubated with PBS (control), indicating that rec-Grp78 is able to specifically bind to the sperm acrosomal region.

Effect of Recombinant Grp78 upon Human Sperm–ZP Interaction

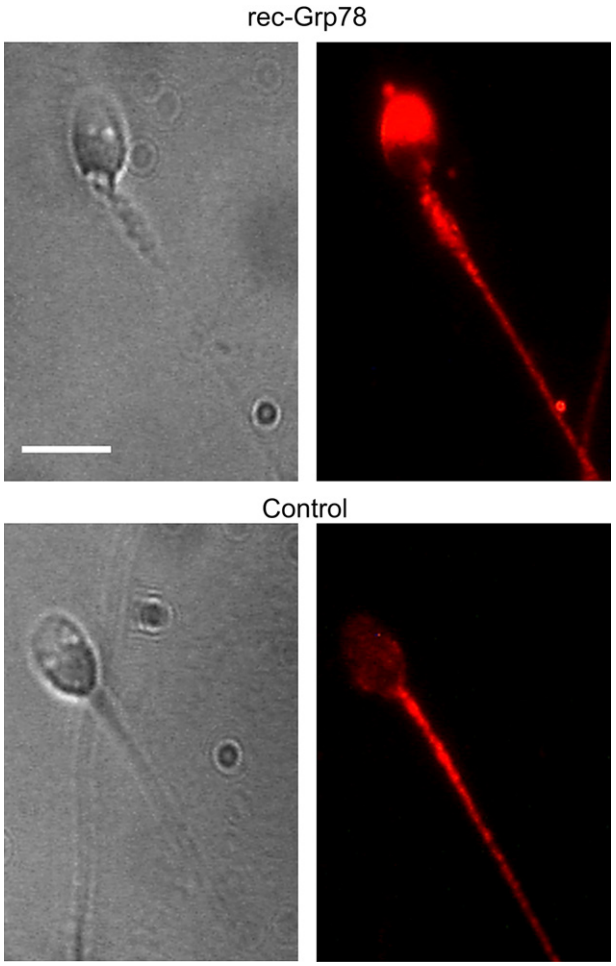
Presence of Grp78 in OF from women in the periovulatory period, and its ability to associate to a sperm region involved in sperm–ZP interaction, led us to propose that Grp78 secreted by oviductal cells may be involved in fertilization. To determine whether Grp78 has a role in sperm–ZP binding, the HZA was performed. When rec-Grp78 (100 and 10 μg/mL) was present during the assay, the number of spermatozoa tightly bound to the ZP was significantly reduced in comparison with the control condition ($P<.05$; Fig. 6A). Incubation with 1 μg/mL of rec-Grp78 or with 100 μg/mL of recombinant β-galactosidase did not affect gamete binding.

Because sperm–ZP interaction depends on sperm motility, sperm acrosomal status and sperm protein tyrosine phosphorylation (38), evaluation of these parameters after sperm exposure to 100 μg/mL of rec-Grp78 was done. No changes in the percentages of motile spermatozoa (rec-Grp78: 93 $\pm$ 2% vs. PBS: 93 $\pm$ 1%, n = 5) or acrosome reacted cells (rec-Grp78: 10 $\pm$ 2% vs. PBS: 13 $\pm$ 2%, n = 3) were observed after treatment with the recombinant protein. Moreover, sperm protein tyrosine phosphorylation patterns were not altered after exposure to rec-Grp78 (data not shown).

The reduced number of sperm bound to ZP in the presence of rec-Grp78 could be attributed to a blockage of sperm

FIGURE 5

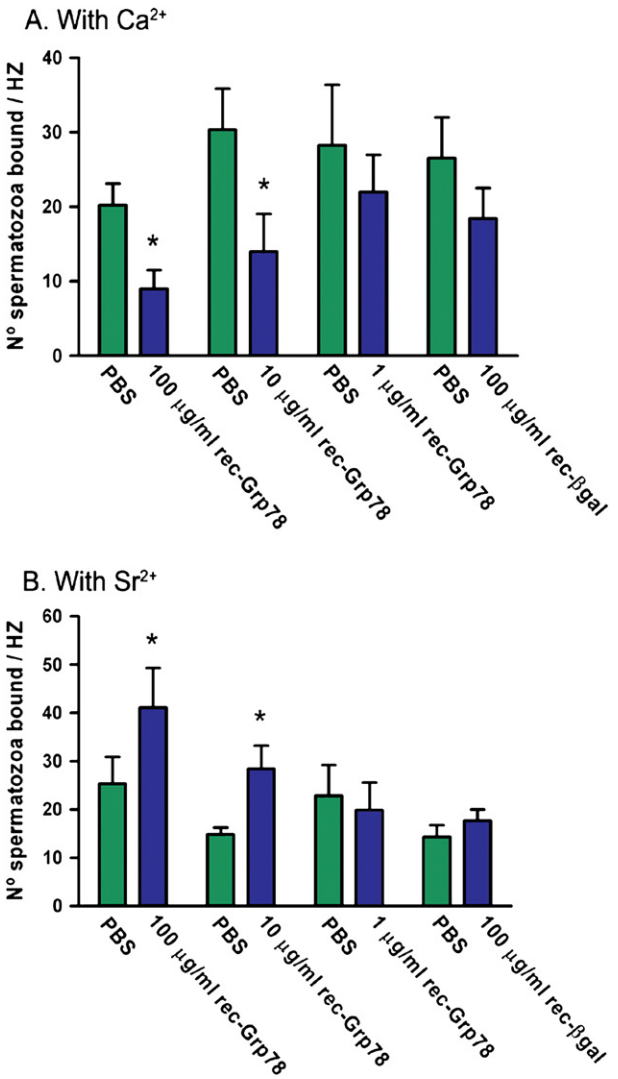
Binding of rec-Grp78 to human spermatozoa. Assessment of rec-Grp78 association to human sperm surface by indirect immunofluorescence. Motile human spermatozoa were incubated for 4 hours under capacitating conditions and exposed to rec-Grp78 (100 μg/mL) or PBS (control) for additional 4 hours. The unbound protein was removed by washing with PBS. Cells were processed for immunocytochemistry as detailed in Figure 4; n = 3 semen samples. In each condition, fluorescence and the corresponding brightfield images are shown. Bar: 5 μm.



Marín-Briggiler. Oviductal Grp78 and sperm-ZP binding. Fertil Steril 2010.

FIGURE 6

Effect of rec-Grp78 on sperm–ZP interaction. Motile spermatozoa were incubated for 4 hours under capacitating conditions and exposed to hemizonae (HZ) in the presence of different concentrations of rec-Grp78. Counterpart HZ was incubated with PBS (control). After a 4-hour incubation period, loosely adherent spermatozoa were removed and cells tightly bound to the outer surface of each HZ were counted. Assays in the presence of 100 μg/mL of recombinant β-galactosidase were also done. Results are expressed as mean ± SEM; n ≥ 7. *$P<.05$ versus control. The experiment was performed in a medium containing Ca^{2+} (**A**) or in a medium in which Ca^{2+} ions had been replaced by Sr^{2+} (**B**).



Marín-Briggiler. Oviductal Grp78 and sperm-ZP binding. Fertil Steril 2010.

binding sites and/or changes in the kinetics of ZP-induced AE. To test these possibilities, the HZA was repeated in a medium in which Ca^{2+} was replaced by strontium (Sr^{2+}) ions; this medium has shown to support the occurrence of human sperm capacitation-related events and ZP-binding but not to induce AE in response to a physiologic stimulus (34). Under this condition, 100 and 10 μg/mL of rec-Grp78 caused a significant increase in the number of spermatozoa bound to the ZP ($P<.05$; Fig. 6B). In the Sr^{2+}-containing medium, similar percentages of motile spermatozoa and acrosome reacted cells, as well as the same levels of protein tyrosine phosphorylation were observed in spermatozoa exposed or not

exposed to the recombinant protein (data not shown). These findings would indicate that rec-Grp78 does not block sperm binding to the ZP, and that it may modulate sperm–ZP interaction, in a Ca^{2+}-dependent manner.

DISCUSSION

The present study was focused on assessing the expression and secretion of Grp78 in the human oviduct, and on determining the involvement of this protein in the fertilization process. The results have shown that the chaperone Grp78 is expressed by OEC, is secreted by these cells in vitro, and is present in native OF with higher protein levels during the periovulatory period. The recombinant Grp78 protein was able to bind to the acrosomal region of capacitated human spermatozoa and to modulate gamete interaction.

Immunohistochemistry studies showed Grp78 localization in the surface of OEC from ampulla and isthmus sections, in agreement with previous findings in the human and bovine models (20, 21). The results from the present investigation revealed that Grp78 expression levels in the ampulla are higher than those of the isthmus. Increased protein synthesis and secretion in the ampulla compared with the isthmus has been reported in animal species (39, 40); considering the classical function of Grp78 in the ER, a higher expression of this chaperone in the ampulla can be anticipated. Our observations contrast with a previous report indicating similar expression of Grp78 in both sections of the human Fallopian tube (21); these differences could account for the stage of the menstrual cycle at which tissues were obtained and/or for the immunodetection protocols used.

The analysis of human native OF and CM from tubal tissue revealed the presence of an extracellular Grp78 form. Grp78 release by cellular damage or by cellular stress associated to culture conditions was discarded, because it lacks the KDEL, a typical sequence present in the ER and plasma membrane chaperone (24, 25). The absence of the KDEL sequence in secreted Grp78 may be explained by alternative splicing of the chaperone RNA under conditions of cellular stress and/or by enzymatic cleavage of the protein at the cell surface, as previously suggested (30). In conjunction, these results support the notion of Grp78 secretion by OEC both in vivo and in vitro, although the mechanisms underlying its release remain to be elucidated.

Results from the present study have shown increased Grp78 protein levels in OF from women in days 12 to 21 of their menstrual cycle. Whether higher Grp78 levels in these fluids are because of a higher protein expression, to an increased secretion by OEC and/or to a decreased degradation in the periovulatory period remains to be determined. Regulation of Grp78 levels throughout the menstrual cycle would be under hormonal control. Regarding its expression, a sixfold increase in Grp78 mRNA levels has been described in bovine OEC during estrous in comparison with diestrous (41), and there is a study indicating that Grp78 gene expression is regulated by estrogens in the mouse uterus (42). Grp78 secretion in the human female reproductive tract might also be regulated by hormones, as reported for other secreted oviductal proteins from several mammalian species (15, 43).

To evaluate the potential association of oviductal Grp78 to human spermatozoa and its involvement in sperm function, in vitro studies using a recombinant protein produced in bacteria were performed. Because Grp78 binding to the sperm surface was monitored using an anti-Grp78 antibody, a previous assessment of the endogenous Grp78 in sperm cells was done. Our results have described that sperm Grp78 is present in the flagellum; this chaperone has been recently identified as an intracellular protein (44). Grp78, as well as other chaperones, have been described in the mammalian male germ line and appear to have a role in initial stages of spermatogenesis (45); future investigations will allow the characterization of endogenous Grp78 tissue origin and function in the mature male gamete. The studies with the recombinant protein have demonstrated that an exogenous Grp78 associates to the human sperm acrosomal cap. The percentage of spermatozoa found to bind rec-Grp78 is in agreement with the small proportion of cells with the ability of undergoing capacitation at a given period of time (46), responding chemotactically to physiologic stimuli (47) and/or binding to recombinant ZP3 protein (48). A tight association of Grp78 to the sperm surface has also been described in bull sperm cells incubated with OEC membrane preparations (20); these results suggest the binding of the oviductal Grp78 to the sperm surface during its passage through the female reproductive tract and/or during its contact with OEC. The association of Grp78 to the sperm head suggests its involvement in gamete interaction.

Our results have shown for the first time that presence of exogenous Grp78 in the incubation medium is able to modulate sperm interaction with the ZP. Sperm incubation with recombinant Grp78 did not alter motility parameters, protein tyrosine phosphorylation, or AE, in agreement with a recent study (21). On the other hand, presence of Grp78 led to a decrease in the number of spermatozoa bound to the ZP when the assay was performed in a Ca^{2+}-containing medium. The recombinant chaperone has been reported to increase the sperm intracellular Ca^{2+} concentrations, and to further elevate the intracellular Ca^{2+} levels reached by sperm upon exposure to progesterone (21). Because the increase in sperm intracellular Ca^{2+} concentrations is an essential step for the occurrence of ZP-induced AE (49), exogenous Grp78 may accelerate the kinetics of acrosomal loss triggered by the ZP. The sperm cells that had undergone AE would have penetrated the ZP, and/or they would have been released from the ZP, resulting in a diminished number of bound spermatozoa. The identification of spermatozoa that had undergone ZP-induced AE may be difficult because they represent a small proportion of cells from the whole population. In an experimental condition in which the induced-AE is abolished, that is, when Ca^{2+} ions are replaced by Sr^{2+} ions (34), an increase in the number of bound spermatozoa was observed in the presence of the recombinant protein. These

latter results would also indicate that Grp78 might facilitate sperm binding to the ZP. Grp78 might cause changes in ZP receptors on the male gamete; in this regard, extracellular Grp78 has been involved in thyroglobulin folding in the lumen of the thyroid gland (50). The effect of exogenous Grp78 upon gamete interaction would be produced by chaperone association to the sperm surface, but the possibility of the Grp78 binding to the ZP must also be considered. In addition to the possible direct effect of cell-free Grp78 on the gametes, the extracellular Grp78 would stimulate cytokine synthesis by the oviductal epithelium, as reported for peripheral blood mononuclear cells and microglia (30, 51). Some of these molecules, in particular interleukin-6, have been demonstrated to induce sperm capacitation and regulate sperm fertilizing ability (52, 53).

The ability of oviductal proteins to bind to the sperm surface and to modulate gamete interaction has been previously described. There are reports indicating that proteins secreted by human OEC associate to spermatozoa, and inhibit sperm binding to the ZP (11, 12). Some of the human oviductal proteins that had this effect are glycodelins F and A (54, 55). On the other hand, the most abundant protein in the oviductal lumen, the oviduct-specific estrogen-dependent glycoprotein, has shown to facilitate gamete interaction [reviewed by (56)]. In vivo, a balance between the negative and positive effects of these oviductal proteins may regulate gamete fertilizing competence.

Grp78 is a member of heat shock protein 70 (Hsp70) family (57), and some of these proteins are expressed at the surface of OEC (17). In particular, HSP70 has been described to be secreted by oviductal cells in response to spermatozoa (16), has been shown to associate to the sperm surface, and has been implicated in the fertilization process (58, 59). To our knowledge, the present study is the first in describing Grp78 secretion by OEC, as well as its participation in gamete interaction. Evidence indicates that the oviduct has an active role in fertilization; the identification of oviductal components with the ability of regulating gamete function would help in the understanding of these processes, and would have implications in the treatment of infertile patients.

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