Mouse Zona Pellucida Glycoproteins mZP2 and mZP3 Undergo Carboxy-Terminal Proteolytic Processing in Growing Oocytes[†]

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ABSTRACT: The extracellular coat, or zona pellucida, of the mouse egg consists of three glycoproteins, called mZP1−3. The glycoproteins are synthesized and secreted concomitantly by growing oocytes during their 2−3-week growth phase. Each of the glycoproteins has a consensus furin cleavage site (-Arg-X-Lys/Arg-Arg-) near the C-terminus of their polypeptide. Here, several approaches were employed to determine whether nascent mZP2 and mZP3 are cleaved at the consensus sites, -Arg-Ser-Lys-Arg- and -Arg-Asn-Arg-Arg-, respectively, prior to secretion. Molecular mass determinations of deglycosylated mZP2 and mZP3 suggest that their polypeptides are ∼9 and ∼7 kDa smaller, respectively, than predicted from exon sequences. Two-dimensional thin-layer chromatographic analyses were also carried out to identify amino acids released from the C-terminus of mZP2 and mZP3 by carboxypeptidase B. On the basis of exon sequences, there are no Arg residues at the predicted C-terminus of the mature glycoproteins. However, for both mZP2 and mZP3, Arg residues were released by carboxypeptidase B, consistent with processing at the consensus furin cleavage site. Furthermore, an antiserum raised against an mZP3 peptide, located downstream of the consensus furin cleavage site, failed to label purified mZP3 on Western immunoblots. The antiserum also failed to label the zona pellucida of oocytes examined by laser scanning confocal microscopy. Collectively, these results strongly suggest that mZP2 and mZP3 are processed at their consensus furin cleavage site prior to secretion and incorporation into the zona pellucida.

All mammalian eggs are surrounded by a thick extracellular coat, called the zona pellucida (ZP)[1] (*1*). The ZP first appears during the oocyte growth phase and increases in thickness as oocytes increase in diameter (*2*). It is well-known that the ZP plays important roles during oogenesis, fertilization, and preimplantation development (*3*). For example, during fertilization the ZP restricts interactions between eggs and sperm from different mammalian species and, following fertilization, prevents fusion of eggs with more than one sperm (i.e., prevents polyspermy).

The mouse egg ZP is ∼6 μm thick, contains ∼3 ng of protein, and consists of three glycoproteins, called mZP1−3 (*4*). All three glycoproteins are heterogeneously glycosylated at both Asn (N-linked) and Ser/Thr (O-linked) residues. Today it is apparent that the ZP of eggs from virtually all mammalian species, including human beings, consist of glycoproteins whose polypeptides are very similar to mZP1−3 (*5*). Even the vitelline layer of eggs from nonmammalian species, including fish, birds, and amphibians, possesses glycoproteins related to mZP1−3.

In mice, mZP1−3 are essential structural components of the ZP (*6*−*8*). For example, growing oocytes and eggs from mice that are homozygous nulls for the *mZP3* gene completely lack a ZP (*9*, *10*), and heterozygous nulls have a thinner than usual ZP (*11*). In addition to their structural role, mZP2 and mZP3 perform other important functions during fertilization. For example, mZP3 serves as a primary receptor for sperm and as an inducer of the acrosome reaction following binding of sperm to ovulated eggs (*12*, *13*). Free-swimming sperm apparently recognize and bind to specific oligosaccharides linked to mZP3 (*12*−*14*), making mammalian fertilization an example of carbohydrate-mediated cellular adhesion.

In mice, ZP glycoproteins are synthesized and secreted exclusively by growing oocytes during a 2−3-week period (*4*). Genes encoding the polypeptides of ZP glycoproteins are expressed exclusively by growing oocytes and are regulated by *cis*-acting sequences located close to the transcription start site and several *trans*-acting factors (*15*−*18*). Here, we have examined the potential utilization of the consensus furin cleavage site of mZP2 and mZP3, a feature common to polypeptides of ZP glycoproteins (*13*), during intracellular processing of the nascent glycoproteins. The results suggest that nascent mZP2 and mZP3 are, indeed, cleaved at their consensus furin cleavage site such that polypeptides of the secreted glycoproteins are considerably smaller than predicted from their exon sequences.

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[1] Abbreviations: ZP, zona pellucida; PBS, phosphate-buffered saline; PVP-40, poly(vinylpyrrolidone)-40; EDTA, ethylenediaminetetraacetic acid; NEM, *N*-ethylmorpholine; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; N-linked, asparagine-linked; O-linked, serine/threonine-linked; C-terminus, carboxy terminus; CBP-B, carboxypeptidase B; N-glycanase, peptide-N^4-(*N*-acetyl-β-glucosaminyl)-asparagine amidase; TFMS, trifluoromethanesulfonic acid; Dans-Cl, 1-(dimethylamino)naphthalene-5-sulfonyl chloride.

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MATERIALS AND METHODS

Isolation of Mouse Oocytes. Growing oocytes were isolated by puncturing ovaries excised from 15−18 day-old Swiss albino female mice (CD-1; Charles River Laboratories) essentially as previously described (*19*). Oocytes were collected with mouth-operated glass micropipets into phosphate-buffered saline (PBS) containing poly(vinylpyrrolidone)-40 (PVP-40). In certain cases, oocytes lacking a ZP were isolated from mice that are homozygous nulls for the *mZP3* gene (*mZP3*$^{-/-}$) essentially as previously described (*9*).

Purification of mZP2 and mZP3. Mouse ovarian homogenates were centrifuged through a Percoll gradient, a band containing ZP was collected, and ZP glycoproteins were purified to homogeneity by HPLC on a size-exclusion column (Bio-Sil, SEC-250), essentially as previously described (*20*). In experiments described here, ∼1000 mouse ovaries (∼500 mice) yielded ∼250 μg of mZP2 and ∼150 μg of mZP3 by this purification procedure.

Enzymatic Deglycosylation. Lyophilized samples of ZP glycoproteins (5−10 μg) were dissolved in 15 μL of 25 mM phosphate−25 mM EDTA, pH 7.6, and boiled for 5 min. After cooling, 5 μL of 7.5% octyl glycoside in 25 mM phosphate−25 mM EDTA, pH 7.6, and 5 μL of N-glycanase (1.25 units; EC 3.5.1.52, Genzyme) were added, and the sample was incubated at 37 °C for 24 h. To stop the reaction, samples were placed at −20 °C and stored frozen prior to use (*21*).

Acid Hydrolysis of Glycosidic Bonds. Samples of ZP glycoproteins (10−20 μg) were lyophilized to dryness in a glass tube with a screw cap. Trifluoromethanesulfonic acid (TFMS; Fluka) was mixed with anisole (Fluka), 9:1 by volume, 50 μL was added to each dried sample, and a stream of N_2 was applied over the surface. Samples were put on ice and kept at 0 °C overnight. The reaction was stopped by slowly adding the sample to a mixture of 500 μL of precooled (−20 °C) pyridine/diethyl ether (Fluka), 1:9 by volume. The precipitated deglycosylated protein was separated from the acid solution by centrifugation at 10000*g* for 10 min. The resulting pellet was redissolved in 0.1 M NH_4HCO_3 and dialyzed extensively, first against NH_4HCO_3 and then against water (*22*). Fetuin (Sigma) and BSA (Sigma) were treated in an identical manner and served as controls.

Digestion by Carboxypeptidase B (CBP-B). Lyophilized samples of ZP glycoproteins (100−200 μg) were dissolved in 19 μL of 0.2 M *N*-ethylmorpholine (NEM, Fluka), pH 8.5, and 1 μL of CBP-B (60 milliunits, DFP-treated; EC 3.4.17.2, Fluka) and incubated at 37 °C for 4 h (*23*). The reaction was stopped by freezing the sample (−20 °C), and it was then subjected to ultrafiltration (Microcon-30; Amicon) to separate released amino acids from protein. Briefly, microconcentrators were first spin-rinsed with 0.1 N NaOH and then water to remove trace amounts of glycerol. Samples were diluted to 400 μL with water and centrifuged through a microconcentrator at 4000*g* at 4 °C for 45 min. The filtrate, which contained amino acids, was lyophilized and kept at −20 °C prior to thin-layer chromatographic analysis.

Two-Dimensional Thin-Layer Chromatography. Lyophilized filtrate containing amino acids was fluorescently labeled by dansylation. A solution of 1-(dimethylamino)naphthalene-5-sulfonyl chloride (Dans-Cl; Fluka) was prepared by mixing Dans-Cl (5 mg/mL in acetone; stored in the dark at 4 °C) and water, 1:1 by volume. Samples were redissolved in 7 μL of 0.2 M $NaHCO_3$, pH 10, to which 3 μL of Dans-Cl was added. The reaction was allowed to proceed at 45 °C for 45 min (*23*). Dansylated samples were lyophilized and stored in the dark at 4 °C.

Two-dimensional thin-layer chromatography on polyamide thin-layer sheets (5 cm × 5 cm; Schleicher and Schuell) was used to separate and identify dansylated amino acids. Two solvent systems were freshly prepared for each analysis; first dimension, 4% formic acid, and second dimension, 20% acetic acid in benzene (*24*). Dansylated samples were redissolved in 2 μL of water just prior to the analysis, and 1 μL was applied to the bottom left-hand corner of the polyamide sheet. Chromatography was carried out in sealed tanks, first in 4% formic acid and, after air-drying the sheet, in 20% acetic acid in benzene at right angle to the direction of movement in the first solvent. The air-dried chromatogram was viewed and photographed under UV light. Standard amino acids (Sigma), a peptide fragment (insulin-like growth factor II, fragment 33−40; Sigma), and lactate dehydrogenase (LDH-5, M_4; Sigma) were used in control experiments.

SDS−PAGE and Immunoblotting. Western immunoblotting was carried out with HPLC-purified ZP glycoproteins (untreated or modified) dissolved in SDS sample buffer. Samples were subjected to SDS−PAGE, transferred to nitrocellulose, and probed with an antibody. Four different rabbit polyclonal antisera were used here: (i) an antiserum directed against mZP2 (#8810); (ii) an antiserum directed against mZP3 (#8811); (iii) an antiserum directed against a peptide (17-mer) representing amino acid residues 306−322 of mZP3 (NH_2-Cys-Ser-Asn-Ser-Ser-Ser-Ser-Gln-Phe-Gln-Ile-His-Gly-Pro-Arg-Gln-Trp-$COOH^{322}$; anti-pep#306-322); (iv) an antiserum directed against a C-terminal peptide (20-mer) representing amino acid residues 347−366 of mZP3 (NH_2-Leu-Gly-Lys-Ala-Asn-Asp-Gln-Thr-Val-Glu-Gly-Trp-Thr-Ala-Ser-Ala-Gln-Thr-Ser-Val-$COOH^{366}$; anti-pep#347-366). In each case, alkaline phosphatase conjugated goat anti-rabbit IgG (Bio-Rad) and NBT/BCIP (Bio-Rad) were used to visualize ZP glycoprotein bands (*25*). To visualize the biotinylated standards (M_r markers), alkaline phosphatase conjugated avidin (Bio-Rad) and NBT/BCIP were used. Antisera were produced at Pocono Rabbit Farms, Canadensis, PA, and peptides were synthesized at the Molecular Resource Facility, New Jersey Medical and Dental School, Newark, NJ.

In some cases, samples were dissolved in PBS and loaded onto nitrocellulose membrane using a Bio-Dot apparatus (Bio-Rad). The membrane was incubated with antiserum anti-pep#347-366 and then developed, as described above for Western immunoblotting.

Laser Scanning Confocal Microscopy. Isolated growing oocytes were fixed in freshly prepared 3.7% paraformaldehyde at room temperature for 15 min and for 30 min in blocking buffer (2% BSA in PBS/PVP-40). Oocytes were then incubated at room temperature for 1 h with either anti-pep#306-322 (1:100) or anti-pep#347-366 (1:100) in blocking buffer, followed by 1 h in the dark with FITC-conjugated goat anti-rabbit IgG (1:50; Pierce). After the oocytes were washed with blocking buffer, the fluorescent signal was stabilized with equilibration buffer (Slowfade Antifade Kit; Molecular Probes) for 15 min in the dark. Oocytes were

Table 1: Molecular Weight Analyses of mZP2 and mZP3

	M_r (kDa) mZP2	M_r (kDa) mZP3
(A) calcd M_r, polypeptide[a] (minus signal sequence)	~75 (679 aa)[c]	~44 (402 aa)
(B) calcd M_r carboxy-terminal peptide[1] (cleavage at consensus furin sequence)	8.6 (78 aa)	7.8 (71 aa)
(C) calcd M_r, polypeptide minus C-terminal peptide[a] (A minus B)	66.1 (601 aa)	36.4 (331 aa)
(D) exptl M_r, polypeptide[b] (TFMS treated-glycoproteins)	~67	~37
(E) exptl M_r, secreted glycoprotein[b]	~120	~83
(F) exptl M_r, N-glycanase-treated secreted glycoprotein[b] (minus N-linked oligosaccharides)	~84	~46
(G) calcd M_r, N-linked oligosaccharides (E minus F)	~36	~37
(H) calcd M_r, O-linked oligosaccharides [E minus (D plus G)]	~17	~9

[a] M_r calculated by using 110 as the average M_r of an amino acid (see Results). [b] M_r determined by SDS−PAGE in the presence of protein M_r standards (see Results). [c] The abbreviation for amino acids is aa.

washed through several drops of PBS/PVP-40, mounted on glass slides with PBS−glycerol, and visualized with a Leica inverted laser scanning confocal microscope. Central optical sections were examined, images processed by using Adobe photoshop software, and pictures printed using a color dye sublimation printer.

RESULTS

Removal of N-Linked Oligosaccharides from ZP Glycoproteins by N-Glycanase. Both mZP2 and mZP3 possess complex-type N-linked oligosaccharides, as well as O-linked oligosaccharides (*26*). It has been reported that mZP2 has six N-linked oligosaccharides and mZP3 has three or four N-linked oligosaccharides (*27*, *28*). To estimate the contribution of N-linked oligosaccharides to the M_rs of mZP2 and mZP3, purified glycoproteins were digested with recombinant N-glycanase. This enzyme catalyzes the hydrolysis of N-linked oligosaccharides at the β-aspartyl−glycosylamine bond between innermost GlcNAc residues and Asn residues that are part of the consensus sequence Asn-X-Ser/Thr of glycoproteins (*29*).

mZP2 and mZP3 were partially deglycosylated with N-glycanase at 37 °C for 24, 48, and 74 h (see Materials and Methods). Aliquots from each digest were subjected to SDS−PAGE, blotted onto nitrocellulose, and stained by Western immunoblotting. After 24 h, both mZP2 and mZP3 exhibited reductions in M_r that did not change after longer exposures to N-glycanase. mZP2 was reduced from ~120 to ~83.6 kDa (data not shown) and mZP3 from ~83 to ~46.4 kDa (Figure 1, lane b). These changes suggest that ~30% and ~45% of the M_rs of mZP2 and mZP3, respectively, are attributable to N-linked oligosaccharides. Since the polypeptides of mZP2 and mZP3 are ~74.7 and ~44.2 kDa, respectively, only ~7.4% (~8.9 kDa) of the M_r of mZP2 and ~2.7% (~2.2 kDa) of the M_r of mZP3 can be attributed to O-linked oligosaccharides (Table 1). It should be noted that all M_rs reported here should be considered as "apparent M_rs", since it is well-known that glycoproteins and glycopeptides migrate anomalously on SDS−PAGE due, in part, to a low charge-to-mass ratio for the molecules (*30*).

Removal of Oligosaccharides from ZP Glycoproteins by Trifluoromethanesulfonic Acid. To estimate the contribution of total oligosaccharide to the M_rs of mZP2 and mZP3, purified glycoproteins were hydrolyzed with TFMS. This reagent removes both O-linked and N-linked oligosaccharides from glycoproteins, leaving only a GlcNAc linked to Asn residues at the consensus sequence Asn-X-Ser/Thr (*31*, *32*).

mZP2 and mZP3 were dissolved in TFMS/anisole and incubated on ice for about 16 h. The reaction was stopped by adding the sample to a mixture of pyridine/diethyl ether, followed by dialysis as described in Materials and Methods. In each experiment, a standard glycoprotein (fetuin; positive control) and protein lacking oligosaccharides (BSA; negative control) were treated in an identical manner. To evaluate the extent of deglycosylation, all samples were dissolved in SDS sample buffer, subjected to SDS−PAGE, and processed for immunoblotting (mZP2 and mZP3) or silver staining (fetuin and BSA).

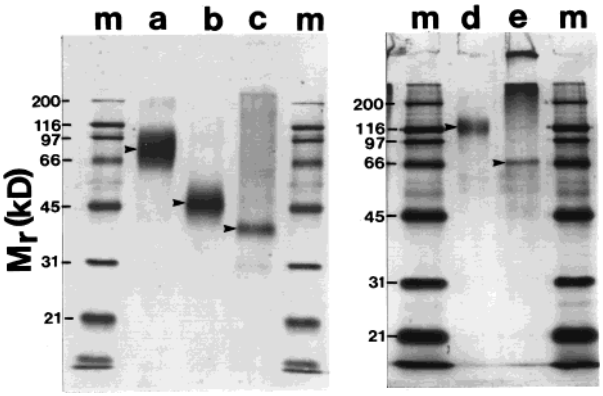

FIGURE 1: SDS−PAGE analysis of ZP glycoproteins treated with either N-glycanase or TFMS. Western immunoblotting was carried out as described in Materials and Methods. Shown are results obtained with (a) purified mZP3 (~200 ng), (b) purified mZP3 (~200 ng) treated with N-glycanase at 37 °C for 24 h, (c) purified mZP3 (~1 μg) treated with TFMS on ice for 16 h, (d) purified mZP2 (~200 ng), and (e) purified mZP2 (~5 μg) treated with TFMS on ice for 16 h. Arrowheads indicate the positions of untreated (a and d) and treated (b, c, and e) ZP glycoproteins. Lanes denoted as m contain biotinylated protein standards (Bio-Rad) for estimating M_r (kDa).

As seen in Figure 1, lane e and lane c, TFMS-treated mZP2 and mZP3 migrate as relatively sharp bands at ~67 and ~37 kDa, respectively. Under similar conditions, TFMS treatment had no detectable effect on BSA (~66 kDa) but lowered the M_r of fetuin from ~68 to ~51 kDa. These results are of interest, since TFMS-treated mZP2 (~67 kDa) and mZP3 (~37 kDa) exhibit M_rs that are significantly lower than those predicted from their exon sequences (i.e., ~75 kDa for mZP2 and ~44 kDa for mZP3), but are in very good agreement with M_rs calculated for polypeptides minus C-terminal peptides (i.e., ~66 kDa for mZP2 and ~36 kDa for mZP3) (Table 1). Therefore, proteolytic cleavage of mZP2 and mZP3 at their consensus furin cleavage site, -Arg-Ser-Lys-Arg^{601}- (amino acids 598−601) and -Arg-Asn-Arg-Arg^{331}- (amino acids 328−331), respectively, could account for the TFMS results (Table 1).

C-Terminal Sequence Analysis of ZP Glycoproteins. In view of the TFMS results described above, C-terminal

analysis of mZP2 and mZP3 polypeptides was carried out. In these experiments, the glycoproteins were exposed to CBP-B, a protease that excises only Arg and Lys residues from the C-terminus of proteins. The released amino acids were fluorescently labeled with Dans-Cl and separated by two-dimensional thin-layer chromatography, as described in Materials and Methods.

The amino acid sequence predicted for the C-terminus of mZP2, based on the sequence of *mZP2* gene exon-18, is -Arg-Phe-Asn-His-COOH^{679} (amino acids 676−679). If the polypeptide was cleaved at the consensus furin cleavage site, the C-terminal sequence would be -Arg-Ser-Lys-Arg-COOH^{601} (amino acids 598−601). The amino acid sequence predicted for the C-terminus of mZP3, based on the sequence of *mZP3* gene exon-8, is -Ser-Leu-Pro-Gln-COOH^{402} (amino acids 399−402). On the other hand, if the polypeptide was cleaved at the consensus furin cleavage site, the C-terminal sequence would be -Arg-Asn-Arg-Arg-COOH^{331} (amino acids 328−331). Therefore, due to the specificity of CBP-B for C-terminal Arg or Lys residues, it should be possible to distinguish between these possibilities.

In preliminary experiments, we used the published pattern of the chromatographic behavior of more than 80 amino acids as a reference (*24*). In addition, several individual dansylated amino acids, including Arg, Lys, and Gly, were subjected to two-dimensional thin-layer chromatography and identified on chromatograms.

Shown in Figure 2 are chromatograms of dansylated amino acids from CBP-B digests of mZP2 and mZP3. In the case of mZP3, two spots are seen in the position assigned to Arg residues (Figure 2A), whereas in the case of mZP2, one spot is seen in the position assigned to Arg residues (Figure 2B). A peptide consisting of eight amino acids, NH_2-Ser-Arg-Val-Ser-Arg-Arg-Ser-Arg-COOH, was employed as a positive control for the release of C-terminal Arg residues by CBP-B (Figure 2F). In addition, several negative control samples were subjected to two-dimensional thin-layer chromatography: (i) mZP2 alone (Figure 2C), (ii) Arg alone (Figure 2D), (iii) Lys alone (Figure 2E), (iv) CBP-B alone (Figure 2G), and (v) CBP-B-treated lactate dehydrogenase (C-terminus, -Glu-Leu-Gln-Phe-COOH) (Figure 2H). Collectively, these results suggest that Arg residues are released from both mZP2 and mZP3 by CBP-B.

Several additional points should be noted. A noticeable spot, representing dansylsulfonic acid (a byproduct of dansylation; blue fluorescence, as opposed to green fluorescence for dansylated amino acids), was observed and provided an additional marker. Another prominent spot observed in mZP2 and mZP3 samples, with and without CBP-B, represented Gly. This spot and several others are contaminants seen in all samples subjected to dialysis and ultrafiltration (e.g., Figure 2A−C) but not seen in unfiltered samples (e.g., Figure 2D,E).

Immunoblot Analysis of the C-Terminus of ZP Glycoproteins. As described in Materials and Methods, three different polyclonal rabbit antisera were used to probe polypeptide epitopes of mZP3 on either Western immunoblots or immunodot blots. These are (i) antiserum #8811 directed against purified mZP3, (ii) antiserum anti-pep#306-322 directed against a peptide corresponding to amino acid residues 306−322 of mZP3, and (iii) antiserum anti-pep#347-366 directed against a C-terminal peptide corresponding to amino acid residues 347−366 of mZP3. A synthetic peptide, consisting of amino acids 347−366 at the C-terminus of mZP3, was used as a positive control in immunodot-blot experiments with anti-pep#347-366.

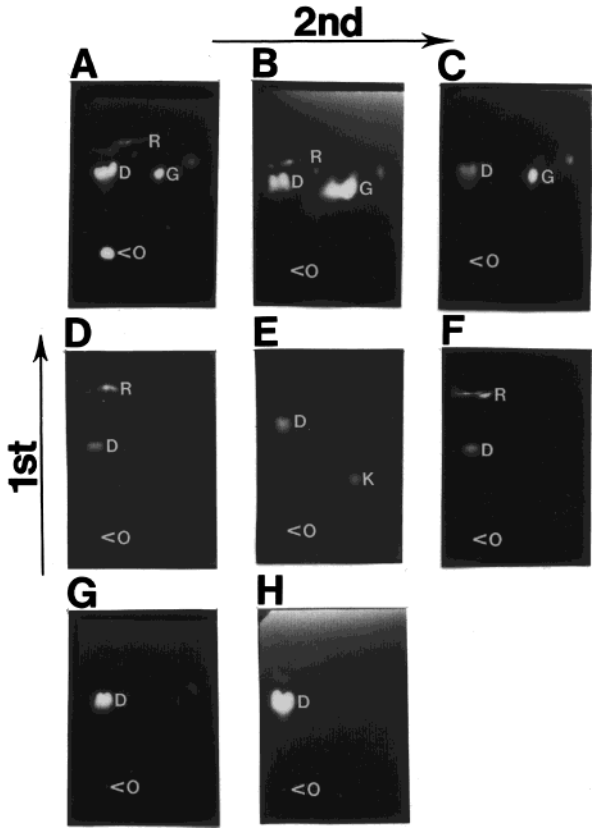

FIGURE 2: Two-dimensional thin-layer chromatograms of dansylated amino acids. Shown are photographs of chromatograms obtained in the manner described in Materials and Methods. Panels: (A) CBP-B (~6.6 pmol) treated purified mZP3 (~480 pmol; ~40 μg); (B) CBP-B (~6.6 pmol) treated purified mZP2 (~833 pmol; ~100 μg); (C) untreated purified mZP2 (~667 pmol; ~80 μg); (D) Arg alone (~574 pmol); (E) Lys alone (~684 pmol); (F) CBP-B (~6.6 pmol) treated peptide with a C-terminal Arg (insulin-like growth factor II, fragment 33−40, Sigma; ~1 nmol); (G) CBP-B alone (~6.6 pmol); (H) CBP-B (~6.6 pmol) treated lactate dehydrogenase with a C-terminal Phe (LDH-5; ~2.8 nmol; ~100 μg). The first and second directions of separation are indicated by arrows. An arrowhead denotes the position of the origin (O). The positions of dansyl chloride (D), Gly (G), Arg (R), and Lys (K) are also indicated where appropriate.

Western immunoblots of purified mZP3 (~250 ng) exhibited staining with antisera directed against mZP3 (#8811; 1:500 dilution) and against amino acids 306−322 of mZP3 (anti-pep#306-322; 1:250 dilution) but no staining with an antiserum directed against amino acids 347−366 at the C-terminus of mZP3 (anti-pep#347-366; 1:250 dilution) (Figure 3B). In this context, it was noted that immunodot-blot assays with antiserum anti-pep#347-366 and synthetic peptide 347−366 (~44−220 pmol) were positive, whereas those with purified mZP3 (~84 pmol) were negative (Figure 3A). These results suggest that the C-terminal epitope of mZP3, encompassing amino acids 347−366, is missing from purified mZP3.

Laser Scanning Confocal Microscopy of mZP3 in Growing Oocytes. Laser scanning confocal microscopy was used to examine the cellular location of mZP3 epitopes recognized by antisera anti-pep#306-322 and anti-pep#347-366. Growing

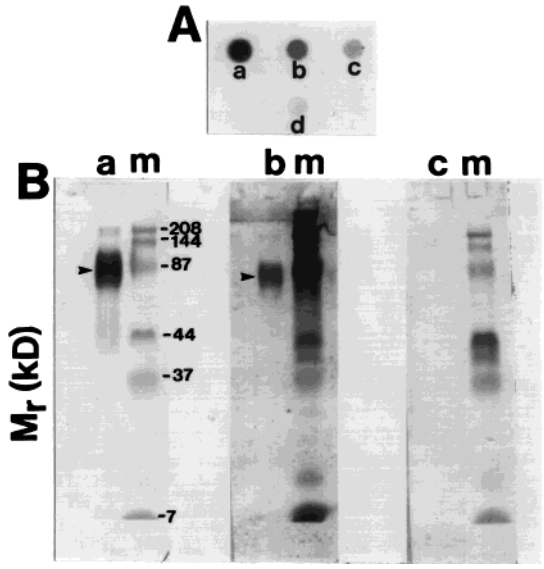

FIGURE 3: Western immunoblots of mZP3 probed with antisera directed against specific mZP3 peptides. Western immunoblotting was carried out as described in Materials and Methods. Panel A: Shown are immunodot-blot assays using anti-pep#347-366 and (a) 500 ng, (b) 250 ng, or (c) 100 ng of peptide 347-366 and (d) purified mZP3 (~84 pmol; ~7 μg). Panel B: Shown are results obtained with purified mZP3 (~250 ng/lane) stained with (a) anti-mZP3#8811 (1:500), (b) anti-pep#306-322 (1:250), or (c) anti-pep#347-366 (1:250). Lanes denoted as m contain prestained protein standards (Amersham) for estimating M_r (kDa).

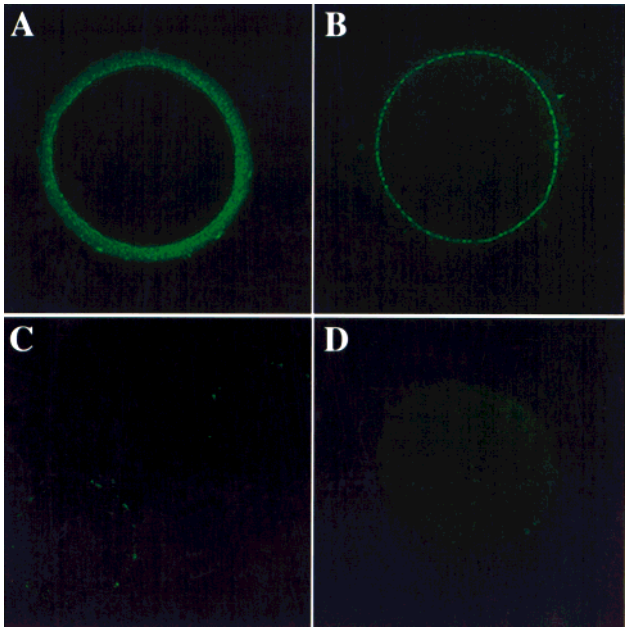

FIGURE 4: Laser scanning confocal microscopy of mZP3 probed with antisera directed against mZP3 and specific mZP3 peptides. Growing oocytes isolated from wild-type (*mZP3*$^{+/+}$; panels A−C) and homozygous null (*mZP3*$^{-/-}$; panel D) mice (see Materials and Methods) were probed with rabbit (A) anti-pep#306-322 (1:100 dilution), (B and D) anti-pep#347-366 (1:100 dilution), or (C) preimmune serum (1:100 dilution). An FITC-conjugated, goat anti-rabbit IgG (1:50 dilution) was used to visualize the oocytes by laser scanning confocal microscopy, as described in Materials and Methods.

oocytes were immunofluroescently labeled, under nonpermeable conditions, and examined as described in Materials and Methods. Oocytes probed with anti-pep#306-322 exhibited intense fluorescence over the ZP (Figure 4A), whereas those probed with anti-pep#347-366 directed against the C-terminus of mZP3 exhibited no signal over the ZP (Figure 4B). However, oocytes treated with anti-pep#347-366 displayed fluorescent spots along the external surface of the plasma membrane, underneath the ZP, suggesting the presence of mZP3 C-terminal epitopes in this location. Oocytes isolated from mice that are homozygous nulls for *mZP3* (*mZP3*$^{-/-}$) and do not synthesize any mZP3 exhibited no staining with anti-pep#347-366 (Figure 4D).

DISCUSSION

Maturation of nascent ZP glycoproteins involves complex cotranslational and posttranslational processing events. For example, the polypeptides possess N-terminal signal sequences for initial transfer of the nascent polypeptides to the endoplasmic reticulum; there, cotranslational addition of high-mannose-type, N-linked oligosaccharides are added at Asn-X-Ser/Thr consensus sequences (*33*−*38*). O-Linked oligosaccharides are added to Ser/Thr residues of ZP glycoproteins in the Golgi, and the glycoproteins are secreted and assembled around growing oocytes. As a result of processing, mouse ZP glycoproteins mZP1, mZP2, and mZP3 have apparent M_rs of ~200 (dimer), ~120, and ~83 kDa, respectively. On the basis of exon sequences, the calculated M_rs of the polypeptides of mZP1, mZP2, and mZP3 are ~70 (monomer), ~76, and ~44 kDa, respectively. These M_rs and various biochemical information indicate that the mature, secreted form of each ZP glycoprotein is extensively glycosylated (*27*, *28*, *38*, *39*).

Recently, it has been reported that an increasing number of biologically active peptides and proteins are produced by cleavage of polypeptide precursors at sites composed of sequential basic amino acid residues. These include many secreted mammalian proteins and peptide hormones, viral envelope glycoproteins, and bacterial exotoxins, as well as other proteins (*40*−*44*). The enzymes responsible for cleavage of the polypeptides belong to a novel family of mammalian subtilisin-like serine endoproteases, of which furin has been studied most extensively (*45*−*47*). Furin is expressed in all tissues and cell lines examined thus far. It appears to be localized predominantly in the trans-Golgi network (TGN), with some enzyme cycling between TGN and the cell surface (*47*−*49*). The ubiquitous expression of the *fur* gene suggests a role for furin in cleavage of proproteins secreted via the constitutive secretory pathway (*50*).

Our first indication that mZP3 was proteolytically processed in the region of its C-terminus came from attempts to purifiy mZP3 from milk of transgenic mice (*51*; E. S. Litscher and P. M. Wassarman, unpublished results). In these experiments, the *mZP3* gene was placed under control of the β-casein promoter, and a short stretch of His residues was added to the C-terminus of the mZP3 polypeptide to aid in purification of the glycoprotein. The presence of a region encoding the His sequence (18 bp insertion; six His residues) was confirmed by PCR analysis. Despite several attempts to purify the glycoprotein on Ni^{2+}−NTA−agarose columns by conventional procedures, none of the material would bind to the column. Subsequently, Western immunoblots of milk mZP3 and a control protein possessing a stretch of His residues revealed that milk mZP3 lacked a

His tag. This suggested that nascent mZP3 was proteolytically processed at its consensus furin cleavage site, thus eliminating the stretch of His residues added to the recombinant polypeptide.

Here, we have investigated C-terminal processing of mZP2 and mZP3 at consensus furin cleavage sites (Arg-X-Lys/Arg-Arg). Although it has been known for some time that both glycoproteins contain a potential recognition site for furin near the C-terminus, it had not been demonstrated that cleavage took place. The inability to detect such processing previously may be attributed to extensive heterogeneous glycosylation of the polypeptides that causes them to migrate as broad bands on SDS−PAGE. mZP2 is synthesized as an ~80 kDa polypeptide, is modified by removal of an N-terminal, 34 amino acid signal sequence, and is glycosylated with N- and O-linked oligosaccharides. mZP3 is synthesized as a ~44 kDa polypeptide, is modified by removal of an N-terminal, 22 amino acid signal sequence, and is glycosylated with N- and O-linked oligosaccharides. Removal of N-linked oligosaccharides from mZP2 and mZP3 by digestion with N-glycanase reduces the apparent M_r of mZP2 from ~120 to ~84 kDa and of mZP3 from ~83 to ~46 kDa (Figure 1; Table 1).

To assess the M_rs of mature mZP2 and mZP3 polypeptides, both glycoproteins were deglycosylated by TFMS, a strong acid that cleaves at acetal linkages. Accordingly, O-linked oligosaccharides are removed completely, and all but the *N*-acetylglucosamine residue at the nonreducing end of N-linked oligosaccharides are removed (*22*). Examination of deglycosylated polypeptides on SDS−PAGE suggests that both mZP2 and mZP3 have lower M_rs than predicted for the polypeptides from their exon sequences. Deglycosylated mZP2 and mZP3 polypeptides are ~67 and ~37 kDa, respectively, whereas the calculated M_rs are ~75 and ~44 kDa, respectively (Table 1). However, were nascent mZP2 and mZP3 polypeptides to be cleaved at their consensus furin cleavage sites, their M_rs would be completely consistent with those M_rs measured experimentally by SDS−PAGE analysis of deglycosylated polypeptides (Figure 5; Table 1). It should be noted that hamster ZP3 (hZP3; 56 kDa) behaves in a similar manner; the deglycosylated hZP3 polypeptide exhibits a significantly lower M_r than that calculated from exon sequences (M. Weetall, E. Litscher, and P. Wassarman, unpublished results).

Support for results presented above comes from C-terminal sequence analyses of mZP2 and mZP3 using CBP-B, a protease specific for C-terminal Arg and Lys residues. The data suggest that Arg residues are located at the C-terminus of each ZP glycoprotein (Figure 2). For example, the final four amino acids of full-length mZP3, predicted from exon sequences, would be Ser-Leu-Pro-GlnCOOH^{402} (amino acids 399−402); no Arg residues. On the other hand, cleavage of mZP3 polypeptide at the consensus furin site would leave the sequence Arg-Asn-Arg-ArgCOOH^{331} (amino acids 328−331) at the C-terminus. As seen in Figure 2, CBP-B released Arg residues from purified mZP3, suggesting that the nascent polypeptide was processed at the consensus furin cleavage site prior to secretion and assembly of the ZP. Similar results were obtained with purified mZP2, consistent with the presence of Arg residues at its C-terminus (Figure 2).

Additional evidence for cleavage of ZP glycoproteins at their consensus furin cleavage sites was obtained by Western

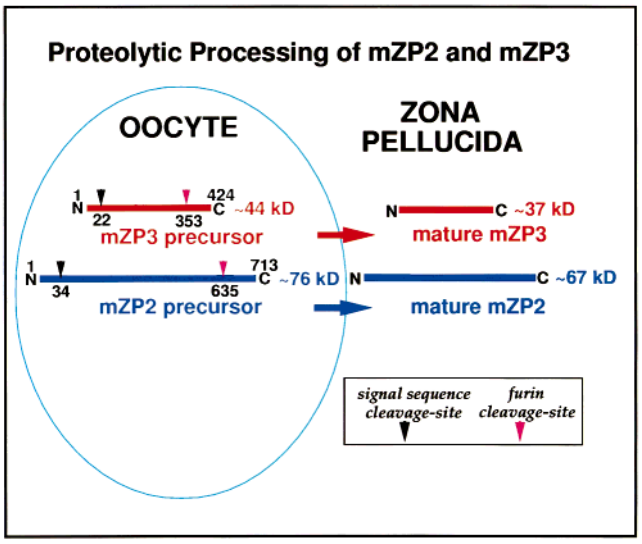

FIGURE 5: Schematic diagram of the proteolytic processing of nascent mZP2 and mZP3 polypeptides during oocyte growth. Shown are the nascent polypeptides of the ZP glycoproteins (mZP2, 713 amino acids; mZP3, M_r 424 amino acids) with their N-terminal signal sequences (mZP2, amino acids 1−34; mZP3, amino acids 1−22) and consensus furin cleavage sites [mZP2, amino acid 635 (amino acid 601 plus signal sequence); mZP3, amino acid 353 (amino acid 331 plus signal sequence)] intact in the growing oocyte but missing from the polypeptides (mZP2, M_r ~67 kDa, 601 amino acids; mZP3, M_r ~37 kDa, 331 amino acids) following secretion from the oocyte and incorporation into the ZP.

immunoblotting of purified mZP3 (Figure 3) and by laser scanning confocal microscopy of growing oocytes (Figure 4). An antiserum directed against a peptide sequence representing amino acids 347−366 at the C-terminus of mZP3 failed to label either purified mZP3 (Figure 3) or the ZP of growing oocytes (Figure 4). On the other hand, an antiserum directed against a peptide sequence representing amino acids 306−322 of mZP3 labeled purified mZP3 (Figure 3) and the ZP of growing oocytes (Figure 4). In this context, experiments carried out with as many as 1000 growing mouse oocytes failed to reveal any labeling with antiserum anti-pep#347-366 on Western immunoblots (data not shown). However, as seen in Figure 4, some labeling of wild-type oocyte plasma membrane with anti-pep#347-366 was observed by laser scanning confocal microscopy, whereas labeling of *mZP3*$^{-/-}$ oocyte plasma membrane was not observed. Whether the labeling is due to the presence of small amounts of full-length mZP3 or the C-terminal fragment of mZP3, containing epitope 347−366, remains to be determined. Indeed, it is possible that proteolytic processing of ZP glycoproteins takes place at the oocyte plasma membrane, a site proposed as a furin processing compartment (*40*, *42*, *49*).

In a number of instances, proteolytic cleavage of secreted and surface glycoproteins is essential for biological activity and is often accompanied by a conformational change of the cleaved molecule (*44*, *52*−*54*). In the case of mZP2 and mZP3, cleavage at the consensus furin cleavage sites would result in loss of a single cysteine residue from each glycoprotein, leaving an even number of cysteine residues in each polypeptide: 20 for mZP2 and 12 for mZP3. Since, in general, secreted glycoproteins have intramolecular disulfides and lack free sulfhydryl groups, cleavage of mZP2 and mZP3 would eliminate a potentially free sulfhydryl

group. Whether this is required for secretion and assembly of ZP glycoproteins remains to be determined.

In conclusion, evidence presented here stongly suggests that nascent mZP2 and mZP3 are proteolytically cleaved at their consensus furin cleavage sites prior to secretion (Figure 5). It will be of interest to determine whether furin or a furin-like convertase (*55*−*57*) or some other endoprotease (*58*), perhaps, oocyte-specific and/or developmentally regulated, is involved in processing of nascent ZP glycoproteins. In addition, it will be of interest to determine whether proteolytic processing is necessary for secretion of the nascent glycoproteins, for their incorporation into the growing oocyte ZP, and for the sperm receptor activity of mZP3.

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REFERENCES

1. Dietl, J., Ed. (1989) *The Mammalian Egg Coat. Structure and Function*, Springer-Verlag, Heidelberg.
2. Wassarman, P. M., Albertini, D. F. (1994) The mammalian ovum, in *The Physiology of Reproduction* (Knobil, E., and Neill, J. D., Eds.) pp 79−122, Raven Press, New York.
3. Yanagimachi, R. (1994) Mammalian fertilization, in *The Physiology of Reproduction* (Knobil, E., Neill, J. D., Eds.) pp 189−317, Raven Press, New York.
4. Wassarman P. M. (1988) Zona pellucida glycoproteins, *Annu. Rev. Biochem. 57*, 415−442.
5. Wassarman, P. M. (1999) Egg zona pellucida glycoproteins, in *Guidebook to the Extracellular Matrix and Adhesion Proteins* (Kreis, T., and Vale, R., Eds.) pp 411−414, Oxford University Press, Oxford (in press).
6. Greve, J. M., Wassarman, P. M. (1985) Mouse egg extracellular coat is a matrix of interconnected filaments possessing a structural repeat, *J. Mol. Biol. 181*, 253−264.
7. Wassarman, P. M., Mortillo, S. (1991) Structure of the mouse egg extracellular coat, the zona pellucida, *Intl. Rev. Cytol. 130*, 85−109.
8. Wassarman, P. M., Liu, C., and Litscher, E. S. (1996) Constructing the mammalian egg zona pellucida: some new pieces of an old puzzle, *J. Cell Sci. 109*, 2001−2004.
9. Liu, C., Litscher, E. S., Mortillo, S., Sakai, Y., Kinloch, R. A., Stewart, C. L., and Wassarman, P. M. (1996) Targeted disruption of the *mZP3* gene results in production of eggs lacking a zona pellucida and infertility in female mice, *Proc. Natl. Acad. Sci. U.S.A. 93*, 5431−5436.
10. Rankin, T., Familiari, M., Lee, E., Ginsburg, A., Dwyer, N., Blanchette-Mackie, J., Drago, J., Westphal, H., and Dean, J. (1996) Mice homozygous for an insertional mutation in the ZP3 gene lack a zona pellucida and are infertile, *Development 122*, 2903−2910.
11. Wassarman, P. M., Qi, H., and Litscher, E. S. (1997) Mutant female mice carrying a single *mZP3* allele produce eggs with a thin zona pellucida, but reproduce normally, *Proc. R. Soc., Biol. Sci. 264*, 323−328.
12. Wassarman, P. M. (1990) Profile of a mammalian sperm receptor, *Development 108*, 1−17.
13. Wassarman, P. M. (1999) Mammalian fertilization: molecular aspects of gamete adhesion, exocytosis, and fusion, *Cell 96*, 175−183.
14. Chen, J., Litscher, E. S., and Wassarman, P. M. (1998) Inactivation of the mouse sperm receptor, mZP3, by site-directed mutagenesis of individual serine residues located at the combining-site for sperm, *Proc. Natl. Acad. Sci. U.S.A. 95*, 6193−6197.
15. Schickler, M., Lira, S. A., Kinloch, R. A., and Wassarman, P. M. (1992) A mouse oocyte-specific protein that binds to a region of mZP3 promoter involved in regulating oocyte-specific expression of the mZP3 gene, *Mol. Cell. Biol. 12*, 120−127.
16. Kinloch, R. A., Lira, S. A., Mortillo, S., Schickler, M., Roller, R. J., and Wassarman, P. M. (1993) Regulation of expression of *mZP3*, the sperm receptor gene, during mouse development, in *Molecular Basis of Morphogenesis* (Bernfield, M., Ed.) pp 19−33, Wiley-Liss, New York.
17. Millar, S. E., Lader, E. S., and Dean, J. (1993) ZAP-1 DNA binding activity is first detected at the onset of zona pellucida gene expression in embryonic mouse oocytes, *Dev. Biol. 158*, 410−413.
18. Liang, L., Soyal, S. M., and Dean, J. (1997) FIGα, a germ cell specific transcription factor involved in the coordinate expression of the zona pellucida genes, *Development 124*, 4939−4947.
19. Moller, C. C., Bleil, J. D., Kinloch, R. A., and Wassarman, P. M. (1990) Structural and functional relationship between mouse and hamster zona pellucida glycoproteins, *Dev. Biol. 137*, 276−286.
20. Litscher, E. S., Wassarman, P. M. (1999) Purification and functional analysis of mouse egg zona pellucida glycoproteins, in *A Comparative Methods Approach to the Study of Oocytes and Embryos* (Richter, J. D., Ed.) pp 10−22, Oxford University Press, Oxford.
21. Litscher, E. S., and Wassarman, P. M. (1996) Characterization of a mouse ZP3-derived glycopeptide, gp55, that exhibits sperm receptor and acrosome reaction-inducing activity *in vitro*, *Biochemistry 35*, 3980−3985.
22. Karp, D. R., Atkinson, J. P., and Shreffler, D. C. (1982) Genetic variation in glycosylation of the fourth component of murine complement, *J. Biol. Chem. 57*, 7330−7335.
23. Yarwood, A. (1989) Manual methods of protein sequencing, in *Protein Sequencing. A Practical Approach* (Findlay, J. B. C., and Geisow, M. J., Eds.) pp 119−145, IRL Press, Oxford.
24. Laatsch, H. (1979) Identification of rare amino acids by microdansylation, *J. Chromatogr. 173*, 398−402.
25. Kinloch, R. A., Mortillo, S., Stewart, C. L., and Wassarman, P. M. (1991) Embryonal carcinoma cells transfected with ZP3 genes differentially glycosylate similar polypeptides and secrete active mouse sperm receptor, *J. Cell Biol. 115*, 655−664.
26. Wassarman, P. M., Bleil, J. D., Florman, H. M., Greve, J. M., Roller, R. J., Salzmann, G. S., and Samuels, F. G. (1985) The mouse egg's receptor for sperm: What is it and how does it work?, *Cold Spring Harbor Symp. Quant. Biol. 50*, 11−19.
27. Greve, J. M., Salzmann, G. S., Roller, R. J., and Wassarman, P. M. (1982) Biosynthesis of the major zona pellucida glycoprotein secreted by oocytes during mammalian oogenesis, *Cell 31*, 749−759.
28. Salzmann, G. S., Greve, J. M., Roller, R. J., and Wassarman, P. M. (1983) Biosynthesis of the sperm receptor during oogenesis in the mouse, *EMBO J. 2*, 1451−1456.
29. Barsomian, G. D., Johnson, T. L., Borowski, M., Denman, J., Ollington, J. F., Hirani, S., McNeilly, D. S., and Rasmussen, J. R. (1990) Cloning and expression of peptide-N^4-(*N*-acetyl-β-D-glucosamyl)asparagine amidase F in *Escherichia coli*, *J. Biol. Chem. 265*, 6967−6972.
30. Leach, B. S., Collawn, J. F., Jr., and Fish, W. W. (1980) Behavior of glycopeptides with empirical molecular weight estimation methods, *Biochemistry 19*, 5734−5741.
31. Edge, A. S. B., Faltynek, C. R., Hof, L., Reichert, L. E., and Weber, P. (1981) Deglycosylation of proteins by trifluoromethanesulfonic acid, *Anal. Biochem. 118*, 131−137.
32. Sojar, H. T., and Bahl, O. P (1987) A chemical method for the deglycosylation of proteins, *Arch. Biochem. Biophys. 259*, 52−57.
33. Kinloch, R. A., Roller, R. J., Fimiani, C. M., Wassarman, D. A., and Wassarman, P. M. (1988) Primary structure of the mouse sperm receptor polypeptide determined by genomic cloning, *Proc. Natl. Acad. Sci. U.S.A. 85*, 6409−6413.
34. Ringuette, M. J., Chamberlin, M. E., Baur, A. W., Sobieski, D. A., and Dean, J. (1988) Molecular analysis of cDNA coding

for ZP3, a sperm binding protein of the mouse zona, *Dev. Biol. 127*, 287−295.
35. Kinloch, R. A., Ruiz-Seiler, B., and Wassarman, P. M. (1990) Genomic organization and polypeptide primary structure of zona pellucida glycoprotein hZP3, the hamster sperm receptor, *Dev. Biol. 142*, 414−421.
36. Chamberlin, M. E., and Dean, J. (1990) Human homologue of the mouse sperm receptor, *Proc. Natl. Acad. Sci. U.S.A. 87*, 6014−6018.
37. Liang, L., Chamow, S. M., and Dean, J. (1990) Oocyte-specific expression of mouse Zp-2: developmental regulation of the zona pellucida genes, *Mol. Cell. Biol. 10*, 1507−1515.
38. Epifano, O., Liang, L., Familari, M., Moos, M. C., Jr., and Dean, J. (1995) Coordinate expression of the three zona pellucida genes during mouse oogenesis, *Development 121*, 1947−1956.
39. Roller R. J., and Wassarman, P. M. (1983) Role of asparagine-linked oligosaccharides in secretion of glycoproteins of the mouse egg's extracellular coat, *J. Biol. Chem. 258*, 13243−13249.
40. Klimpel, K. R., Molloy, S. S., Thomas, G., and Leppla, S. H. (1992) Anthrax toxin protective antigen is activated by a cell surface protease with the sequence specificity and catalytic properties of furin, *Proc. Natl. Acad. Sci. U.S.A. 98*, 10277−10281.
41. Tsuneoka, M., Nakayama, K., Hatsuzawa, K., Komada, M., Kitamura, N., and Mekada, E. (1993) Evidence for involvement of furin in cleavage and activation of diphteria toxin, *J. Biol. Chem. 268*, 26461−26465.
42. Abrami, L., Fivaz, M., Decroly, E., Seidah, N. G., Jean, F., Thomas, G., Leppla, S. H., Buckley, J. T., and van der Goot, F. G. (1998) The pore-forming toxin proaerolysin is activated by furin, *J. Biol. Chem. 273*, 32656−32661.
43. Logeat, F., Bessia, C., Brou, C., LeBail, O., Jarriault, S., Seidah, N. G., and Israel, A. (1998) The notch 1 receptor is cleaved constitutively by a furin-like convertase, *Proc. Natl. Acad. Sci. U.S.A. 95*, 8108−8112.
44. Volchkov, V. E., Feldmann, H., Volchkova, V. A., and Klenk, H. D. (1998) Processing of the ebola virus glycoprotein by the proprotein convertase furin, *Proc. Natl. Acad. Sci. U.S.A. 95*, 5762−5767.
45. Barr, P. J. (1991) Mammalian subtilisins: the long-sought dibasic processing endoproteases, *Cell 66*, 1−3.
46. Nakayama, K. (1997) Furin: a mammalian subtilisin/Kex2p-like endoprotease involved in processing of a wide variety of precursor proteins, *Biochem. J. 327*, 625−635.
47. Molloy, S. S., Anderson, E. D., Jean, F., and Thomas, G. (1999) Bi-cycling the furin pathway: from TGN localization to pathogen activation and embryogenesis, *Trends Cell Biol. 9*, 28−35.
48. Molloy, S. S., Thomas, L., VanSlyke, J. K., Stenberg, P. E., and Thomas, G. (1994) Intracellular trafficking and activation of the furin proprotein convertase: localization to the TGN and recycling from the cell surface, *EMBO J. 13*, 18−33.
49. Liu, G., Thomas, L., Warren, R. A., Enns, C. A., Cunningham, C. C., Hartwig, J. H., and Thomas, G. (1997) Cytoskeletal protein ABP-280 directs the intracellular trafficking of furin and modulates proprotein processing in the endocytic pathway, *J. Cell Biol. 139*, 1719−1733.
50. Hosaka, M., Nagahama, M., Kim, W., Watanabe, T., Hatsuzawa, K., Ikemizu, J., Murakami, K., and Nakayama, K. (1991) Arg-X-Lys/Arg-Arg motif as a signal for precursor cleavage catalyzed by furin within the constitutive pathway, *J. Biol. Chem. 266*, 12127−12130.
51. Litscher, E. S., Liu, C., Echelard, Y., and Wassarman, P. M. (1999) Zona pellucida glycoprotein mZP3 produced in milk of transgenic mice is active as a sperm receptor, but can be lethal to newborns, *Transgenic Res.* (in press).
52. McCune, J. M., Rabin, L. B., Feinberg, M. B., Lieberman, M., Kosek, J. C., Reyes, G. R., and Weissman, I. L. (1988) Endoproteolytic cleavage of gp160 is required for the activation of human immunodeficiency virus, *Cell 53*, 55−67.
53. Bullough, P. A., Hughson, F. M., Skehel, J. J., and Wiley, D. C. (1994) Structure of influenza haemagglutinin at the pH of membrane fusion, *Nature 371*, 37−43.
54. Chan, D. C., Fass, D., Berger, J. M., and Kim, P. S. (1997) Core structure of gp41 from the HIV envelope glycoprotein, *Cell 89*, 263−273.
55. Nakayama, K., Kim, W.-S., Torii, S., Hosaka, M., Nagakawa, T., Ikemizu, J., Baba, T., and Murakami, K. (1992) Identification of the fourth member of the mammalian endoprotease family homologous to the yeast Kex2 protease: its testis-specific expression, *J. Biol. Chem. 267*, 5897−5900.
56. Seidah, N. G., Day, R., Hamelin, J., Gaspart, A., Collard, M. W., and Chrétien, M. (1992) Testicular expression of PC4 in the rat: molecular diversity of a novel germ cell-specific Kex2/subtilisin-like proprotein convertase, *Mol. Endocrinol. 6*, 1559−1570.
57. Torii, S., Yamagishi, T., Murakami, K., and Nakayama, K. (1993) Localization of Kex2-like processing endoproteases, furin and PC4, within mouse testis by in situ hybridization, *FEBS Lett. 316*, 12−16.
58. Blobel, C. P. (1999) Role of metalloprotease-disintegrins in cell−cell interactions and in the cleavage of TNFα and Notch, *Adv. Dev. Biochem. 5*, 163−196.

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