

Proteasomal Interference Prevents Zona Pellucida Penetration and Fertilization in Mammals¹

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ABSTRACT

The ubiquitin-proteasome pathway has been implicated in the penetration of ascidian vitelline envelope by the fertilizing spermatozoon (Sawada et al., *Proc Natl Acad Sci U S A* 2002; 99:1223–1228). The present study provides experimental evidence demonstrating proteasome involvement in the penetration of mammalian zona pellucida (ZP). Using porcine *in vitro* fertilization as a model, penetration of ZP was completely inhibited by specific proteasomal inhibitors MG-132 and lactacystin. Three commercial rabbit sera recognizing 20S proteasomal core subunits β -1i, β -2i, α -6, and β -5 completely blocked fertilization at a very low concentration (i.e., diluted 1/2000 to 1/8000 in fertilization medium). Neither proteasome inhibitors nor antibodies had any effects on sperm-ZP binding and acrosome exocytosis in zona-enclosed oocytes or on fertilization rates in zona-free oocytes, which were highly polyspermic. Consistent with a possible role of ubiquitin-proteasome pathway in ZP penetration, ubiquitin and various α and β type proteasomal subunits were detected in boar sperm acrosome by specific antibodies, immunoprecipitated and microsequenced by MALDI-TOF from boar sperm extracts. Antiubiquitin-immunoreactive substrates were detected on the outer face of ZP by epifluorescence microscopy. This study therefore provides strong evidence implicating the ubiquitin-proteasome pathway in mammalian fertilization and zona penetration. This finding opens a new line of acrosome/ZP research because further studies of the sperm acrosomal proteasome can provide new tools for the management of polyspermia during *in vitro* fertilization and identify new targets for contraceptive development.

acrosome, acrosome reaction, contraceptive, early development, exocytosis, fertilization, gamete biology, proteasome, sperm, ubiquitin, zona pellucida, ZP-3

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INTRODUCTION

The ubiquitin-proteasome pathway is an evolutionarily conserved, substrate-specific pathway of protein degradation in eukaryotic cells [1, 2]. Posttranslational protein modification by ubiquitin occurs via covalent attachment of a 76 amino acid (AA) ubiquitin residue to a lysine (K) residue within the substrate's AA sequence, via ubiquitin's C-terminal glycine (G76) residue. This reaction is catalyzed by a set of ubiquitin activating- (UBA) and ubiquitin-conjugating (UBC) enzymes. Because the ubiquitin molecule carries seven Lys residues of its own, this initial monoubiquitination is often followed by the ligation of additional ubiquitin molecules to the first, substrate-bound ubiquitin monomer and results in formation of di-, tri-, tetra- or polyubiquitin chains [3]. Monoubiquitination serves a variety of purposes, including, but not limited to, lysosomal protein degradation, endocytosis of membrane receptors, signal transduction, and transcriptional control (reviewed in [4, 5]). Polyubiquitination facilitates protein docking to 26S proteasome, a multisubunit proteolytic holoenzyme with affinity for polyubiquitinated proteins. The 26S proteasome is typically composed of a 20S catalytic core capped on one or both ends with a 19-S regulatory subunit or with an 11S activator complex [6, 7]. The barrel-shaped 20S proteasomal core is composed of four concentric rings comprising seven α -type (outer rings) and seven β -type (inner rings) subunits. In immunoproteasomes and in some other types of proteasomes, the constitutively expressed subunits β -1, β -2, and β -5 are replaced by inducible subunits β -1i, β -2i, and β -5i.

Ubiquitinated protein substrates are deubiquitinated in the 19-S regulatory complex and inserted in the lumen of the 20S core. Within the lumen of the 20S core, the substrate protein is dissected into small peptides of 3–23 amino acids and released in the cytoplasm, where proteolysis is completed by a set of cytosolic endopeptidases (reviewed in [5, 6, 8]). The liberated polyubiquitin chains are not degraded by the proteasome but rather are disassembled into individual ubiquitin molecules by specific deubiquitinating enzymes (DUA), returned to the cytoplasm, and reused for modification of other proteins [9].

Ubiquitination and proteasomal degradation of various substrates was documented in both the cytoplasm and the nucleus [5, 10]. Intriguingly, studies of mammalian reproduction yielded evidence that ubiquitin can also occur on the cell surface and in the extracellular space. Ubiquitin was detected in the ovarian follicular fluid [11], seminal plasma [12], and the epididymal fluid [13, 14] and on the

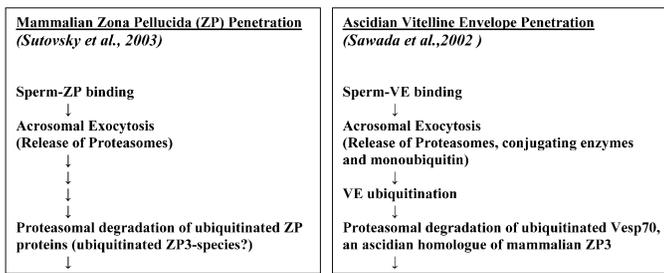


FIG. 1. Comparison of the present model of porcine/mammalian ZP penetration (left) with the established model of ubiquitin-proteasome-dependent penetration of vitelline envelope in ascidians (right, after [22]). The main difference is that the ubiquitinated proteins are present on the ZP surface before fertilization in mammals, while the ascidian spermatozoa actively ubiquitinate ascidian ZP3-analogue after acrosomal exocytosis.

surface of defective spermatozoa passing through the epididymis [14]. Relevant to the present work, the ubiquitination and proteasomal degradation of sperm receptor on the surface of the egg vitelline envelope occurs during ascidian fertilization [15–18]. Furthermore, specific proteasomal inhibitors block the penetration of mouse [19] and pig [20] egg vitelline coat, zona pellucida (ZP).

The ability of the mammalian spermatozoa to bind to and pass through ZP has been ascribed to a set of sperm-surface receptors and proteolytic trypsin-like enzymes (reviewed in [21]) residing in the sperm acrosomal cap, which undergoes exocytosis [22] upon binding to the ZP3-sperm receptor of the ZP matrix [23]. Targeted mutations and knockouts of several candidate acrosomal enzymes and sperm surface receptors did not eliminate the ability of mouse sperm to bind to and penetrate/digest the zona (reviewed in [24, 25]). This suggests that a proteolytic system other than serine proteases exists in the sperm acrosome and contributes to the digestion of ZP during fertilization.

We first observed the ability of proteasomal inhibitors lactacystin and MG-132 to block zona penetration in mammals during our studies using these inhibitors to block the degradation of the paternal mitochondria inside the zygotic cytoplasm [20]. For this to occur, proteasomal inhibitors had to be added to fertilization medium only after the spermatozoa passed through the zona and entered the oocyte cytoplasm. This unexpected observation corroborated the findings of Sawada et al. [15, 16], showing that the ascidian acrosomal exudates contain ubiquitin and proteasomes that are deployed during fertilization, to ubiquitinate and degrade the ascidian homologue of mammalian ZP3, HrVC70, on the ascidian egg vitelline envelope. More recent studies provided further support for the participation of sperm proteasomes in ascidian [17, 18], porcine [26, 27], and human fertilization [28, 29]. Our earlier studies [30] showed that, besides ubiquitin and proteasomal subunits, the mammalian sperm acrosome contains the ubiquitin-conjugating enzyme E2.

Following up on the above studies, the present article provides evidence that mammalian fertilization and ZP penetration rely on the proteolytic activity of the sperm acrosomal proteasomes. In contrast with ascidians, where spermatozoa seem to actively ubiquitinate the egg surface, mammalian eggs may acquire ubiquitinated ZP proteins before fertilization, i.e., during oogenesis. These respective models of porcine and ascidian ZP penetration are presented in Figure 1. Our data furnish an important piece in the

puzzle of mammalian fertilization and identify new contraceptive targets and infertility markers.

MATERIALS AND METHODS

Antibodies

Proteasome subunit sampler pack and the following rabbit sera against proteasomal subunits were purchased from Affinity Research Products Ltd. (Mammhead, UK): Anti- β -2i (MECL1/LMP-10) subunit serum (cat. #PW8150;) [31], raised against synthetic peptide based on the mouse β -2i amino acid sequence of AA 218–236 (NVDACVITAG GAKLQRALST PTEPVQRAGR; [32]), used at 1/200 for immunofluorescence and immunogold transmission electron microscopy (TEM), 1/4000 for Western blotting, and 1/2000 to 1/8000 for inhibiting *in vitro* fertilization. This antibody was shown by the manufacturer to recognize the processed β -2i subunit of ~25 kDa, the unprocessed MECL1 precursor protein of ~32 kDa, and several other, larger, posttranslationally modified isoforms of this subunit [33]. Serum designated α/β (cat. # PW 8155) was raised against proteasomal preparation isolated from human red blood cells [7], thus recognizing a set of bands between 20 and 30 kDa in human erythrocyte lysate, and additional, higher molecular weight (MW) bands in other cell lysates. Such a multiband pattern is due to the fact that multiple α - and β -type subunits, namely α -5, α -6, α -7, β -1, β -5, β -5i, and β -7, are recognized by this serum. This serum was designed for immunoprecipitation and also used for such purposes in our studies. Anti- β -1i-subunit serum (cat. #PW 8205) was raised against a recombinant, full-length sequence of murine β -1i (LMP2/Ring12) subunit [34] and recognizes proteins of 20, 26, and 36 kDa and other higher MW protein complexes. The α/β , β -1i and β -2i antisera were used at a dilution of 1/200 for immunofluorescence and immunogold TEM and at 1/4000 for Western blotting.

Ubiquitin on the outer face of porcine ZP was detected by antibody KM691 (dilution 1/200 for immunofluorescence, 1/1000 for Western; purchased from Kamyia Biomedical Company, Seattle, WA) raised against the whole molecule of recombinant human ubiquitin. Pronuclei were counterstained using rabbit polyclonal antibody AB 1690 (dilution 1/100), recognizing ubiquitinated proteins in porcine embryonic nuclei [35], purchased from Chemicon International Inc. (Temecula, CA). This antibody also recognized ubiquitinated proteins in the outer layer of ZP on ovarian tissue sections (see Fig. 4F).

The acrosomal tyrosine kinase c-Yes [36] was detected using rabbit serum against a recombinant peptide corresponding to the evolutionarily conserved sequence of human c-Yes C-terminus (dilution, 1/100), purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit antiproteasomal antibody Ab-2 (dilution, 1/100) [37] was purchased from Neomarkers Inc. (Union City, CA).

Secondary antibodies used for immunofluorescence studies included the red fluorescent, TRITC-conjugated and the green-fluorescent, FITC-conjugated goat anti-mouse IgG, goat anti-mouse IgM and goat anti-rabbit IgG from Zymed Inc. (South San Francisco, CA) diluted to 1/80. DNA was counterstained with blue-fluorescent 4',6'-diamidino-2-phenylindole (DAPI) (Molecular Probes Inc., Eugene, OR). For colloidal gold TEM, 10 nm gold-conjugated goat anti-rabbit IgG was purchased from Electron Microscopy Science (Washington, PA) and used at a dilution of 1/10.

Gametes and *In Vitro* Fertilization

Ovaries from prepubertal gilts were collected at a local slaughterhouse and transported to the laboratory for the isolation of oocytes from the 3- to 6-mm large antral follicles. Cumulus cell-oocyte-complexes (COCs) were selected under a dissecting microscope for the presence of multilayered cumulus oophorus and washed repeatedly in HEPES-buffered Tyrode lactate (TL-HEPES) medium containing 0.1% (w/v) polyvinyl alcohol (PVA). Groups of 50 COCs were matured for 22 h at 39°C and 5% CO₂ in drops of serum-free modified tissue culture medium (TCM 199; Gibco, Grand Island, NY) supplemented with 3.05 mM glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 10 ng/ml epidermal growth factor, 0.5 μ g/ml FSH, 0.5 μ g/ml LH, 0.1% (w/v) PVA, 75 μ g/ml penicillin-G, and 50 μ g/ml streptomycin sulfate [38]. After this time, COCs were washed in modified TCM 199 without FSH or LH and matured for an additional 20 h. Before fertilization, cumulus cells were removed by vortexing COCs in TL-HEPES medium containing 0.1% (w/v) hyaluronidase and were washed in modified Tris-buffered medium (mTBM) consisting of 113.1 mM NaCl, 3 mM KCl, 7.5 mM CaCl₂, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate, 2 mM caffeine, and 0.2% (w/v) BSA. Fertilization was performed in 50- μ l drops of mTBM medium. Cryopreserved semen was thawed in 10 ml of Dulbecco PBS (Gibco) supplemented with 0.1%

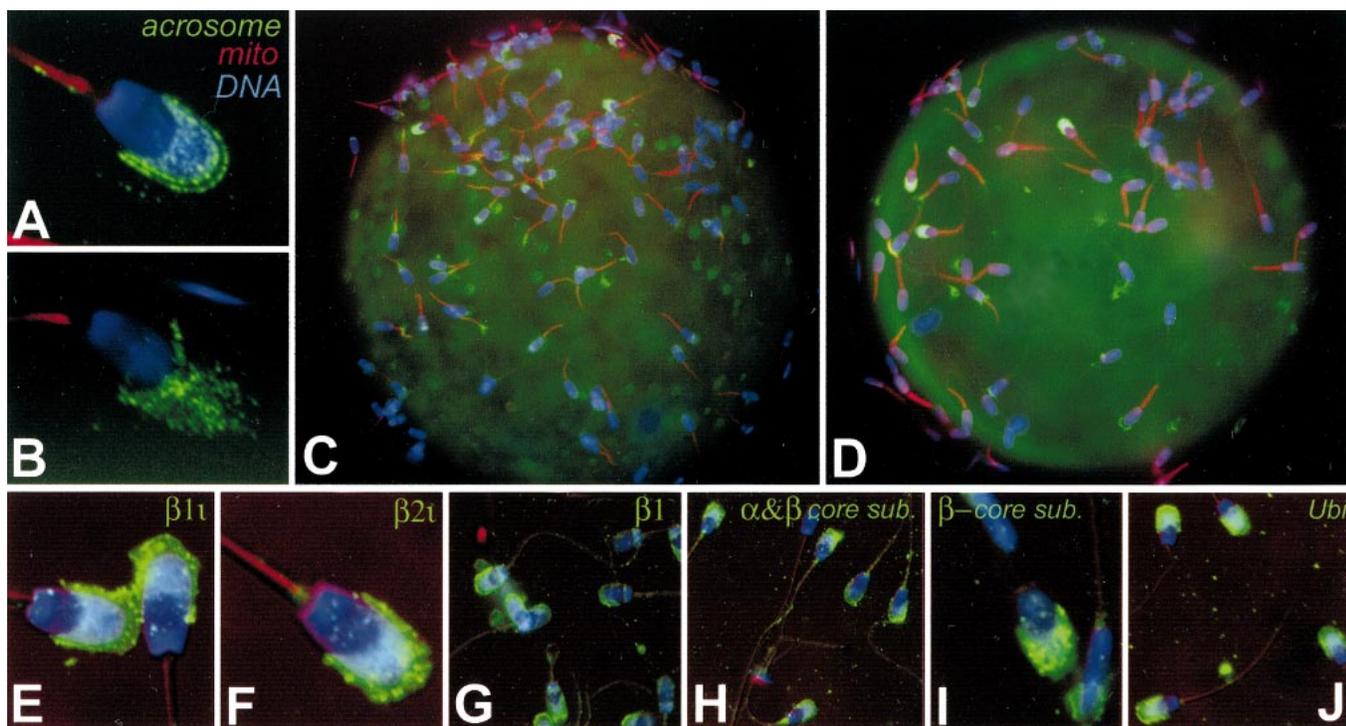


FIG. 2. Sperm-zona binding in the MG-132-treated oocytes and the components of ubiquitin-proteasome system in porcine gametes. **A**) Boar spermatozoon with intact acrosome labeled with antibody against resident tyrosine kinase c-Yes (green). **B**) Remnants of c-Yes labeling (green) after acrosome dispersion on the zona surface. **C**) An oocyte coincubated with spermatozoa for 20 h in the presence of 10 μ M MG-132 shows only three spermatozoa with intact acrosomes (green). **D**) Control oocyte with three acrosome-intact spermatozoa (green) at 20 h PI. Spermatozoa in **A–D** were pre-labeled with MitoTracker CMTM Ros (red). **E–I**) Detection of boar sperm proteasomes by antibodies against subunits β -1i, β -2i, various α and β subunits (serum $\alpha\beta$), and two different antibodies against proteasomal core subunits (all in green). **J**) Ubiquitin (green) was detected by polyclonal antiubiquitin Ab 1690. **A, B, and F**) Original magnification $\times 2200$, (**C–D**) original magnification $\times 400$, (**E**) original magnification $\times 1800$, (**G–H**) original magnification $\times 700$, (**I**) original magnification $\times 1400$, and (**J**) original magnification $\times 800$.

(w/v) BSA. To determine fertilization by the presence of sperm tails and pronuclei inside the fertilized eggs, spermatozoa were pre-labeled with vital mitochondrial probe MitoTracker CMTM Ros as described previously ([18]), and the resultant zygotes were counterstained with DNA stain DAPI as described below. Spermatozoa were washed two times by centrifugation and added to the fertilization drops to a final concentration of 5×10^5 spermatozoa/ml. Six hours after in vitro fertilization (IVF), the presumptive zygotes were washed three times in a serum-free TALP HEPES and processed for immunofluorescence, immunogold TEM, or Western blotting. Zona-free oocytes for Western blotting were obtained by 30-sec treatment with protein-free TALP-HEPES containing 0.5% (w/v) Pronase (Protease, Sigma, St. Louis, MO).

Proteasomal Inhibitor and Antibody Treatments for IVF

To examine the role of the ubiquitin-proteasome pathway in sperm-zona interactions, the effect of antiproteasomal antibodies α/β , β -1i, and β -2i and specific inhibitors of proteasomal proteolytic activity, MG-132 (Z-Leu-Leu-Leu-CHO; reversible inhibitor; Biomol Research Labs Inc., Plymouth Meeting, PA) and lactacystin ($C_{15}H_{24}N_2O_7S$; irreversible inhibitor; Biomol Research Labs Inc.), was evaluated. IVF was performed as described above with the addition of MG-132 (10 μ M) or lactacystin (10 μ M or 100 μ M) or antibodies α/β , β -1i, or β -2i (dilution 1/2000, 1/4000, 1/8000; Affinity Research, Mamhead, UK) in the fertilization medium. Controls without inhibitor included addition of the appropriate solvent (MG-132: 100% ethanol; lactacystin: H_2O) or rabbit serum against ubiquitin C-terminal hydrolase PGP 9.5 from the same company distributing antiproteasomal sera (Affinity Research). PGP 9.5 is an enzyme in the ubiquitin pathway that is found in the boar sperm acrosome but is not directly involved in proteasomal function. All control reagents were used at dilutions equivalent to the inhibitors during IVF. Additional controls for antibody block included antibody omission and addition of an equivalent amount of preimmune rabbit serum. To further confirm that antiproteasomal antibodies from fertilization medium bound to the acrosomal region of zona-bound spermatozoa, oocytes with intact zonae were fixed in formaldehyde, incubated with anti-rabbit IgG-TRITC, and examined under epi-

fluorescence microscope. No permeabilization was used in this procedure. These and other controls were submitted as supplemental data for manuscript review, but are not shown in the published version.

Additional experiments were performed to identify the exact step of fertilization at which the block occurred. Because the inclusion of MG-132, lactacystin, or antiproteasomal antibodies was observed to completely prevent fertilization of zona-enclosed oocytes, IVF was performed using zona-enclosed (ZP+) and zona-free (ZP-) oocytes in the presence or absence of MG-132 and antiproteasomal antibodies. Removal of the zona pellucida was accomplished by a brief (30-sec) exposure of oocytes to 0.5% pronase (Sigma). Oocytes were washed extensively and ZP- oocytes were allowed to recover from enzymatic treatment for 1 h before insemination. For IVF, ZF-oocytes were coincubated with 1×10^5 sperm/ml while zona-enclosed/zona-intact oocytes were treated as described above. Oocytes were fixed 12 h postinsemination (PI) in 25% acetic acid/ethanol and stained with 1% orcein to determine the incidence of fertilization. In some experiments, oocytes were evaluated by immunofluorescence microscopy to detect the presence of pronuclei and sperm tails in the zygotic cytoplasm. Zona-enclosed and zona-free embryos were also fixed at 6 or 20 h PI and processed for immunofluorescence analysis to determine if the inhibition of proteasome activity by MG-132 specifically blocked sperm-zona interaction, inhibited sperm-oolemma fusion, or affected sperm motility, viability, or the ability of spermatozoa to undergo the acrosomal exocytosis.

To evaluate sperm-zona binding and sperm acrosomal status, zona-enclosed oocytes were fixed and the tyrosine kinase c-Yes, known to be present in the subacrosomal and outer acrosomal layer of perinuclear theca in spermatozoa with an intact acrosome [36], was evaluated by immunofluorescence. The number of spermatozoa bound to the zona pellucida was determined after staining with DAPI. Five eggs per group were counterstained with an antibody against the acrosomal tyrosine kinase c-Yes (see *Immunofluorescence*) and DAPI and photographed at 60 \times magnification. ZP-bound acrosome-reacted and acrosome-intact spermatozoa were counted and the values were expressed as a percent (%) of acrosome-intact sperm/egg. Treatment results were compared by χ^2 test and by general linear model procedures of SAS 8.2.

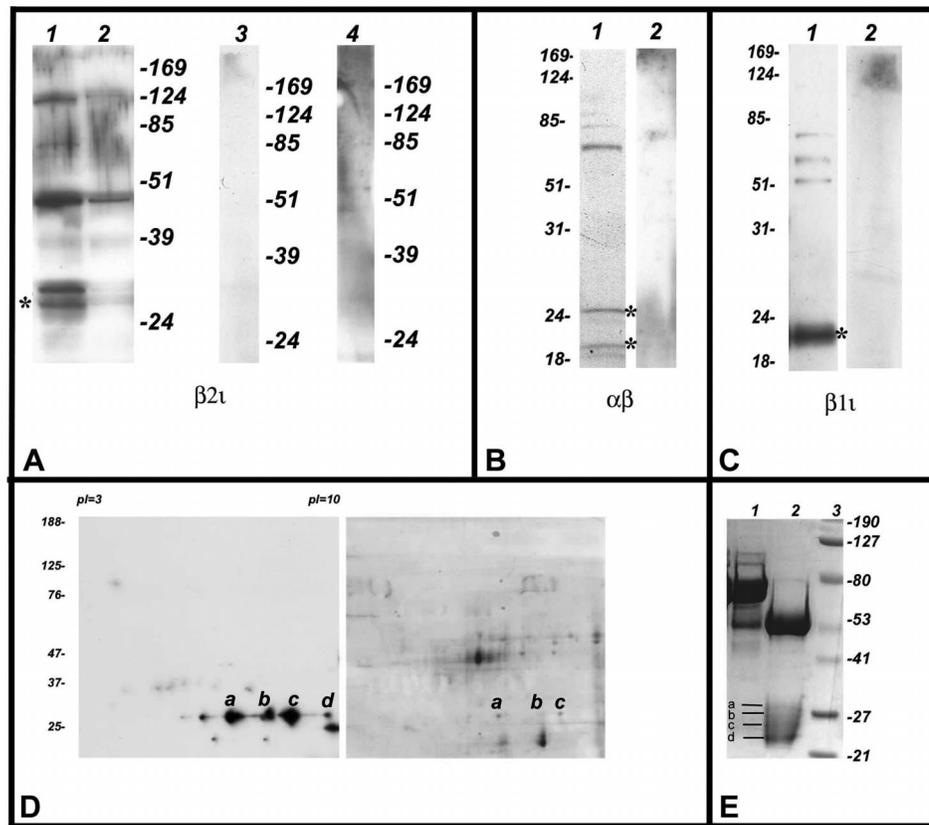


FIG. 3. Immunodetection of proteasomal subunits β -2i (A), various α - and β -type core subunits (B), and subunit β 1i (C) in boar sperm extracts. A) Inducible 20S proteasomal core subunit β -2i was detected as the expected set of bands above 25 kDa in the intact spermatozoa (lane 1), but the immunoreactivity was significantly reduced in the acrosomeless sperm heads (lane 2; some sperm heads retained acrosomes after this treatment), and was completely absent from whole-sperm extracts incubated with a nonimmune rabbit serum (lane 3), or with an immune, anti- β -2i serum saturated with synthetic β -2i peptide (lane 4). B) Serum prepared against the whole, purified proteasomes, expected to recognize several individual α and β subunits, detected several major bands as expected (lane 1), while the nonimmune serum showed no reaction (lane 2). C) Subunit β -1i was detected as a major, expected band of \sim 20 kDa using immune serum (lane 1), but not using a preimmune serum (lane 2). As specified by the distributor of antibodies (Biomol/Affiniti Research Products), additional proteasomal subunit bands are invariably found in various cell type lysates, as also seen here in the sperm lysates. Such bands are thought to be unprocessed subunit-precursor molecules and molecules of appropriate proteasomal subunits not completely separated from other proteasomal subunit molecules within the proteasome. The band corresponding to the mass of processed, nonpost-translationally modified subunit is marked with an asterisk in A–C. D, E) The specificity of the fertilization-blocking antiproteasomal antibodies was further confirmed by two-dimensional gel electrophoresis and Western blotting and by immunoprecipitation and MALDI-TOF protein sequencing. D) two-dimensional Western of subunit β -2i. Four major spots (a–d) trail at the same mass corresponding to the mass of β -2i in one-dimensional Western shown in A. E) One-dimensional SDS-PAGE of boar sperm proteins immunoprecipitated with antiproteasomal antibody α/β and analyzed by MALDI-TOF protein sequencing of four bands (lane 2, bands a–d) in the expected 20- to 30-kDa range of proteasomal core subunits. These bands were identified as: proteasomal subunit β -5 (band d); rabbit Ig gamma heavy chain (band c), mix of rabbit Ig kappa-chain and proteasomal subunit α 6 (band b), and rabbit Ig kappa-chain (band a). Lane 1: antibody α/β without coinubation with sperm extracts; lane 3: MW markers. While the expected rabbit IgG breakdown products were identified in the range of proteasomal subunits, the intact heavy and light chains are visible above 50 kDa.

Immunofluorescence on Ovarian Tissue Sections, Isolated Spermatozoa, and Whole-Mounted Zygotes

Pieces of ovarian tissue were fixed in 4% paraformaldehyde, washed, and embedded in paraffin following conventional histochemical protocols. Four-micron-thick paraffin tissue sections were cut, placed on microscopy slides, dewaxed by xylene, rehydrated in a 100–70% ethanol series and water, pretreated for 20 min in citric acid buffer (pH 6.0) using a steamer, and blocked and processed with antibodies on a histological tray filled with a water-soaked tissue. Spermatozoa were pelleted from semen samples by centrifugation, attached to poly-L-lysine coated coverslips, fixed for 40 min in 2% formaldehyde and processed by passage from well to well in four-well Petri dishes. Zona-free and zona-intact oocytes were fixed in a solution of 2% formaldehyde in PBS without attachment to slides or coverslips, handled using Unapette pipettes (Beckton Dickinson) attached to a 1-cc syringe, and processed by passage through individual wells of a nine-well Pyrex glass plate (Fischer Scientific). All samples were processed with the antibodies described above and in the *Results* section. In a general protocol, spermatozoa on coverslips and unattached oocytes were permeabilized for 40 min or overnight in 0.1% Triton X-100, while the slides with paraffin sections were not permeabilized. All samples were blocked in 5% normal goat serum (NGS; Sigma) in PBS with 0.1% TX-

100. Subsequent incubations and washes were performed using a labeling solution of neutral PBS enriched with 1% NGS and 0.1% TX-100. Coverslips were overlaid for 40 min (whole cells mounted on coverslips) or 2 h (tissue sections mounted on slides) with diluted primary antibodies (in labeling solution) listed above and in the *Results* section. After a brief wash in labeling solution, appropriate fluorescently conjugated anti-mouse IgG or anti-mouse IgM were mixed with a differentially fluorescent anti-rabbit IgG and with 2.5 μ g/ml DAPI (Molecular probes Inc.) and incubated with the samples for 30–40 min. Negative P controls (some of the data not shown) were performed as described above, except that the first antibody was replaced by a nonimmune serum (all antibodies) or by an antibody that was immunosaturated with a custom-made peptide (SynPep, Dublin, CA) corresponding to amino acids 218–236 of murine MECL1 (MECL1 serum only). Identical peptide was reported by the manufacturer as an antigen used for raising this immune serum. Antiubiquitin antibodies used in negative control slides were immunosaturated with purified bovine erythrocyte ubiquitin (Sigma). Following a wash in labeling solution, coverslips and zygotes were mounted in the VectaShield medium on microscopy slides, sealed with a transparent nail polish, and viewed with a Nikon Eclipse 800 epifluorescence microscope using appropriate filter sets and differential interference contrast (DIC) optics. Similarly, tissue sections on microscopy slides were covered with VectaShield and sealed under large

TABLE 1. Inhibition of porcine fertilization by proteasomal inhibitors MG-132 and lactacystin.

Treatment ^a	No. oocytes examined	No. fertilized (%)
Control	41	32 (78.1%)
MG-132 (10 μ M)	65	0 (0)
Lactacystin (10 μ M)	35	19 (54.3%)
Lactacystin (100 μ M)	22	0 (0)

^a IVF was performed using zona-intact oocytes in the absence (control) or presence of the proteasome inhibitors MG-132 and lactacystin.

coverslips. Images were acquired with a CoolSnap CCD HQ camera (Roper Scientific, Tucson, AZ) and Metamorph software (Universal Imaging Corp., Downingtown, PA), archived on recordable compact disks, edited using Adobe Photoshop 5.5 (Adobe Systems, Mountain View, CA), and printed on an Epson Stylus 1280 photo printer.

Transmission Electron Microscopy and Colloidal Gold Immunolabeling

For conventional electron microscopic embedding, the zygotes were fixed in a mixture of 2% paraformaldehyde and 0.6% glutaraldehyde in cacodylate buffer, postfixed in 1% osmium tetroxide, dehydrated by an ascending ethanol series (30–100%) and embedded in PolyBed 812 resin. For colloidal gold labeling, zona-intact zygotes were fixed at 6 h PI in 4% formaldehyde as described for immunofluorescence, washed, permeabilized, blocked, and incubated for 2 h with rabbit sera against proteasomal subunits or with serum against tyrosine kinase c-Yes. Negative controls (some not shown) were performed by a nonimmune serum. After a wash, the zygotes were incubated for 1 h with colloidal gold-conjugated goat anti-rabbit IgG (dilution 1/10 in PBS with 1% NGS and 0.1% TX-100), washed again, and refixed in paraformaldehyde and glutaraldehyde (see below) for electron microscopic embedding.

Ultrathin sections were prepared on a Leica Ultracut UCT ultramicrotome, placed on 100 mesh copper grids and stained in two steps with uranyl acetate and lead citrate. Serial sections were examined and photographed in a Jeol 1200 EX electron microscope. Ten oocytes were processed for each antibody and for the ultrastructural studies of unprocessed/unlabeled zygotes. Negatives were scanned by an Umax Magic Scan flatbed scanner, recorded on a recordable compact disk, and printed on an Epson Stylus 1280 photo printer using Adobe Photoshop 5.5 software.

SDS-PAGE and Western Blotting

Boar sperm proteins were extracted by grinding in extraction buffer (50 mM Tris, 20 mM imidazole, 1 mM EDTA, 5 mM benzamide HCl, 5 μ g/ml leupeptine, 1 μ g/ml pepstatin A, 100 mM PMSF, and 0.1% SDS), centrifuged at 17 000 \times g for 30 min. Then β -mercaptoethanol, glycerol, SDS were added to the supernatants, achieving the final concentrations as that of the loading buffer. Electrophoretic separation was achieved on a 10% gel, and proteins were transferred to PVDF membranes using Mini-Tank Electrobloater (Owl Scientific, Woburn, MA). The membranes were blocked with 1% casein solution. After incubating overnight with the primary antiproteasomal antibodies and then with the appropriate secondary antibodies, the signals were detected with alkaline phosphatase reaction using Vectastain ABC-AmP kit (Vector Lab Inc., Burlingame, CA). Negative controls were performed by a nonimmune serum (all antibodies) or by an immune serum that was immunosaturated with a custom-made peptide (SynPep) corresponding to amino acids 218–236 of murine MECL1 (MECL1 serum only).

For two-dimensional SDS-PAGE, spermatozoa were washed with cold PBS and centrifuged to obtain a tight pellet. The supernatant was removed and the pellet was suspended in ReadyPrep extraction reagent-3 (Bio-Rad Lab Inc., Hercules, CA) supplemented with 1 mM PMSF and Sigma protease inhibitor cocktail (Sigma). The suspension was briefly ground in an Eppendorf tube with a motorized grinder. The extracts were centrifuged at 13 000 \times g for 30 min at 4°C. The protein concentration of the supernatant was measured with Bradford reagent (Bio-Rad) and then adjusted to 1 mg/ml by adding the required volume of the extraction buffer.

Isoelectric focusing was performed on 11-cm ReadyStripe IPG stripes with pH range 3–10 (Bio-Rad) by using Protean IEF Cell (Bio-Rad) and following the manufacturer's protocol. Electrophoresis was performed on Criterion Tris-HCl 10% SDS gels (Bio-Rad). The gels were either stained with Coomassie blue stain or blotted to Immobilon-P PVDF membranes

TABLE 2. Inhibition of sperm-zona penetration by a peptide-specific antibody recognizing proteasomal subunit β -2i and other antiproteasomal antibodies.

	β -2i-1 dilution	n	Mean fertilization % \pm SEM
Zona-intact ^a	No antibody	87	54.02 \pm 14.7 ^b
	Preimmune s.	85	57.0 \pm 14.8 ^b
	β -2i 1/2000	91	0 \pm 0 ^c
	β -2i 1/4000	87	2.0 \pm 1.2 ^c
	β -2i 1/8000	87	2.6 \pm 1.5 ^c
	PGP 9.5 1/500	174	62.7 \pm 13.7 ^d
	β -1i 1/1000	47	0 ^d
	β -1i 1/2000	71	7.9 \pm 7.9 ^d
Zona-free ^a	α/β 1/2000	48	0 ^d
	Preimmune s.	47	69.9 \pm 13.4 ^e
	β -2i 1/2000	52	40.2 \pm 4.2 ^e

^a IVF was performed using zona-intact or zona-free oocytes in the absence (control) or in the presence of antiproteasomal antibodies or an amount of rabbit preimmune serum equivalent to lowest dilution (i.e., highest concentration) of antiproteasomal antibodies.

^b $P > 0.1$ between preimmune serum and no serum/no antibody.

^c $P < 0.01$ between each of the antibody dilutions and controls.

^d $P < 0.01$ between PGP 9.5 antibody and dilutions of β -1i and α/β antibodies.

^e $P > 0.1$ between preimmune serum and β -2i in ZF oocytes.

(Millipore, Bedford, MA) for Western blotting by using Trans-Blot SD Electrophoretic transfer cell (Bio-Rad). Further processing of the PVDF membranes was performed according to the method as described for the one-dimensional Western blotting.

Immunoprecipitation and MALDI-TOF Protein Sequencing

According to manufacturer's data (Biomol/Affiniti Research), the antibody α/β was the only one suitable for immunoprecipitation using Seize X Protein G immunoprecipitation kit (Pierce, Rockford, IL). The immunoprecipitated sperm proteins, presumed to be a mix of rabbit IgG fragments and various proteasomal subunits, were separated on 1D-SDS-PAGE, stained with Coomassie blue, and five distinguishable bands (see Fig. 3E) in the 20–30 kDa range of most processed proteasomal subunits were individually excised, destained, reduced with dithiothreitol (DTT), alkylated with iodoacetamide (IAA), and trypsinized overnight. The digests were desalted on C₁₈ZipTips and peptides were eluted in 10 μ l of 700/290/10 CH₃(CN/H₂O)/88% HCOOH (v/v/v). The eluted peptides were analyzed by MALDI TOF MS (Applied Biosystems Voyager System 6266). Spectra were submitted to Mascot search and searched against the National Center for Biotechnology Information database.

RESULTS

Proteasomal Inhibitors Block Zona Penetration by the Boar Spermatozoa Without Affecting Sperm-Zona Binding and Acrosome Reaction

The working hypothesis of the present investigation presumed a mechanism in which boar spermatozoa release proteolytically active proteasomes onto the surface of porcine oocyte ZP during acrosomal exocytosis (Fig. 1). This study was followed by experiments designed to determine the fertilization step at which the inhibitors exert the block. Both MG-132 and lactacystin prevented fertilization when added at the time of insemination (Table 1). The experiment was repeated four times with highly consistent results. In these trials, MG-132 prevented fertilization in 100% of oocytes. Lactacystin, which is known to be less potent than MG-132 and irreversible [39], reduced the fertilization rate by ~25% at 10 μ M concentration and caused a 100% fertilization block at 100 μ M concentration (Table 1). Further experiments were performed with MG-132 only, due to its known proteasome specificity and reversibility [40] and the

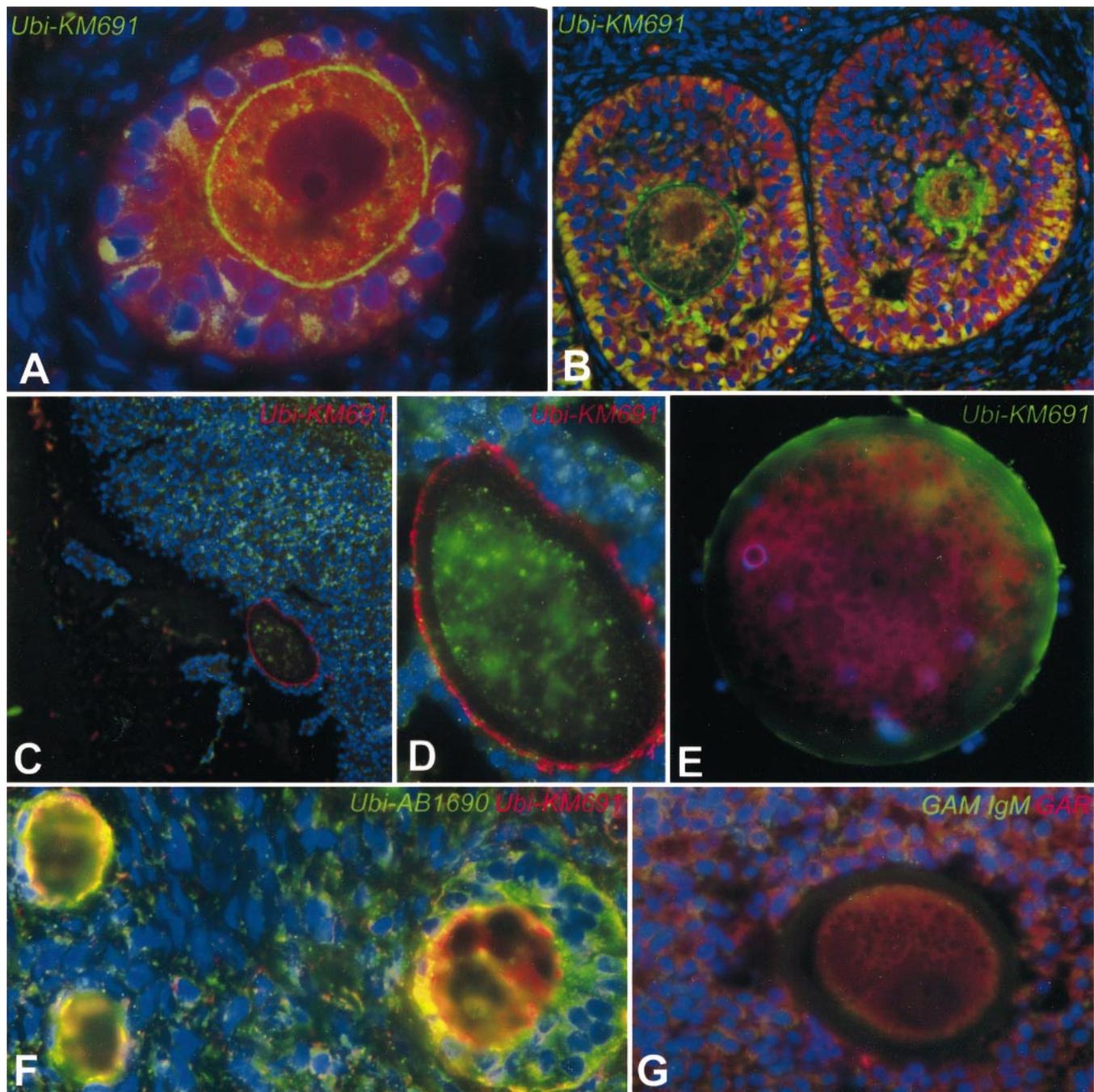


FIG. 4. Ubiquitin immunoreactive proteins on the outer face of porcine zona pellucida. Ubiquitin was detected either by mouse monoclonal antibody KM 691 (A, B, E—green, C, D, F—red); or by rabbit serum AB1690 (E—red, F—green). Tissue sections of preantral (A, B, F, G) and antral (C, D) porcine ovarian follicles are shown, along with specimens of whole-mounted, full grown oocytes (E, germinal vesicle stage is shown). Follicular granulosa cells in some of these paraffin sections (A, B) were counterstained with antibody against mitochondrial protein prohibitin (red). E Red labeling with anti-ubiquitin Ab1690 outlines nucleolus inside the germinal vesicle of this oocyte, showing that antiubiquitin antibodies easily bypass ZP of fixed ova. Ubiquitin immunoreactivity is not seen in a negative-control tissue section treated by combining the omission of KM691 and incubation with preimmune rabbit serum, followed by appropriate fluorescently conjugated immunoglobulins (G). DNA in all images was counterstained with DAPI (blue). A) Original magnification $\times 500$, (B) original magnification $\times 200$, (C) original magnification $\times 80$, (D and G) original magnification $\times 300$, (E) original magnification $\times 400$, and (F) original magnification $\times 350$.

concern that lactacystin might partially inhibit the activities of some of the nonproteasomal proteases that could be present in the acrosome, namely that of cathepsin A [41]. It was established earlier that MG-132 and related inhibitors do not block the activity of serine proteases [33, 42]. To rule out that MG-132 affects sperm viability, sperm-zona binding or acrosomal exocytosis, oocytes fertilized in the presence of 10 μM MG-132 were fixed with their zona

pellucida intact at 20 h PI. Sperm heads bound to ZP were detected with DAPI and intact acrosomes were detected by antibodies against tyrosine kinase c-Yes, known to reside in the subacrosomal and outer acrosomal layer of perinuclear theca of acrosome-intact spermatozoa (Fig. 2, A–D). Because the spermatozoa in this experiment were prelabeled with MitoTracker CMTM Ros, the sperm mitochondrial sheaths were rendered detectable inside the fertilized oo-

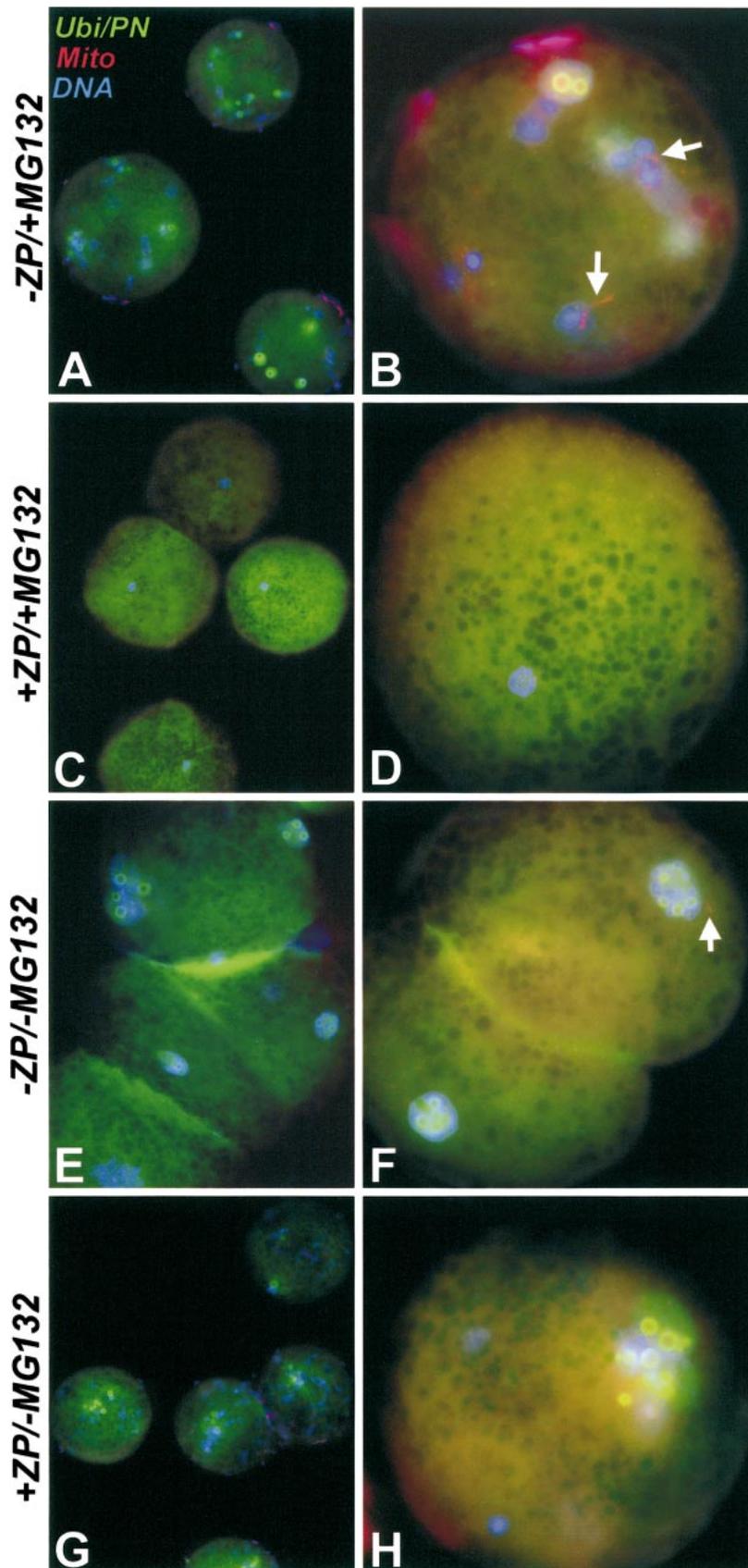


FIG. 5. Proteasomal inhibitors block in vitro fertilization in zona intact oocytes, but not in zona-free oocytes. MG-132 had no effect on in vitro fertilization of zona-free oocytes (A, B), but inhibited the penetration of zona-intact oocytes (C, D). Appropriate zona free/MG-132-free (E, F) and zona intact/MG-132-free (G, H) controls were performed. Pronuclear and meta-phase-II DNA was counterstained with DAPI. Pronuclei and nucleoli inside the fertilized ova were labeled with antibody Ab 1690, recognizing ubiquitin-tail fusion ribonucleoproteins in the nucleolus precursor bodies of porcine pronuclei. A and C) Original magnification $\times 150$, (B, D, F, and H) original magnification $\times 350$, (E) original magnification $\times 200$, and (G) original magnification $\times 100$.

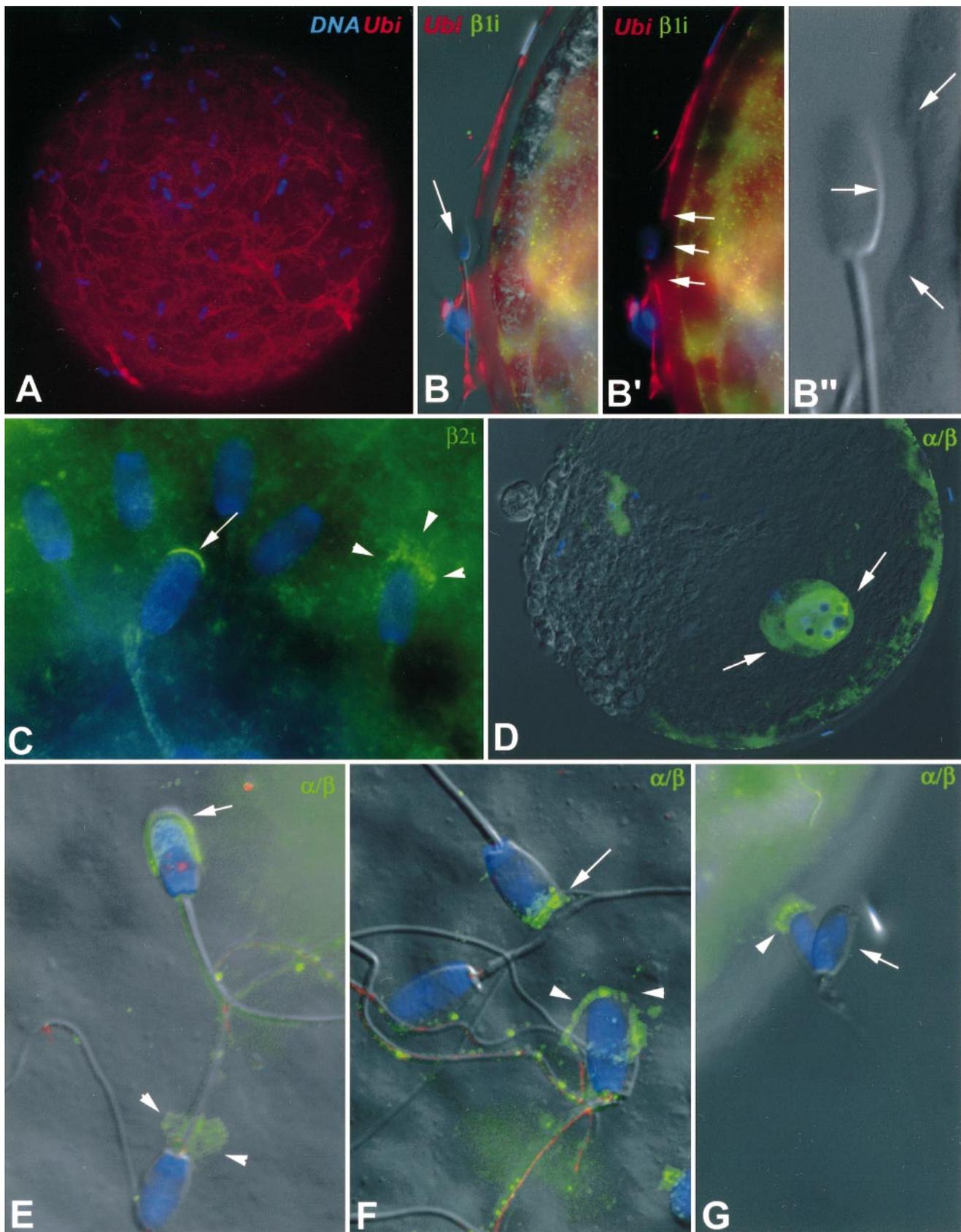


FIG. 6. Interaction of sperm proteasomes with the ubiquitin immunoreactive ZP proteins as visualized by antiubiquitin and antiproteasome immunofluorescence combined with DIC imaging of boar spermatozoa on the ZP surface 6 h postinsemination. **A**) A mesh-like pattern of ubiquitin labeling on the surface of ZP in the fertilized zygotes, with reduced fluorescence underneath and around the ZP-bound spermatozoa. **B**) A lateral view of a ZP-bound spermatozoon (arrow). **B'**) The ubiquitin-immunoreactive layer (red) is digested underneath the spermatozoon (arrows). **B''**) A vault (arrows) left by the digested ZP matrix underneath the sperm head, as visualized by DIC optics. **C**) Proteasomal subunit β -2i (green) in the intact acrosome (arrow) and in the rejected acrosomal shroud (arrowheads) of the ZP-bound spermatozoa. **D–G**) Detection of proteasomal subunits (green) α/β (**D–F**) and β 1i (**G**)

cytes. Similar to previous experiments with MG-132, the inhibitor prevented fertilization completely (0% fertilization in MG-132 group vs. 77% fertilization in control). However, there was no significant difference in the number of sperm bound per egg (99.3 vs. 93.3 sperm/egg in control) or in the percentage of acrosome-intact, zona-bound sperm (2.01% vs. 2.14% in control).

Proteasomal Subunits, Present in the Boar Sperm Acrosome, Can Be Inactivated by Peptide-Specific Antibodies

Taking into account our inhibitor studies and previous reports confirming that proteasomal subunits are present in the mammalian/human sperm acrosome [43] as well as the evidence implicating the ubiquitin-proteasome system in the acrosomal activity of invertebrate animals [15–18; 44], we investigated whether proteasomes are present in the boar sperm acrosome. Immune sera against various 20S proteasomal core subunits of type α and β and monospecific sera against the inducible 20S core subunits β 1i and β 2i, indeed rendered a prominent signal in the sperm acrosome (Fig. 2, E–I). Ubiquitin was also detected in the acrosome (Fig. 2J). The same proteasomal subunits were further detected by Western blotting in the extracts of boar sperm heads and whole boar spermatozoa (Fig. 3, A–C). No such reactions were observed in control blots performed using preimmune rabbit serum (Fig. 3, A–C), and reduced immunoreactivity was found in the β -2i serum immunosaturated with a peptide corresponding to that used for immunization (Fig. 3A). Consistent with the proposed role of proteasomes in zona penetration, even extremely low doses of antibodies against inducible proteasomal subunits β -1i and β -2i and multiple constitutive α/β subunits of the 20S proteasomal core reliably blocked fertilization in zona-intact but not in the zona-free oocytes (Table 2).

The specificity of the binding of antiproteasomal sera to the acrosomal region of zona-bound spermatozoa was confirmed by the incubation of zona-intact zygotes fertilized in the presence of antiproteasomal sera (as listed above), with goat anti-rabbit IgG-TRITC (data not shown). The specificity of all three fertilization-blocking antibodies was further confirmed by two-dimensional gel electrophoresis and Western blotting (Fig. 3D) and by immunoprecipitation followed by MALDI-TOF protein sequencing of resultant spots (Fig. 3E). While the β -2i protein had a tendency to aggregate into multiple bands on one-dimensional SDS-PAGE, such an electrophoretic migration pattern was not present on two-dimensional gels, where four major spots of β -2i trailed at a close pI range at the 24–29 kDa level and only a few very weak spots, attributed to a posttranslational modification (see [33]), migrated between 30 and 50 kDa (Fig. 3, D and E). This pattern of β -2i protein and preprotein migration is in agreement with previous two-dimensional electrophoretic studies using the same antibody [31, 33]. Immunoprecipitation with antibody α/β yielded a set of

five distinguishable bands in the expected 20–30 kDa range of processed proteasomal subunits in addition to higher mass IgG heavy chain (Fig. 3E). The first band was a highly significant, top match ($P < 0.05$ in Mascot search) for proteasomal subunit β 5. The second band was a match for subunit α 6 apparently mixed with rabbit immunoglobulin (the second significant match in that band). The remaining two bands matched with high probability ($P < 0.05$) the rabbit Ig gamma heavy chain and rabbit Ig kappa-light chain, respectively. These were the expected products of rabbit IgG breakdown during the immunoprecipitation procedure.

Ubiquitinated Proteins Are Present on Outer Face of Porcine Zona Pellucida

If proteasomal inhibitors and antibodies block zona penetration (Table 1 and 2) and both proteasomal subunits and components of ubiquitin pathway are present in the acrosome (Fig. 2, E–J), either the ubiquitination-susceptible proteins/sperm receptors should be present on the outer layer of ZP, as shown in the ascidian vitelline envelope [15–18], or there should be proteins/sperm receptors on the ZP that are already ubiquitinated before the contact with the sperm acrosomal exudates. Using antibody KM 691 against recombinant human ubiquitin, we screened paraffin sections of porcine ovarian tissue (Fig. 4, A–D, F, G) as well as whole mounts of porcine COCs isolated from antral ovarian follicles (Fig. 4E; see also Fig. 6, A and B). A distinct, dense layer of ubiquitin-cross-immunoreactive proteins were detected in the outer layer of porcine zona in the oocytes from both preantral and antral follicles on both ovarian tissue sections (Fig. 4, A–D, E, F) and on the whole mount preparations (Figs. 4E and 6, A, B, O). The most striking feature of these ubiquitin-immunoreactive proteins was the fact that they were detected mainly on the outer face of the ZP, which is in contrast with the homogeneous distribution of major ZP components, ZP 1, 2, and 3 throughout the mammalian zona [24, 25]. Certain types of sugar residues present in zona glycoproteins are concentrated on the inner face of the ZP, while others are dispersed evenly throughout it [45]. This localization of ubiquitin immunoreactivity was not due to the limited penetration of antiubiquitin antibodies through the ZP because such data were obtained by surface staining of tissue sections. Furthermore, an unrelated polyclonal antiubiquitin AB1690 also recognized this layer of ubiquitinated proteins on the outer face of the ZP (Fig. 4F). Such an immunoreactivity was not found in negative controls, including the omission of KM691 and incubation with preimmune rabbit serum followed by appropriate fluorescently conjugated immunoglobulins (Fig. 4G) or the incubation of ovarian sections with immunosaturated antiubiquitin antibodies (see *Materials and Methods*). The same antibodies recognized multiple bands above 120 kDa, consistent with the ubiquitinated species of major ZP proteins on Western blots of porcine ova and zygotes (data not shown).

Zona Removal Abolishes the Effect of Proteasomal Inhibitors and Antiproteasomal Antibodies

To ascertain the fertilizing capability of boar spermatozoa treated with proteasomal inhibitors, porcine oocytes were stripped of ZP and inseminated with acrosome-intact spermatozoa in the presence or absence of MG-132 (Fig. 5, A–H). As expected, MG-132 inhibited fertilization in the zona-intact oocytes but had no effect on the zona-free oo-

(G) in the ZP-bound spermatozoa with the intact acrosomes (C, E, F; arrows), the acrosomes undergoing exocytosis (C, F, G; arrowheads), and the rejected acrosomal shrouds (C, E; arrowheads). Note the accumulation of proteasomes inside the apposed pronuclei (arrows in D). DNA was counterstained with blue-fluorescent DAPI. (A) Original magnification $\times 450$, (B and B') original magnification $\times 400$, (B'') original magnification $\times 2500$, (C) original magnification $\times 1800$, (D) original magnification $\times 550$, (E) original magnification $\times 2000$; (F) original magnification $\times 2200$, and (G) original magnification $\times 1600$.

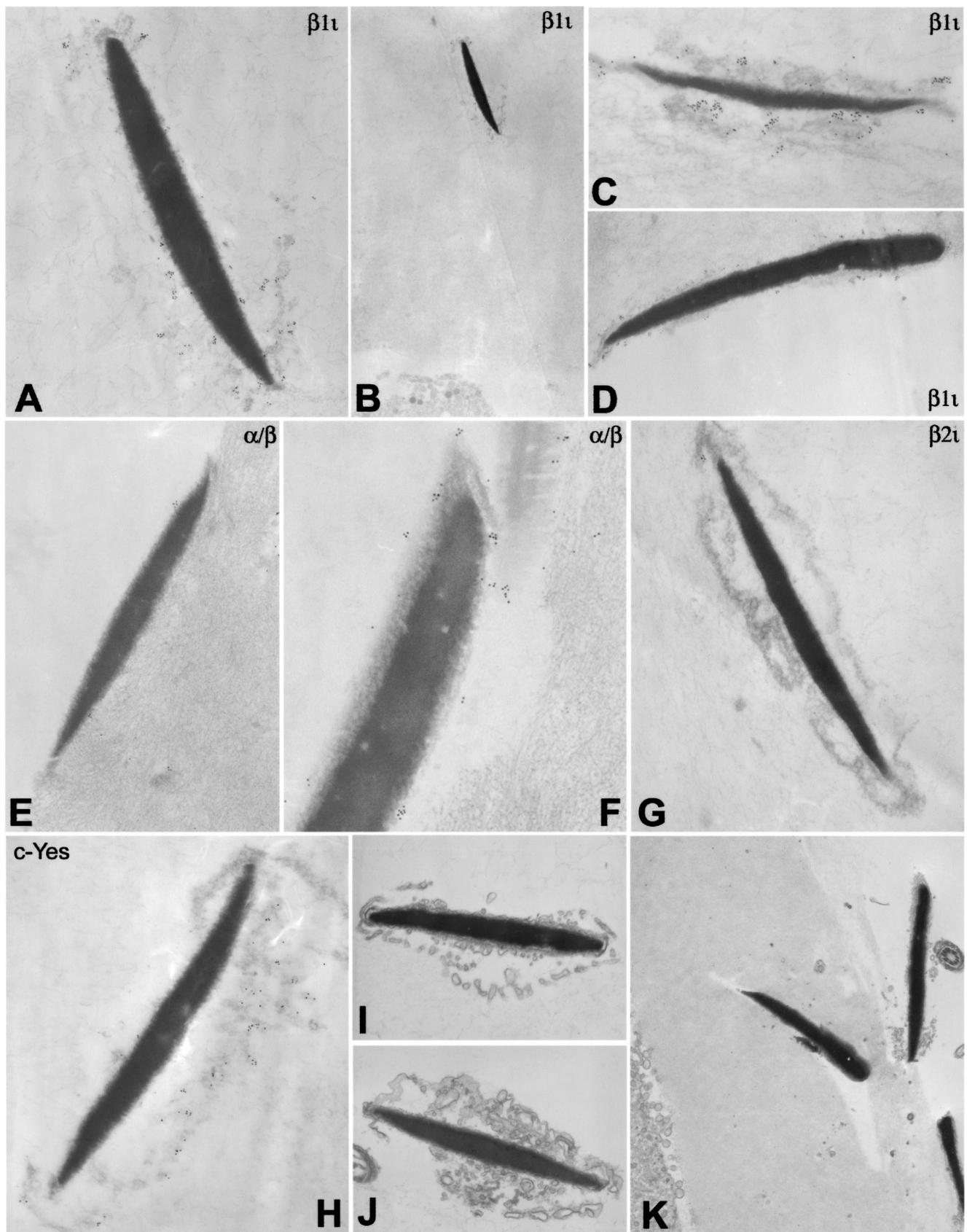


FIG. 7. Immunoelectron-microscopic detection of proteasomal subunits during acrosome exocytosis. **A–G** Colloidal gold labeling of subunits $\beta 1i$ (**A–D**), α/β (**E, F**), and $\beta 2i$ (**G**) is present in both acrosomal matrix and on the inner acrosomal membrane. **H**) Acrosomal marker, tyrosine kinase *c-Yes* is confined mainly to the complex of the outer acrosomal membrane and outer-acrosomal perinuclear theca. **I–K**) Conventional electron microscopy showing the ultrastructure of acrosomal exocytosis during porcine fertilization. Fixation and permeabilization procedures for colloidal gold labeling did not preserve the structure of such acrosomal membrane vesicles in **A–H**. **A** and **H**) Original magnification $\times 36\,000$, (**B**) original magnification $\times 8000$, (**C, I,** and **J**) original magnification $\times 20\,000$, (**D**) original magnification $\times 22\,000$, (**E** and **G**) original magnification $\times 25\,000$, (**F**) original magnification $\times 72\,000$, and (**K**) original magnification $\times 14\,000$.

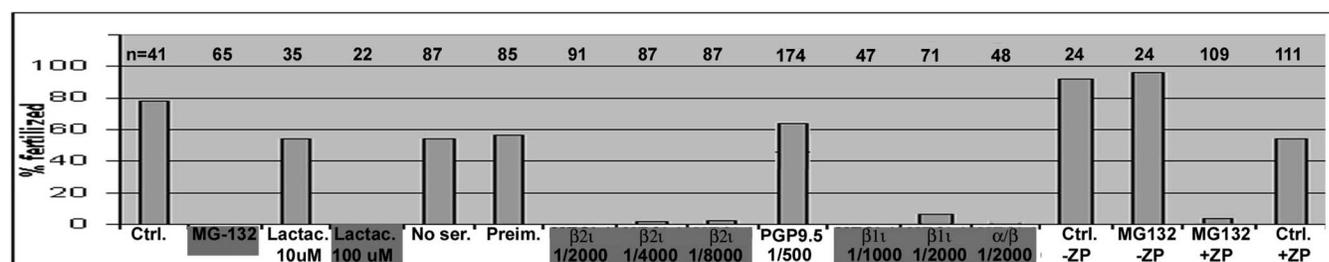


FIG. 8. Diagram summarizing the fertilization rates obtained in three sets of IVF experiments including proteasomal inhibitor experiments, antiproteasomal antibody experiments, and zona removal experiments. Multiple repeats were performed within each set of experiments.

cytes, 92–96% of which were fertilized, most of them in a polyspermic fashion (Fig. 5, A–H). MG-132 had no effect on the fertilization of zona-intact oocytes when either oocytes or spermatozoa were preincubated with this reagent, washed extensively, and used for insemination (data not shown). Similarly, antiproteasomal antibodies did not prevent fertilization of zona-free ova (Table 2).

Proteasomes Are Released from the Acrosome During Acrosomal Exocytosis

We next attempted to visualize the interaction of sperm-acrosomal proteasomes with the ubiquitin immunoreactive ZP proteins by immunofluorescence combined with DIC microscopy imaging of zygotes 6 h PI. A mesh-like pattern of ubiquitin labeling was seen on the ZP surface of fertilized zygotes, with reduced fluorescence underneath and around the ZP-bound spermatozoa (Fig. 6A). In a lateral view (Fig. 6, B and B'), the ubiquitin-immunoreactive layer under the ZP-bound spermatozoa was missing (Fig. 6B', arrows), producing a vault under the sperm head that was clearly discernible under DIC optics (Fig. 6B', arrows). Proteasomal subunits previously detected in the intact sperm acrosome (Fig. 1) were also found in the ZP-bound spermatozoa with the intact acrosomes (Fig. 6, C, E, F; arrows), in the acrosomes undergoing exocytosis (Fig. 6, C, F, G; arrowheads) and in the rejected acrosomal shrouds (Fig. 6, C, E; arrowheads). Consistent with the presence of proteasomal subunits and with the activity of the ubiquitin system in the somatic cell nucleus [10, 46], both the α - and β -type proteasomal subunits were detected in the pronuclei inside the fertilized zygotes (Fig. 6D).

The proteasomal subunits were also detected by colloidal gold TEM in the acrosomal matrix (Fig. 7, A–C) and on the inner acrosomal membrane (Fig. 7, D–G) of the ZP-bound spermatozoa undergoing acrosomal exocytosis and vesiculation (Fig. 7, I–K). As a positive control, the anti-Yes antibody (see Fig. 1, A and B, for immunofluorescence) recognized mainly the complex of the outer acrosomal membrane and outer acrosomal perinuclear theca (Fig. 7H).

DISCUSSION

Collectively, the data obtained in the course of this study, as summarized in Figure 8, provide evidence that the proteasomal inhibitors and antiproteasomal antibodies effectively block zona penetration by boar spermatozoa without affecting sperm motility, sperm-zona binding, and acrosomal exocytosis. Three lines of evidence support the role of proteasome in the penetration of mammalian ZP. First, proteasomal subunits are present in the acrosome of human [43, 47–49], boar (this study), and mouse (unpublished results) spermatozoa. Second, ubiquitin-immunoreactive pro-

teins can be detected on the outer face of the pig ZP (this study), and sequential ubiquitination and proteasomal degradation of the putative sperm receptor protein HrVC70 has been shown on ascidian vitelline envelope, an analogue of mammalian ZP [15–18]. Finally, zona penetration had been blocked by proteasomal inhibitors (this study and [19, 26]) and antiproteasomal antibodies (this study) in mammals and by proteasomal inhibitors in invertebrates [16, 44].

Careful consideration has been given to the possibility that lactacystin, MG-132, and their derivatives could inhibit proteolytic activities of nonproteasomal proteases. Studies conducted to date conclude that nonproteasomal serine proteases, including chymotrypsin, trypsin, or papain, are not affected by the above peptides [39, 42]. These peptides are indeed designed to bind specifically to the catalytic subunit of the proteasome via X/MB1 proteasomal subunit [42], a motif not present in nonproteasomal proteases. The only report suggesting that lactacystin could partially inhibit the activity of cathepsin A [41] was the reason for focusing on the inhibitory action of MG-132 in our fertilization studies. The experiments on sperm mitochondrial degradation [20, 27], in which MG-132 was added at 6 h after insemination to prevent the degradation of sperm mitochondria incorporated inside the fertilized zygote, demonstrated full reversibility of this treatment. The experiments with fertilization of zona-free ova (this study) showed that MG-132 had no effect on the fertilizing ability of boar spermatozoa in vitro. Further support for our results is provided by a report that ALLN, a proteasomal inhibitor closely related to MG-132, blocks mouse fertilization without affecting sperm capacitation or acrosomal exocytosis [19]. Additional evidence is being accumulated in the laboratory, showing that MG-132 blocks zona penetration during bovine and murine fertilization (unpublished results). Similarly, the first evidence of proteasomal activity in human sperm acrosome has been published [28, 29]. While the studies by Morales et al. [28, 29] seem to indicate that proteasomal inhibition may actually block acrosomal exocytosis, it should be considered that these authors used clasto-lactacystin- β -lactone, an irreversible inhibitor that could affect acrosomal functions activated early during exocytosis, i.e., before actual zona digestion.

Altogether, we have to consider three scenarios of proteasomal involvement in sperm-zona interactions during fertilization: 1) The ascidian model according to Sawada et al. [15], in which proteasomes, ubiquitin, and ubiquitin-conjugating enzymes are released from the sperm acrosome to sequentially ubiquitinate and degrade sperm receptor on the vitelline envelope. 2) The porcine model (present study; also applicable to bovine fertilization), in which only proteasomes are released by acrosomal exocytosis to degrade ubiquitinated ZP proteins, already deposited onto ZP surface during oogenesis. 3) The human model according to

Morales et al. [28, 29], proposing a role for acrosomal proteasomes in acrosomal exocytosis and/or dispersion of the acrosomal matrix rather than the actual zona digestion. While the ascidian and porcine models are supported by functional fertilization studies and thus could be considered more reliable, the human model does not rule out that sperm proteasomes could be involved in both acrosomal exocytosis and zona degradation. With regard to abundant O-linked glycosylation of ZP proteins [23, 48], it is worth noting the recent discovery of a unique, E3-type ubiquitin-conjugating enzyme Fbx2, which recognizes trimmed, mannose-enriched sugar residues of glycoproteins rather than a specific sequence of a substrate's amino acids, as a signal for substrate-ligase binding and ubiquitin ligation [49]. The possibility of a similar ubiquitin-conjugating enzyme being involved in the degradation of the outer ZP layer by sperm acrosomal proteasomes corroborates the studies of Benoff et al. [50] showing that the sperm-ZP binding involves the interactions of sperm surface mannose receptors with mannose ligands on the ZP.

While major zona proteins ZP1, ZP2, and ZP3 are thought to be produced exclusively by the oocyte, the ubiquitin-immunoreactive proteins appear predominantly on the cumulus-facing side of ZP and the immunoreactivity also appears at the basal surface of the cumulus innermost layer of cells, the corona radiata. This suggests that the ubiquitinated proteins of ZP may not be exclusively of germ cell origin or at least that the ubiquitin moiety added to them is not. The presence of ubiquitin in the follicular fluid [11] indirectly supports such observations. The ubiquitinated substrate in ascidian vitelline envelope, HrVC70, was shown to be the ascidian homologue of human ZP3 [15]. If a parallel can be drawn between mammals and ascidians/tunicates, our findings will open a new area of research, providing an alternative explanation for the mechanisms of sperm-zona binding and zona penetration during mammalian fertilization, and possibly, a new contraceptive target. Even if one is very cautious interpreting the present data, it is conceivable that some ZP proteins on the outer face of the ZP are posttranslationally modified by ubiquitination, perhaps in a manner similar to varied ZP-protein glycosylation [23]. While ZP3 and other ZP proteins are believed to be secreted mainly by the ovum, ZP3 alpha mRNA appears to be present at high levels in porcine ovarian follicular cells [51]. Thus, ubiquitinated ZP proteins could be deposited directly onto the ZP surface in this and other species. This would render such ZP proteins sensitive to proteasomal degradation and provide an alternative mechanism for species specificity of fertilization at the same time. While ZP3 analogues are thought to be responsible for the species specificity of sperm-zona recognition in mammals [48], human spermatozoa do not bind to the zona of transgenic mice expressing human ZP3 [52]. This may be due to improper or missing posttranslational modification. Given the tremendous variability of ubiquitin-ubiquitin and ubiquitin-protein conjugation [1], it is not surprising that ubiquitination and proteasomal degradation is highly substrate specific [2]. The fate of ubiquitinated substrates is tightly regulated by the order of ubiquitination (monoubiquitination or tetra/polyubiquitination), by the site of ubiquitin chain initiation on the initial substrate-ligated ubiquitin molecule (lysine residues K7, 27, 29, 31, 33, 48, 63) in the ubiquitin-molecule [1] and by the participation of multiple unique ubiquitin conjugating-enzymes with well-defined substrate specificity. This leaves room for the species-specificity of many cellular events, including sperm

mitochondrial degradation [53–55] and possibly in the sperm-zona interactions. Along this line, pig ZP immunoreactivity is observed with only certain antiubiquitin antibodies, a well-known pattern in ubiquitin research, where such probes are proven to be very selective toward certain orders and epitopes of ubiquitin chain formation (e.g., K29, K48, K63-polyubiquitin chain-specific antibodies; [1]).

In conclusion, the present work shows that sperm-ZP penetration during mammalian fertilization can be prevented by specific proteasomal inhibitors and antiproteasomal antibodies. These observations identify the sperm acrosome-borne proteasome as a potentially highly sensitive target for nonhormonal, peptide, drug, or vaccine-based contraceptives. In addition to contraceptive development, the targeting of sperm 26S-proteasome or 20S proteasomal core may provide excellent tools for investigating the timing of sperm-zona interactions during *in vitro* fertilization and eventually provide the tools for management of polyspermia in IVF and embryo-transfer programs.

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REFERENCES

- Pickart CM. Polyubiquitin chains. In: Peters J-M, Harris JR, Finley D (eds.), *Ubiquitin and the Biology of the Cell*. New York: Plenum Press; 1998:19–63.
- Laney JD, Hochstrasser M. Substrate targeting in the ubiquitin system. *Cell* 1999; 97:427–430.
- Hershko A, Ciechanover A. The ubiquitin system. *Annu Rev Biochem* 1998; 67:425–479.
- Conaway RC, Brower CS, Conaway JW. Emerging roles of ubiquitin in transcription regulation. *Science* 2002; 296:1254–1258.
- Glickman MH, Ciechanover A. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev* 2002; 82:373–428.
- Hochstrasser M. New proteases in a ubiquitin stew. *Science* 2002; 298:549–552.
- Tanaka K, Tsurumi C. The 26S proteasome: subunits and functions. *Mol Biol Rep* 1997; 24:3–11.
- Kohler A, Bajorek M, Groll M, Moroder L, Rubin DM, Huber R, Glickman MH, Finley D. The substrate translocation channel of the proteasome. *Biochimie* 2002; 83:325–332.
- Voges D, Zwickl P, Baumeister W. The 26S proteasome: a molecular machine designed for controlled proteolysis. *Annu Rev Biochem* 1999; 68:1015–1068.
- Chen M, Rockel T, Steinweger G, Hemmerich P, Risch J, Von Mikecz A. Subcellular recruitment of fibrillarin to nucleoplasmic proteasomes: implications for processing of a nucleolar autoantigen. *Mol Biol Cell* 2002; 13:3576–3587.
- Einspanier R, Schuster H, Schams D. A comparison of hormone levels in follicle-lutein-cyst and in normal bovine ovarian follicles. *Theriogenology* 1993; 40:181–188.
- Lippert TH, Seeger H, Schieferstein G, Voelter W. Immunoreactive ubiquitin in human seminal plasma. *J Androl* 1993; 14:130–131.
- Hermo L, Jacks D. Nature's ingenuity: bypassing the classical secretory route via apocrine secretion. *Mol Reprod Dev* 2002; 63:394–410.
- Sutovsky P, Moreno R, Ramalho-Santos J, Dominko T, Thompson WE, Schatten G. A putative, ubiquitin-dependent mechanism for the recognition and elimination of defective spermatozoa in the mammalian epididymis. *J Cell Sci* 2001; 114:1665–1675.
- Sawada H, Sakai N, Abe Y, Tanaka E, Takahashi Y, Fujino J, Kodama E, Takizawa S, Yokosawa H. Extracellular ubiquitination and proteasome-mediated degradation of the ascidian sperm receptor. *Proc Natl Acad Sci U S A* 2002; 99:1223–1228.

16. Sawada H, Takahashi Y, Fujino J, Flores SY, Yokosawa H. Localization and roles in fertilization of sperm proteasomes in the ascidian *Halocynthia roretzi*. *Mol Reprod Dev* 2002; 62:271–276.
17. Sakai N, Sawada H, Yokosawa H. Extracellular ubiquitin system implicated in fertilization of the ascidian, *Halocynthia roretzi*: isolation and characterization. *Dev Biol* 2003; 264:299–307.
18. Sakai N, Sawada MT, Sawada H. Non-traditional roles of ubiquitin-proteasome system in fertilization and gametogenesis. *Int J Biochem Cell Biol* 2004; 36:776–784.
19. Wang HM, Song CC, Duan CW, Shi WX, Li CX, Chen DY, Wang YC. Effects of ubiquitin-proteasome pathway on mouse sperm capacitation, acrosome reaction and in vitro fertilization. *Chin Sci Bull* 2002; 47:127–132.
20. Sutovsky P, McCauley TC, Sutovsky M, Day BN. Early degradation of paternal mitochondria in domestic pig (*Sus scrofa*) is prevented by selective proteasomal inhibitors lactacystin and MG-132. *Biol Reprod* 2003; 68:1793–1800.
21. Primakoff P, Myles DG. Penetration, adhesion, and fusion in mammalian sperm-egg interaction. *Science* 2002; 296:2183–2185.
22. Gerton G. Function of the sperm acrosome. In: Hardy D (ed.), *Fertilization*. San Diego: Academic Press; 2002:265–302.
23. Wassarman PM, Litscher ES. Sperm-egg recognition mechanisms in mammals. *Curr Top Dev Biol* 1995; 30:1–19.
24. Cardullo RA, Thaler CD. Function of the egg's extracellular matrix. In: Hardy DM (ed.), *Fertilization*. San Diego: Academic Press; 2002: 119–152.
25. Talbot P, Shur BD, Myles DG. Cell adhesion and fertilization: steps in oocyte transport, sperm-zona pellucida interactions, and sperm-egg fusion. *Biol Reprod* 2003; 68:1–9.
26. Sun QY, Fuchimoto D, Nagai T. Regulatory roles of ubiquitin-proteasome pathway in pig oocyte meiotic maturation and fertilization. *Theorigenology* 2004; 62:245–255.
27. Sutovsky P, van Leyen K, McCauley T, Day BN, Sutovsky M. Degradation of the paternal mitochondria after fertilization: implications for heteroplasmy, ART and mtDNA inheritance. *Reprod Biomed Online* 2004; 8:24–33.
28. Morales P, Kong M, Pizarro E, Pasten C. Participation of the sperm proteasome in human fertilization. *Hum Reprod* 2003; 18:1010–1017.
29. Morales P, Pizarro E, Kong M, Jara M. Extracellular localization of proteasomes in human sperm. *Mol Reprod Dev* 2004; 68:115–124.
30. Sutovsky P, Moreno R, Ramalho-Santos J, Dominko T, Simerly C, Schatten G. Ubiquitinated sperm mitochondria, selective proteolysis and the regulation of mitochondrial inheritance in mammalian embryos. *Biol Reprod* 2000; 63:582–590.
31. Groettrup M, Stander S, Stohwasser R, Kloetzel PM. The subunits MECL-1 and LMP2 are mutually required for incorporation into the 20S proteasome. *Proc Natl Acad Sci U S A* 1997; 94:8970–8975.
32. Hayashi M, Ishibashi T, Tanaka K, Kasahara M. The mouse genes encoding the third pair of beta-type proteasome subunits regulated reciprocally by IFN-gamma: structural comparison, chromosomal localization, and analysis of the promoter. *J Immunol* 1997; 159:2760–2770.
33. Eleuteri AM, Kohanski RA, Cardozo C, Orlowski M. Bovine spleen multicatalytic proteinase complex (proteasome). Replacement of X, Y, and Z subunits by LMP7, LMP2, and MECL1 and changes in properties and specificity. *J Biol Chem* 1997; 272:1824–1831.
34. Groettrup M, Kraft R, Kostka S, Stander S, Stohwasser R, Kloetzel PM. A third interferon-gamma-induced subunit exchange in the 20S proteasome. *Eur J Immunol* 1996; 26:863–869.
35. McCauley T, Sutovsky M, van Leyen K, Lai L, Day BN, Prather RS, Oko R, Sutovsky P. Unique aspects of zygotic development in pig after in vitro fertilization and cloning. *Biol. Reprod* 2002; 66(suppl 1):311.
36. Leclerc P, Goupil S. Regulation of the human sperm tyrosine kinase c-Yes. Activation by cyclic adenosine 3',5'-monophosphate and inhibition by Ca(2+). *Biol Reprod* 2002; 67:301–307.
37. Thompson WE, Powell JM, Whittaker JA, Sridaran R, Thomas KH. Immunolocalization and expression of prohibitin, a mitochondrial associated protein within the rat ovaries. *Anat Rec* 1999; 256:40–48.
38. Abeysdeera LR, Day BN. Fertilization and subsequent development in vitro of pig oocytes inseminated in a modified tris-buffered medium with frozen-thawed ejaculated spermatozoa. *Biol Reprod* 1997; 57: 729–734.
39. Goldberg AL, Stein R, Adams J. New insights into proteasome function: from archaeobacteria to drug development. *Chem Biol* 1995; 2: 503–508.
40. Lee DH, Goldberg AL. Proteasome inhibitors: valuable new tools for cell biologists. *Trends Cell Biol* 1998; 8:397–403.
41. Ostrowska H, Wojcik C, Wilk S, Omura S, Kozlowski L, Stoklosa T, Worowski K, Radziwon P. Separation of cathepsin A-like enzyme and the proteasome: evidence that lactacystin/beta-lactone is not a specific inhibitor of the proteasome. *Int J Biochem Cell Biol* 2000; 32:747–757.
42. Fenteany G, Standaert RF, Lane WS, Choi S, Corey EJ, Schreiber SL. (1995). Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science* 1995; 268: 726–731.
43. Bialy LP, Ziemba HT, Marianowski P, Fracki S, Bury M, Wojcik C. Localization of a proteasomal antigen in human spermatozoa: immunohistochemical electron microscopic study. *Folia Histochem Cytobiol* 2001; 39:129–130.
44. Matsumura K, Aketa K. Proteasome (multicatalytic proteinase) of sea urchin sperm and its possible participation in the acrosome reaction. *Mol Reprod Dev* 1991; 29:189–199.
45. Aviles M, Okinaga T, Shur BD, Ballesta J. Differential expression of glycoside residues in the mammalian zona pellucida. *Mol Reprod Dev* 2000; 57:296–308.
46. Rivett AJ. Intracellular distribution of proteasomes. *Curr Opin Immunol* 1998; 10:110–114.
47. Wojcik C, Benchaib M, Lornage J, Czyba JC, Guerin JF. Proteasomes in human spermatozoa. *Int J Androl* 2000; 23:169–177.
48. Wassarman PM. Profile of a mammalian sperm receptor. *Development* 1990; 108:1–17.
49. Yoshida Y, Chiba T, Tokunaga F, Kawasaki H, Iwai K, Suzuki T, Ito Y, Matsuoka K, Yoshida M, Tanaka K, Tai T. E3 ubiquitin ligase that recognizes sugar chains. *Nature* 2002; 418:438–442.
50. Benoff S, Hurley IR, Mandel FS, Cooper GW, Herschlag A. Induction of the human sperm acrosome reaction with mannose-containing neoglycoprotein ligands. *Mol Hum Reprod* 1997; 3:827–837.
51. Kolle S, Sinowatz F, Boie G, Totzauer I, Amselgruber W, Plendl J. Localization of the mRNA encoding the zona protein ZP3 alpha in the porcine ovary, oocyte and embryo by nonradioactive in situ hybridization. *Histochem J* 1996; 28:441–447.
52. Dean J. Oocyte-specific genes regulate follicle formation, fertility and early mouse development. *J Reprod Immunol* 2002; 53:171–180.
53. Kaneda H, Hayashi JI, Takahama S, Taya C, Fischer-Lindahl K, Yonekawa H. Elimination of paternal mitochondrial DNA in intraspecific crosses during early mouse embryogenesis. *Proc Natl Acad Sci U S A* 1995; 92:4542–4546.
54. Shitara H, Hayashi JI, Takahama S, Kaneda H, Yonekawa H. Maternal inheritance of mouse mtDNA in interspecific hybrids: segregation of the leaked paternal mtDNA followed by the prevention of subsequent paternal leakage. *Genetics* 1998; 148:851–857.
55. Sutovsky P, Moreno R, Ramalho-Santos J, Dominko T, Simerly C, Schatten G. Ubiquitin tag for sperm mitochondria. *Nature* 1999; 402: 371–372.