Secretion of Mouse ZP3, the Sperm Receptor, Requires Cleavage of Its Polypeptide at a Consensus Furin Cleavage-Site†

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Received September 27, 2000; Revised Manuscript Received November 22, 2000

ABSTRACT: The mouse egg extracellular coat, or zona pellucida, consists of three glycoproteins, called mZP1−3. Each glycoprotein possesses a consensus sequence recognized by the furin family of proprotein convertases. Previously, it was reported that mZP2 and mZP3 are cleaved at their consensus furin cleavage-sites located near the C-terminus of the polypeptides [Litscher, E. S., Qi, H., and Wassarman, P. M. (1999) *Biochemistry 38*, 12280−12287]. Here, use of site-directed mutagenesis of the *mZP3* gene and a specific inhibitor of furin-like enzymes revealed that secretion of nascent mZP3 from transfected cells is dependent on cleavage of mZP3 at its consensus furin cleavage-site. The dependence of secretion on cleavage represents a novel function for furin family enzymes.

All mammalian eggs are surrounded by a thick extracellular coat, the zona pellucida (ZP).[1] The mouse egg ZP is composed of three glycoproteins, called mZP1−3, that are organized into cross-linked filaments possessing a structural periodicity (*1*−*4*). Similar glycoproteins are found in the ZP of eggs from all mammals, including human beings (*4*). Furthermore, the extracellular coat, or vitelline envelope, surrounding eggs from fish, birds, and frogs also contains glycoproteins that are homologous with ZP glycoproteins (*5*).

mZP3 functions as a sperm receptor and inducer of the sperm acrosome reaction during fertilization in mice (*4*). The oligosaccharides of mZP3 are essential for binding of sperm and induction of the acrosome reaction, i.e., adhesion of sperm to eggs in mice is a carbohydrate-mediated event. mZP3 (∼83 kDa apparent M_r) is synthesized exclusively by growing oocytes over a 2−3-week period as an ∼44 kDa M_r polypeptide (424 amino acids) to which asparagine- (N-) linked and serine/threonine- (O-) linked oligosaccharides are added (*3*). Prior to secretion, the mZP3 polypeptide is proteolytically processed to remove an N-terminal signal-sequence (22 amino acids) encoded by exon-1 and a C-terminal peptide (71 amino acids) encoded by exon-8 of *mZP3* (8 exons). The latter processing occurs at a consensus furin cleavage-site (CFCS) that is common to all ZP glycoproteins (*6*). For mZP3, the CFCS is located 34 amino acids upstream from a predicted transmembrane domain.

Furin and other members of the proprotein convertase (PC) family (PACE4, PC6, PC7, PC1/3, PC2, and PC4) are calcium-dependent serine endoproteases related to yeast protease Kex2P and bacterial subtilisin (*7*). PCs cleave on the C-terminal side of paired basic amino acids, while furin has an added requirement for a basic amino acid at the P4 position (*8*). The range of substrates for PCs is remarkably diverse, including hormones, growth factors, receptors, viral glycoproteins, and bacterial toxins (*9*). In general, PCs function in activating their substrates (*10*). For example, cleavage of viral glycoproteins and bacterial toxins is required for activation of their fusogenic properties; an action that may prevent intracellular activation of the proteins (*11*−*13*). In some cases, protein multimerization is prevented and exit from the endoplasmic reticulum (ER) is delayed in the absence of furin cleavage (*10*, *14*−*17*). In other cases, the role of cleavage is unclear since noncleavable mutant polypeptides retain functional activity (*16*, *18*). To date, a role for furin enzymes in regulating secretion has not been demonstrated.

Previously, we reported that mZP3 from egg ZP is cleaved at its CFCS (−Arg−Asn−Arg−Arg−), resulting in removal of a 71-amino acid C-terminal peptide encoded by *mZP3* exon-8 (*6*). Here, we examined the relationship between C-terminal cleavage and secretion of nascent mZP3 by stably and transiently transfected cells. Results of the investigation strongly suggest that the presence of the C-terminal peptide encoded by *mZP3* exon-8 is necessary for secretion, but failure to cleave the peptide at its CFCS prevents secretion of nascent mZP3 from cells. Uncleaved nascent mZP3 accumulates in the ER and possesses high-mannose-type, not complex-type, N-linked oligosaccharides. Therefore, in addition to its well documented role in protein activation, furin may play a role in regulating protein secretion.

† The research was supported in part by a grant from the NIH (HD-35105).

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[1] Abbreviations: ZP, zona pellucida; CFCS, consensus furin cleavage-site; PC, proprotein convertase; ER, endoplasmic reticulum; EC, embryonal carcinoma; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; MCS, multiple cloning site; SDS−PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RT, room temperature; N-linked, asparagine-linked; O-linked, serine/threonine-linked; C-terminus, carboxy-terminus; LSCM, laser scanning confocal microscopy; DPC, decanoylated peptidyl chloromethylketone; PSB, protein sample buffer; HRP, horseradish peroxidase; *N*-Gly, peptide-N^4-(*N*-acetyl-β-glucosaminyl)-asparagine amidase; Endo H, endo-β-*N*-acetylglucosaminidase H.

10.1021/bi002275x CCC: $20.00
Published on Web 01/03/2001

EXPERIMENTAL PROCEDURES

mZP3 Expression Constructs. Flag/mZP3. Construct *PGK/mZP3* was described previously (*19*). It consists of the entire *mZP3* coding region, as well as polyadenylation and termination signals, placed under the control of the *pgk-1* promoter (*20*). *PGK/mZP3* contains two *Sal* I sites; one in the *pBluescript SK* (+) phagemid MCS (Stratagene) and one in the genomic *mZP3* gene. The *Sal* I site in the MCS would interfere with further steps and was eliminated. *Sal* I-/+ *PGK/mZP3* was generated by digesting *PGK/mZP3* with *Cla* I/*Xho* I. The ends were filled with T4 polymerase, and blunt ends were ligated. A *Sal* I/*Xba* I fragment (1.7 kb) of *PGK/mZP3* was ligated into a *pGEM-11zf(+)* plasmid (Promega) that had been digested with *Sal* I/*Xba* I. This fragment contains the last three exons of mouse genomic *ZP3*, including the polyadenylation and termination sequences, as well as the coding region for the CFCS and the hydrophobic domain. This plasmid, called *Sal* I/*Xba* I *PGK/mZP3 pGEM-11zf(+)*, was digested with *Apa* I. The *Apa* I site is found in a poorly conserved region upstream of the hydrophobic tail of mZP3 but downstream of the CFCS. A double-stranded oligonucleotide containing the complete in frame *Flag* sequence, with a sticky end complimentary to an *Apa* I site, was ligated to the *Apa* I site of *Sal* I/*Xba* I *PGK/mZP3 pGEM-11zf(+)*. The sequences for the sense and antisense *Flag* inserts were 5′-CGACTACAAGGACGACGATGACAAGGGGCC-3′ and 5′-CCTTGTCATCGTCGTCCTTGTAGTCGGGCC-3′, respectively. Each primer (0.5 μg) was present in T4 ligase buffer with ATP and T4 kinase. The mixtures were boiled for 2 min and combined. The combined mixtures were placed in a 68 °C heating block that was turned off and allowed to cool for 3 h. *Flag* primers (100 ng) were then ligated to *Apa* I digested *Sal* I/*Xba* I *PGK/mZP3pGEM-11zf(+)* which had been gel-purified and then treated with calf intestinal phosphatase (New England Biolabs). The resulting plasmid was called *Flag*$^+$*Sal* I/*Xba* I *PGK/mZP3 pGEM-11zf(+)*. The accuracy of the insertion was assessed by DNA sequencing. *Sal* I-/+/*Flag*$^+$ *PGK/mZP3* was generated by digesting *Sal* I-/+ *PGK/mZP3* with *Sal* I/*Not* I and purifying the fragment lacking this 1.7-kb insert. A *Sal* I/*Not* I fragment from *Flag*$^+$*Sal* I/*Xba* I *PGK/mZP3 pGEM-11zf(+)* was ligated into the *Sal* I/*Not* I site of *Sal* I-/+ *PGK/mZP3*.

*Flag/*Δ*F-mZP3.* This construct was generated by using Quick Change Site-Directed Mutagenesis (QCSDM; Stratagene) on *Flag*$^+$*Sal* I/*Xba* I *PGK/mZP3* to convert the CFCS from −Arg−Asn−Arg−Arg− to a noncleavable form −Arg−Asn−Ala−Ala− (Volchkov et al., 1998). The sense and antisense primers used were 5′-GCTAGTTTCTCGAAACGCCGCGCACGGTATGTCGGGG-3′ and 5′-CCCCGACATACCGTGCGCGGCGTTTCGAGAAACTAGC-3′, respectively (Genset Oligos). The mutation was confirmed by DNA sequencing. A *Sal* I/*Not* I fragment from this mutated plasmid was ligated into an 8-kb gel purified *Sal* I/*Not* I fragment of *Sal* I-/+*PGK/mZP3* creating *Flag/*Δ*F-mZP3*.

Flag/Stop-mZP3. This construct was generated by using QCSDM on *Flag*$^+$*Sal* I/*Xba* I *PGK/mZP3* to place a stop-codon immediately after the CFCS of mZP3. Specifically, −Arg−Asn−Arg−Arg−His−Val was converted to −Arg−Asn−Arg−Arg−Stop−Val− using sense and antisense primers 5′-CTCGAAACCGCAGGTAGGGTATGTCGGGGACACG-3′ and 5′-CGTGTCCCCGACATACCCTACCTGCGGTTTCGAG-3′, respectively. The mutation was confirmed by DNA sequencing. A *Sal* I/*Not* I fragment from this mutated plasmid was ligated into an 8-kb gel-purified *Sal* I/*Not* I fragment of *Sal* I-/+*PGK/mZP3* creating *Flag/Stop-mZP3*.

CMV-Flag/mZP3. A partial *mZP3* cDNA, lacking both the 5′- and the 3′- ends, was obtained from the ATCC (no. 63163). Overhanging primers were utilized to generate by PCR a full length cDNA, including a 5′ Kozak sequence, a 3′ Flag epitope, and a termination sequence. The 5′ primers used were 5′-CGTCAAGCTATTTCCTCTTCCTTTGTCTCTGCTG-3′ and 5′-CCGGATCCACCATGGCGTCAAGCTATTTCCTCTTCCT-3′. The 3′ primers used were 5′-GGGATACAAGGTAGGAAGAGGAGTGACACTTCCTGGTGA-3′ and 5′-CCGGTACCTTTTATTGCGGAAGGGATACAAGGTAGGAAGAG-3′ (Genset Oligos). The full-length *mZP3*, including the coding sequence for Flag, was cloned into *pCR 3.1* (Invitrogen) which has an upstream CMV promoter.

*CMV-Flag/*Δ*F-mZP3.* PCR mutagenesis by QCSDM was used on *CMV-Flag/mZP3* to convert the CFCS, −Arg−Asn−Arg−Arg−, to a noncleavable form, −Arg−Asn−Gly−Glu− (*13*). The primers used were 5′-CTAGTTTCTCGAAACGGCGAGCACGTGACCGATGAAGCTG-3′ and 5′-CAGCTTCATCGGTCACGTGCTCGCCGTTTCGAGAAACTAG-3′. The mutation was confirmed by DNA sequencing.

CMV-Flag/Stop-mZP3. PCR mutagenesis by QCSDM was used on *CMV-Flag/mZP3* to insert a stop-codon immediately after the CFCS. Nucleotide mutagenesis converted the sequence −Arg−Asn−Arg−Arg−His−Val− to −Arg−Asn−Arg−Arg−Stop−Val−. The primers used were 5′-CGAAACCGCAGGTAAGTGACCGATGAAGCTGATG-3′ and 5′-CATCAGCTTCATCGGTCACTTACCTGCGGTTTCG-3′. The mutation was confirmed by DNA sequencing.

Cell Culture. Embryonal carcinoma (EC) cells (F9) and 293T cells were cultured in D-MEM (GIBCO-BRL) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and 3 μg/mL Gentamicin (EC culture medium) (*21*). Transfected EC cell lines were selected in the same culture medium supplemented with G418 (0.4 mg/mL) and were then cultured in the presence of G418 (0.2 mg/mL). Standard EC and 293T cell culture procedures were followed (*22*). All cells were cultured at 37 °C, in 5% CO_2 in air and a humidified atmosphere.

Production of Stably Tranfected EC Cell Lines. EC cells were cotransfected with one of the *Flag/mZP3* constructs and pKJ-1, a neomycin resistance plasmid (*19*). The *Flag/mZP3* vectors were digested with *Pvu* I and *Not* I and the *Flag/mZP3* fragments, which include the *pgk-1* promoter, were gel-purified with the GFX PCR DNA and Gel Band Purification kit (Amersham). pKJ-1 was linearized with *EcoR* I and *Hind* III and gel-purified with the GFX PCR DNA and Gel Band Purification kit (Amersham). Digested *PGK/mZP3* inserts (30 μg) were each mixed with pKJ-1 insert (3 μg). For each transfection, an 80% confluent plate (100 mm diameter) of EC cells was trypsinized and the cells resuspended in 10 mL of EC culture medium. Cells were spun at 500 rpm at 4 °C for 5 min. Supernatant was removed and

the pellet of cells was resuspended in 500 μL of ice-cold PBS. The solution containing the *Flag/mZP3* construct of interest and pKJ-1 was added to the resuspended cells and the mixture was kept on ice for 5 min prior to electroporation. Electroporation was performed (BioRad Gene Pulser; 0.4 cm cuvette, 625−875 V/cm, 250−500 μF, pulse time of ∼6.9 ms). The cuvette was immediately placed on ice for 10 min. Cells were then mixed with 10 mL of standard EC cell culture medium, plated onto gelatin-coated, tissue culture plates, and cultured at 37 °C. A day later, cells were washed with PBS and fresh cell culture medium was added. A day later, cells were washed with PBS and culture medium, supplemented with G418 (0.4 μg/mL), was added. The culture medium was changed every other day. After approximately 9 days, individual colonies were visible. Plates were washed with PBS and trypsinized. As colonies became loose, they were collected, plated, and grown.

Production of Transiently Transfected 293T Cells. The various *CMV*/*mZP3* constructs were introduced into 293T cells using Lipofectamine Plus (Gibco BRL), essentially as described by the manufacturer. Briefly, 293T cells were grown to 75% confluency on 35-mm tissue culture dishes. Each of the *CMV*/*mZP3* constructs (1.6 μg) was mixed with Plus reagent (14 μL) and D-MEM (100 μL) and then incubated at RT for 15 min. To this, a premixed solution of D-MEM (100 μL) and Lipofectamine (4 μL) was added. The mixture was incubated for an additional 15 min and was then added to the 293T cells in 800 μL of fresh EC cell culture medium. Cells were incubated for 4 h and, after fresh culture medium (2 mL) was added, were grown for an additional 30 h. Cells were then washed twice with PBS (pH 7.3) and incubated for 18 h in D-MEM.

Western Immunoblot Analyses. For collection of culture medium and cell lysates, 75% confluent plates were washed twice with PBS (pH 7.3) and incubated for 18 h in D-MEM without any supplements. After 18 h, the culture medium was collected and filtered. Cells were washed twice with PBS and then lysis buffer (1% Triton X-100, 1 mM iodoacetamide, 10 mM Tris-Cl, pH 7.5, 0.14 M NaCl, 0.025% sodium azide, and 1.5× protease inhibitor cocktail [Boehringer Mannheim]) was added for 20 min at 4 °C. Cells were scraped off the plate, collected, and stored at −20 °C. Prior to use, cell lysates were spun for 5 min at 14 000 rpm and the supernatants used for analysis.

Culture medium and cell lysates were adjusted to 2% SDS/10% glycerol/50 mM DTT/0.05% bromphenol blue (protein sample buffer, PSB) and boiled for 3−5 min. When the volume of culture medium was greater than 20 μL, samples were concentrated using a Centricon-30 (Amicon). Aliquots of treated culture medium and cell lysates were run on 10% SDS−PAGE gels and transferred to nitrocellulose membrane (Amersham Pharmacia Biotech). The membrane was processed for Western immunoblotting by first incubating in 5% fat-free milk/TBS (0.02 M Tris-Cl, 0.5 M NaCl, pH 7.5) for 1 h at RT. The membrane was then incubated in the presence of either polyclonal antibody no. 8811 (1:10 000 dilution; Pocono Rabbit Farms) directed against mZP3 (anti-mZP3) or monoclonal antibody M2 (10 μg/mL; Sigma) directed against Flag (anti-Flag). Incubations with antibodies were carried out in 0.5% fat-free milk/TBS for 1 h at RT, and the membranes were thoroughly washed with TBS. Membranes were then exposed to either HRP-conjugated goat anti-rabbit (Bio-Rad) or HRP-conjugated goat anti-mouse (Organon Teknida Corp.) secondary antibody (1:5000 dilution in 0.5% fat-free milk/TBS) and incubated for 1 h at RT. Proteins were visualized with either Lumi-Light Western Blotting Substrate (Boehringer-Mannheim) or ECL+Plus (Amersham Life Sciences). Quantitation of band intensities was carried out with NIH Image Version 1.61 (http://rsb.info.nih.gov). Lysates were first compared and equalized. The amount of secreted mZP3 was compared between samples and adjusted for the relative amounts seen in cell lysates. In some cases, membranes were stripped by washing in distilled water for 5 min, 0.2 M NaOH for 5 min, and distilled water for 5 min, all at RT. Membranes were then blocked and probed as described.

Furin Inhibitor Assays. Stably transfected and untransfected cells were grown to 75% confluence. Culture medium was removed and replaced with complete culture medium supplemented with 52 μg/mL (∼70 μM) decanoylated peptidyl chloromethylketone (DPC; Bachem) and incubated for 4 h. Culture medium containing DPC was removed, cells were washed twice with PBS, and D-MEM containing 52 μg/mL DPC was added. Cells were cultured for 8 h and DPC was added (52 μg/mL) for an additional 4 h. Culture medium and cell lysates were collected as previously described.

Glycosidase Treatment. Culture medium (100 μL) and lysates (10 μL) from cells stably transfected with *Flag/mZP3* were digested with either endoglycosidase H (Endo H; New England Biolabs) or N-glycosidase F (N-Gly; Boehringer Mannheim). Endo H treatments were carried out in denaturing buffer containing 5% SDS and 10% β-mercaptoethanol (final concentrations, 0.5% SDS and 1% β-mercaptoethanol), samples were boiled for 10 min, and one-tenth volume of 10× G5 buffer (0.5 M sodium citrate, pH 5.5) and 1500 U of Endo H were added. The samples were incubated at 37 °C for 4 h, boiled for 5 min in PSB, and run on 10% SDS−PAGE gels. N-Gly treatments were carried out by adding 20 μL of buffer A (25 mM sodium phosphate, 25 mM EDTA, pH 7.6) to the samples and boiling for 5 min. After boiling, 5 μL buffer B (buffer A + 7.5% octyl-β-glucoside) and 2.5 U of N-Gly were added. The samples were incubated at 37 °C for 18 h, boiled for 5 min in PSB, and run on 10% SDS−PAGE gels.

Immunofluorescence. Stably transfected and untransfected EC cells were cultured overnight on 0.1% gelatin-coated coverslips. Coverslips were washed in PBS (pH 7.3), and cells were fixed in 2% formaldehyde in PBS at RT for 25 min. Coverslips were washed again with PBS, permeabilized in cold acetone (−20 °C) for 4 min, washed with PBS, and then placed in 3% BSA/PBS for 30 min. A 3% BSA/PBS solution containing anti-mZP3 (1:10 000 dilution), anti-Flag (M2; 10 μg/mL), or anti-KDEL (StressGen Biotech.; 1:300 dilution) was placed over the coverslips for 1 h. Coverslips were washed with PBS and placed in either FITC-conjugated goat anti-rabbit (1:50 dilution; Molecular Probes) or Texas Red-conjugated goat anti-mouse secondary antibody (1:50 dilution; Molecular Probes) for 30 min. After washing the coverslips in PBS, they were placed for 5 min in a DAPI solution, rinsed in PBS, and then placed in Slowfade Antifade equilibration buffer (Molecular Probes) for 15 min. Coverslips were washed with PBS, and cells were mounted onto glass slides containing a drop of Slowfade Antifade reagent in glycerol (Molecular Probes). Imaging was performed by

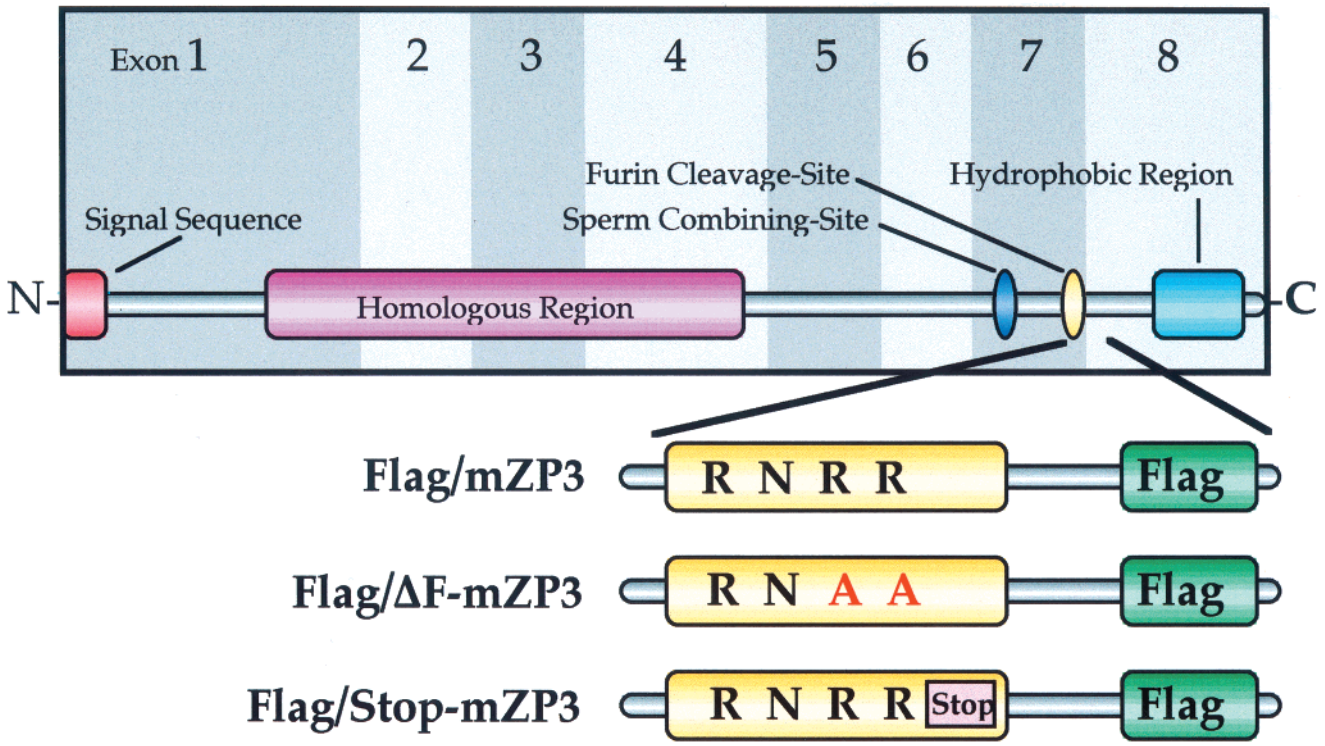

FIGURE 1: Diagrammatic representation of three protein constructs encoded by plasmids introduced into EC cells by electroporation as described in Experimental Procedures. The constructs are designated as *Flag/mZP3*, *Flag/ΔF-mZP3*, and *Flag/Stop-mZP3*. Shown are the positions of certain regions (e.g., homologous region [amino acids 70−240], furin cleavage-site [amino acids 350−353], and C-terminal hydrophobic region [amino acids 388−409]), the position of the Flag epitope between the furin cleavage-site and hydrophobic region, and the sites of amino acid mutagenesis at the furin cleavage-site of mZP3 polypeptide. Also shown are the boundaries of the eight exons encoding mZP3. With the exception of the furin cleavage-site and sperm combining-site, the polypeptide is drawn to scale. R, Arg; N, Asn; A, Ala.

laser scanning confocal microscopy (LSCM) using a Leica TCS-SP (UV) microscope.

Experiments in which cells transfected with *Flag/mZP3* were cultured in the presence of furin inhibitor DPC prior to staining were performed as follows. Cells that were 75% confluent were trypsinized and plated as described above. At 15 h after trypsinization, cells were grown in culture medium in the presence of DPC (52 μg/mL; ~70 μM) for 6 h. After 6 h, DPC was added to cells again (52 μg/mL) and the cells were incubated for an additional 4 h. At the end of this period, cells were stained as described above.

RESULTS

Production of Transfected Cell Lines. A schematic diagram of three protein constructs encoded by plasmids introduced into EC (stably transfected) and 293T (transiently transfected) cells is presented in Figure 1. The location of *Flag* (encoding Asp−Tyr−Lys−[Asp]$_4$−Lys) in *mZP3* exon-8 was selected for two reasons. First, the site was located downstream of the CFCS but upstream of a hydrophobic region predicted to be a transmembrane domain (amino acids 388−409), thereby allowing polypeptide encoded by exons 1−7 and exon-8 to be probed independently. Second, the region between the CFCS and the hydrophobic domain encoded by exon-8 of *mZP3* (amino acids 354−387) exhibits length variation between species. Consequently, it seemed very likely that addition of eight amino acids (i.e., Flag epitope) to this region would not affect mZP3-folding and processing.

Secretion of Flag/mZP3. Western immunoblotting was used to compare culture medium from EC cells transfected

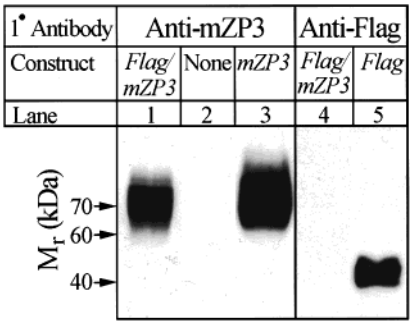

FIGURE 2: Western immunoblots probed with antibodies directed against either mZP3 or Flag. Antibodies were obtained and Western immunoblotting was carried out as described in Experimental Procedures. Shown are immunoblots (anti-mZP3) of culture medium from untransfected EC cells (lane 2) and EC cells stably transfected with either *Flag/mZP3* (lane 1) or *mZP3* (lane 3). Also shown are immunoblots (anti-Flag) of culture medium from EC cells stably transfected with *Flag/mZP3* (lane 4) and of a control protein tagged with Flag (lane 5).

with *Flag/mZP3*, EC cells transfected with *mZP3* alone (*19*), and untransfected EC cells (Figure 2). Probing with a polyclonal antibody directed against mZP3 (anti-mZP3) resulted in a robust signal for culture medium from both *Flag/mZP3* (Figure 2, lane 1) and *mZP3* (Figure 2, lane 3) transfected EC cells but not from untransfected EC cells (Figure 2, lane 2). An anti-Flag monoclonal antibody failed to recognize secreted Flag/mZP3 (Figure 2, lane 4) but did recognize a control protein (Sprouty; ~40 kDa M_r) tagged with Flag (Figure 2, lane 5). This suggests that the Flag epitope was removed from nascent mZP3 prior to secretion.

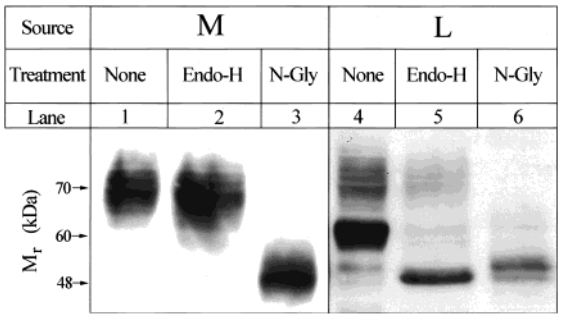

FIGURE 3: Western immunoblots probed with an antibody directed against mZP3. Western immunoblotting and glycosidase digestions of EC cells stably transfected with *Flag/mZP3* were carried out as described in Experimental Procedures. Shown are immunoblots of untreated samples (None) and samples digested with either endoglycosidase-H (Endo-H) or N-glycanase (N-Gly) from culture medium (M) and cell lysates (L). It should be noted that only the band in lane 4 is susceptible to digestion by Endo-H (lane 5).

It should be noted that the secreted forms (~72 kDa apparent M_r) of Flag/mZP3 and mZP3 migrated similarly, suggesting that posttranslational processing of mZP3 was unaffected by addition of the Flag epitope.

Treatment of Flag/mZP3 with Glycosidases. Glycosidases were used to examine whether Flag/mZP3 found in EC cell lysates represented a form of the glycoprotein that had not yet exited the ER. In this context, it was reported previously that nascent mZP3 is glycosylated with high-mannose-type N-linked oligosaccharides in the ER and that these are processed to complex-type oligosaccharides in the Golgi prior to secretion of mature mZP3 (*3*, *23*, *24*). The glycosidases Endo-H and N-glycanase (N-Gly) specifically cleave N-linked oligosaccharides of glycoproteins (*25*). Endo H cleaves only high-mannose-type oligosaccharides, whereas N-Gly cleaves both high-mannose-type and complex-type/hybrid-type oligosaccharides. Therefore, these glycosidases were used to examine the stage of intracellular processing of nascent glycoproteins.

As seen in Figure 3 (lane 1), Flag/mZP3 in culture medium from EC cells migrated as a relatively broad band with an apparent M_r of ~72 kDa. Whereas Endo-H treatment failed to affect the apparent M_r of secreted Flag/mZP3 (Figure 3, lane 2), N-Gly caused a dramatic reduction to ~47 kDa M_r (Figure 3, lane 3). Flag-mZP3 from EC cell lysates migrated as a relatively narrow band with an apparent M_r of ~60 kDa (Figure 3, lane 4). Treatment of this form of the glycoprotein with either Endo-H (Figure 3, lane 5) or N-Gly (Figure 3, lane 6) resulted in a significant decrease in apparent M_r (Endo-H, ~48 kDa; N-Gly, ~50 kDa). These results suggest that the intracellular form of Flag/mZP3 possesses high-mannose-type N-linked oligosaccharides, while the secreted form possesses complex-type N-linked oligosaccharides.

Microscopy of Cells Transfected with Flag/mZP3. Results of the glycosidase experiments described above suggest that the intracellular form of Flag/mZP3 may be associated with ER. Accordingly, laser scanning confocal microscopy (LSCM) was carried out with stably transfected EC cells, as described in Experimental Procedures, to examine the intracellular location of Flag/mZP3. Antibodies directed against the Flag epitope (anti-Flag; monoclonal) and against mZP3 (anti-mZP3; polyclonal) were used. As seen in Figure 4, panels A and D, fluorescence from both antibodies colocalized to a fine layer surrounding the nucleus and appeared in a loose reticular pattern extending from the nucleus. Untransfected EC cells exhibited only background levels of fluorescence (Figure 4, panels C and F). These results are consistent with localization of nascent Flag/mZP3 to the ER. To determine whether this was the case, untransfected and transfected EC cells were probed with an antibody (anti-KDEL; monoclonal) directed against ER and with anti-mZP3. As seen in Figure 4, panels B and E, fluorescence from both antibodies colocalized in a manner very reminiscent of the pattern seen with anti-Flag and anti-mZP3 (Figure 4, panels A and D).

Treatment of Transfected Cells with DPC. DPC is a modified peptide that binds covalently to the substrate binding site of PC enzymes and inhibits their activity (*26*). To determine whether cleavage of mZP3 polypeptide at its CFCS is essential for secretion, EC cells transfected with *Flag/mZP3* were treated with DPC (Arg−Val−Lys−Arg), as described in Experimental Procedures. Cell lysates and culture medium were collected and analyzed by Western immunoblotting as before. When probed with anti-Flag, a narrow band was seen in cell lysates (apparent M_r ~60 kDa) but not in culture medium from cells incubated with and without DPC (data not shown). It was noted that a slightly stronger signal was seen in lysates of cells incubated with the inhibitor. The immunoblot was then stripped and reprobed with anti-mZP3. As seen in Figure 5, the presence of DPC caused a reduction in secretion of mZP3 into culture medium, whereas there was a detectable increase in levels of mZP3 in cells; the latter was confirmed by LSCM (Figure 6). Therefore, secretion of mZP3 from transfected EC cells was significantly reduced (>20-fold; Figure 5) by incubating transfected cells in the presence of DPC.

Synthesis and Secretion of Flag/ΔF-mZP3 and Flag/Stop-mZP3. Two mutated forms of *Flag/mZP3*, designated *Flag/ΔF-mZP3* and *Flag/Stop-mZP3*, were used to assess the potential role of mZP3 C-terminal peptide in secretion of mZP3. Stably transfected EC cell lines were produced using the constructs described in Figure 1 and cell lysates and culture medium were subjected to Western immunoblotting as before.

When probed with anti-Flag, signal was detected only in lysates from cells transfected with *Flag/mZP3* and *Flag/ΔF-mZP3* (Figure 7, panel A). These results suggest that the Flag epitope was removed from Flag/mZP3 prior to its secretion and are consistent with previous findings on mZP3 processing in mouse eggs (*6*). mZP3 in both lysates migrated as a narrow band with an apparent M_r of ~60 kDa, consistent with results in Figure 3. No signal was detected when lysates from cells transfected with *Flag/Stop-mZP3* were probed with anti-Flag. Similarly, no signal was detected in culture medium from cell lines carrying *Flag/mZP3*, *Flag/ΔF-mZP3* or *Flag/Stop-mZP3* when probed with anti-Flag (Figure 7, panel A).

The immunoblots were stripped and then reprobed with anti-mZP3 (Figure 7, panel B). As before, mZP3 that migrated with an apparent M_r of ~72 kDa was detected in culture medium from *Flag/mZP3* transfected cells. However, no mZP3 was detected in culture medium from cells transfected with *Flag/Stop-mZP3* and only trace amounts were present in culture medium from cells transfected with *Flag/ΔF-mZP3*. These results suggest that either the absence of a C-terminal peptide (i.e., *Flag/Stop-mZP3)* or failure to

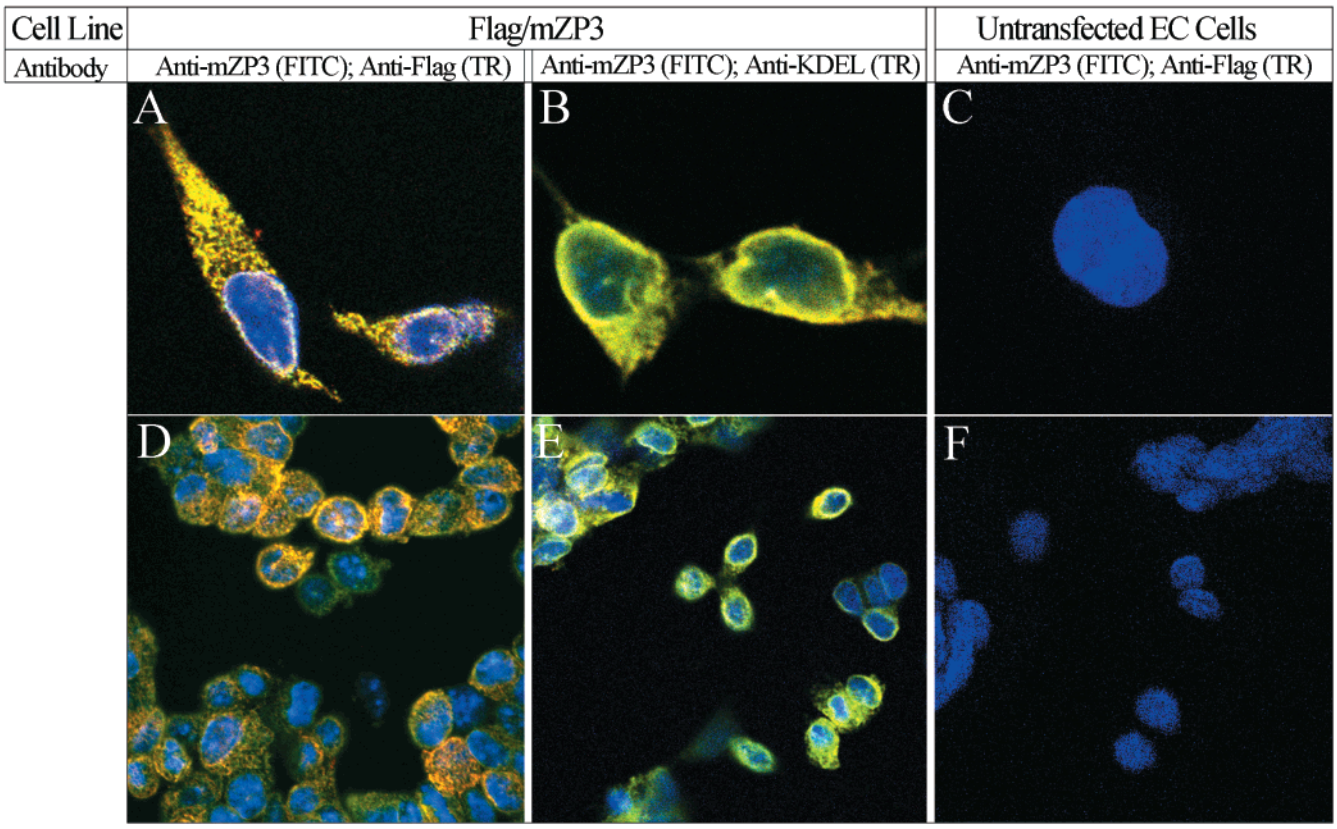

FIGURE 4: Laser scanning confocal microscopy of EC cells probed with antibodies directed against Flag and mZP3, or ER and mZP3. Cells stably transfected with *Flag/mZP3* (panels A and D, and B and E) and untransfected cells (panels C and F) were permeabilized and probed with both rabbit anti-mZP3 (FITC goat anti-rabbit) and mouse anti-Flag (TR anti-mouse) (panels A and D, and C and F) or with anti-KDEL (TR anti-mouse) (panels B and E) antibodies, as described in Experimental Procedures. In all cases, nuclei were stained with DAPI (blue), as described in Experimental Procedures. Panels A and D: Note the colocalization (yellow) of FITC (green) and TR (red). Panels C and F: Note the lack of staining of untransfected cells. Panels B and E: Note the loose reticular pattern of staining similar to that seen in panels A and D.

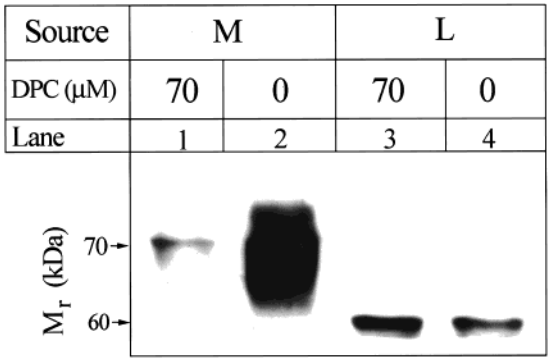

FIGURE 5: Western immunoblots probed with an antibody directed against mZP3. Western immunoblotting and use of the PC enzyme inhibitor DPC with EC cells stably transfected with *Flag/mZP3* are described in Experimental Procedures. Shown are immunoblots of culture medium (M) and cell lysates (L) from cells cultured in the absence or presence (~70 μM) of DPC. It should be noted that secretion of large amounts of mZP3 into the culture medium (lane 2) was inhibited by DPC (lane 1).

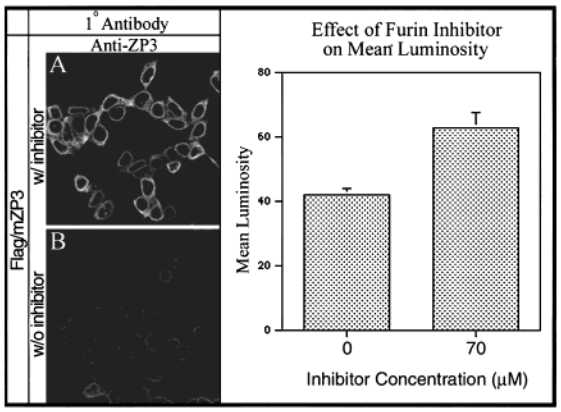

FIGURE 6: Laser scanning confocal microscopy of EC cells probed with an antibody directed against mZP3. Cells stably transfected with *Flag/mZP3* were incubated in the presence (panel A) or absence (panel B) of the furin inhibitor DPC and were then permeabilized and probed with a rabbit anti-mZP3 (FITC goat anti-rabbit) antibody, as described in Experimental Procedures. Also shown is a histogram depicting the mean fluorescence intensity (± SEM) for 12 fields of cells grown in the presence (~70 μM) and absence of DPC. The significance of the difference was confirmed by the Wilcoxin−Mann−Whitney Exact test ($p \leq 0.0008$).

cleave the C-terminal peptide (i.e., *Flag/ΔF-mZP3*) prevents secretion of nascent mZP3.

Although the signal either was not detected or was very weak in culture medium from EC cells transfected with mutated forms of *Flag/mZP3*, cell lysates presented a robust signal. When lysates of cells transfected with *Flag/ΔF-mZP3* or *Flag/Stop-mZP3* were probed on immunoblots with anti-mZP3, bands at ~60 and ~38 kDa M_r, respectively, were detected (Figure 7, panel B). Occasionally, a very faint signal at ~72 kDa M_r was detected in lysates from cells stably transfected with *Flag/ΔF-mZP3*. Similar results were obtained using transiently transfected human kidney (293T) cells (CMV-promoter; see Experimental Procedures), as well

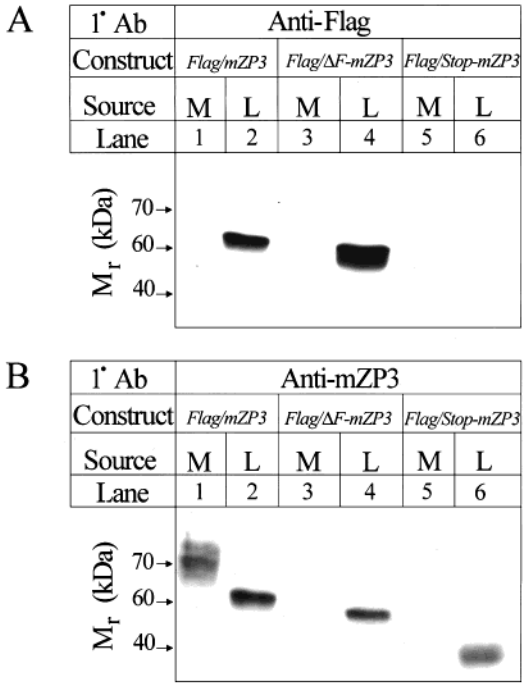

FIGURE 7: Western immunoblots probed with antibodies directed against either Flag (panel A) or mZP3 (panel B). Western immunoblotting was carried out as described in Experimental Procedures. Panel A: Shown are immunoblots of culture medium (M) and cell lysates (L) from EC cells stably transfected with *Flag/mZP3* (lanes 1 and 2), *Flag/*Δ*F-mZP3* (lanes 3 and 4), or *Flag/Stop-mZP3* (lanes 5 and 6). Bands were detected only with cell lysates from cells transfected with *Flag/mZP3* (lane 2) and *Flag/*Δ*F-mZP3* (lane 4). No bands were detected in either culture medium or cell lysates from cells transfected with *Flag/Stop-mZP3* (lanes 5 and 6). Panel B: Shown is the same gel as in panel A, stripped and probed with anti-mZP3. Bands were detected with both culture medium and cell lysates from cells transfected with *Flag/mZP3* (lanes 1 and 2) and with cell lysates only from cells transfected with either *Flag/*Δ*F-mZP3* (lane 4) or *Flag/Stop-mZP3* (lane 6). No bands were detected in culture medium from cells transfected with *Flag/*Δ*F-mZP3* (lane 3) or *Flag/Stop-mZP3* (lane 5).

as with a mixed population of stably transfected EC cells (data not shown). These results suggest that, in cells expressing mutated forms of *Flag/mZP3*, nascent mZP3 failed to exit the ER.

*Microscopy of Cells Transfected with Flag/*Δ*F-mZP3 and Flag/Stop-mZP3.* Results of LSCM of EC cells stably transfected with either *Flag/*Δ*F-mZP3* or *Flag/Stop-mZP3* are shown in Figure 8. Cells were incubated with anti-Flag and anti-mZP3, as described in Experimental Procedures. Fluorescent labeling of EC cells transfected with *Flag/*Δ*F-mZP3* appeared indistinguishable from labeling seen with cells transfected with *Flag/mZP3* (Figure 4). Both antibodies colocalized to a reticular region surrounding the nucleus and the pattern of fluorescence resembled that seen with anti-KDEL directed against ER. A similar pattern of fluorescence was also seen when anti-mZP3 was used with cells transfected with *Flag/Stop-mZP3.* As expected, anti-Flag failed to label these cells.

DISCUSSION

Production of mature ZP glycoproteins by growing oocytes involves both co-translational and posttranslational processing events. For example, the polypeptides possess an N-terminal signal sequence for initial transfer of nascent

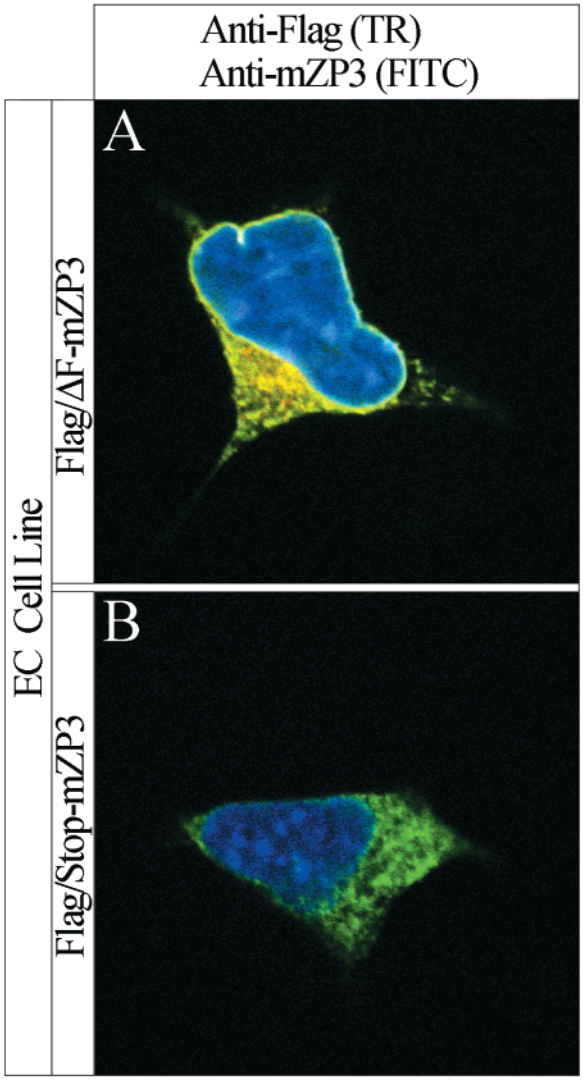

FIGURE 8: Laser scanning confocal microscopy of EC cells probed with antibodies directed against Flag and mZP3. Cells stably transfected with either *Flag/*Δ*F-mZP3* (panel A) or *Flag/Stop-mZP3* (panel B) were permeabilized and probed with both rabbit anti-mZP3 (FITC goat anti-rabbit) and mouse anti-Flag (TR anti-mouse) antibodies, as described in Experimental Procedures. In both cases, nuclei were stained with DAPI (blue), as described in Experimental Procedures. Panel A: Note the colocalization (yellow) of FITC (green) and TR (red). Panel B: Note the absence of TR (red) staining.

polypeptides to the ER. There, co-translational addition of high-mannose-type, N-linked oligosaccharides occurs at Asn−X−Ser/Thr consensus sequences (*27*−*32*). O-Linked oligosaccharides are added to Ser/Thr residues of ZP glycoproteins in the Golgi and the glycoproteins are secreted by growing oocytes. Consequently, mouse ZP glycoproteins mZP1, mZP2, and mZP3 have apparent M_r values of ∼200 (dimer), ∼120, and ∼83 kDa, respectively. On the basis of exon sequences, the calculated M_r values of the polypeptides of mZP1, mZP2, and mZP3 are ∼70 (monomer), ∼76, and ∼44 kDa, respectively. These M_r values, together with other biochemical information, indicate that the mature, secreted forms of ZP glycoproteins are extensively glycosylated (*23*, *24*, *32*−*34*). It should be noted that female mice that are homozygous null mutants for *mZP3* (i.e., *mZP3*$^{-/-}$) fail to deposit a ZP around growing oocytes and are infertile (*35*−*37*).

Previously, it was determined that, in addition to removal of an N-terminal signal sequence and N- and O-linked glycosylation of polypeptide, mZP2 and mZP3 undergo C-terminal proteolytic processing at their CFCS in growing oocytes (*6*). In the case of mZP3, this results in removal of the C-terminal 71 amino acids encoded by *mZP3* exon-8 prior to incorporation of the glycoprotein into the ZP. Some evidence suggested that proteolytic processing of ZP glycoproteins took place at the oocyte plasma membrane (*6*), a site proposed as a furin processing compartment (*11*, *12*, *38*). These observations raised the possibility that such proteolytic processing may be necessary for secretion of nascent mZP3 from growing oocytes, as well as from cells transfected with *mZP3* (*19*, *39*−*41*).

Results reported here strongly suggest that, indeed, proteolytic cleavage of the C-terminal peptide of mZP3 at the CFCS by a member of the PC family of enzymes is required for secretion to occur. The localization and processing of the PC family of enzymes, especially furin, have been studied extensively (*7*−*9*, *42*). Furin is synthesized as a pro-protein and undergoes intramolecular cleavage in the ER with release of a small peptide (*7*). The latter remains noncovalently associated with furin and inhibits its catalytic activity until the enzyme reaches the *trans*-Golgi. Autolytic cleavage of furin is a prerequisite for its exit from the ER (*43*). In the decreased pH environment of the *trans*-Golgi, the peptide is released from furin and a second cleavage event occurs, thereby maintaining furin in an active state (*44*). The activation and localization of furin applies to other members of the PC family, with the exception of PC2 that requires the activity of a second protein (7B2) and is activated in a late post-Golgi compartment (*45*).

EC cells stably transfected with wild-type *mZP3* secrete relatively large amounts of functional mZP3 into the culture medium (*19*, *39*, *41*). Similar results were obtained here with EC cells transfected with *Flag/mZP3* (Figure 2). Results of glycosidase treatments, LSCM, and Western blot analyses (Figures 3 and 4) indicate that the predominant intracellular form of Flag/mZP3 localizes to the ER as an ∼60 kDa M_r species possessing high-mannose-type oligosaccharides. On the other hand, Flag/mZP3 that has transited through the Golgi is ∼72 kDa M_r and possesses complex-type N-linked oligosaccharides. These results suggest that exit from the ER may be the rate-limiting step in secretion of Flag/mZP3 by EC cells. EC cells stably transfected with *mZP3* mutated at its CFCS synthesize but fail to secrete mZP3. Such is the case for mutants containing either a stop-codon (*Flag/Stop-mZP3*; Figure 7, panel B, lanes 5 and 6) or altered amino acid-codons (*Flag/*Δ*F-mZP3*; Figure 7, panel B, lanes 3 and 4) at the CFCS. Furthermore, EC cells stably transfected with wild-type *mZP3 (Flag/mZP3)* and incubated in the presence of DPC, a peptide inhibitor of PC enzymes, also fail to secrete mZP3 (Figure 5). These results suggest that the peptide encoded by *mZP3* exon-8 must be present (i.e., *Flag/Stop-mZP3)*, but eventually removed (i.e., *Flag/*Δ*F-mZP3* and DPC inhibition) for secretion of nascent mZP3 to take place.

As discussed above, secretion of mZP3 from EC cells into culture medium was severely affected by mutations in the CFCS region. On the other hand, the levels of mutant mZP3 in EC cells, assessed with an anti-mZP3 probe, differed dramatically for *Flag/Stop-mZP3* and *Flag/*Δ*F-mZP3* transfected cells. Intracellular levels of mZP3 were approximately equivalent for cells expressing *Flag/mZP3* or *Flag/*Δ*F-mZP3*. Levels of mZP3 were substantially lower in cells expressing *Flag/Stop-mZP3*. This was also the case for 293T cells transiently transfected with *mZP3* cDNAs containing Flag and the same mutations at the CFCS, as well as for mixed populations of as many as 100 colonies of stably transfected EC cells (see Experimental Procedures; data not shown). These findings suggest that polypeptide encoded by *mZP3* exon-8 may aid in the folding of nascent mZP3; in its absence, nascent mZP3 is rapidly degraded intracellularly. This may be attributable to a chaperone-like function of the predicted transmembrane domain (amino acids 388−409) in this region of mZP3 polypeptide, as recently reported for Kex2p (*46*).

Furin and other members of the PC family are found in the *trans*-Golgi and distal regions of the secretory pathway, including the plasma membrane (*8*, *38*, *47*). Therefore, it might be expected that any intracellular accumulation of either mutant mZP3 encoded by *Flag/*Δ*F-mZP3*, or *Flag/mZP3* synthesized in the presence of DPC, would be in the *trans*-Golgi. Unexpectedly, the uncleaved species of nascent mZP3 (i.e., containing Flag and high-mannose-type, N-linked oligosaccharides) were found associated with the ER not Golgi (Figures 3 and 4). However, this finding is consistent with several recent reports, including one on chicken low-density lipoprotein receptor-related protein (LRP) that is a substrate for furin (*48*). Mutation of the CFCS of LRP also delayed its exit from the ER, suggesting to the investigators that the site may be necessary for binding of a chaperone that assists in folding of nascent LRP. Apparently, failure to bind a chaperone cannot account for retention of mutant mZP3 in the ER since it was also retained there when Flag/mZP3, containing a wild-type CFCS, was synthesized in the presence of DPC (Figure 5). It has been reported previously that N-linked oligosaccharides are not essential for secretion of nascent mZP3 by growing oocytes (*24*). Therefore, the inability to process high-mannose-type oligosaccharides of mZP3 in the presence of DPC is unlikely to contribute to the intracellular retention of Flag/ΔF-mZP3, Flag/Stop-mZP3, and Flag/mZP3.

A potential mechanism for accumulation of mutant mZP3 in the ER comes from studies of MHC class II synthesis. Nascent MHC-II is found in the ER as an $\alpha\beta$ heterodimer complexed with invariant chain (Ii) that functions to shuttle MHC-II from the ER to the Golgi (*49*, *50*). In the absence of Ii, exit of MHC-II from the ER is severely retarded, such that very little MHC-II is found on the plasma membrane (*51*). If there is a comparable shuttle protein for nascent mZP3 that binds to uncleaved polypeptide (i.e., similar to Ii) and is present in limiting amounts, it could account for accumulation of mutant mZP3 in the ER. Since the mutated CFCS cannot be cleaved, the shuttle protein would not be released from mZP3 in the *trans*-Golgi, or at subsequent sites, and nascent mZP3 would accumulate in the ER in its absence. This mechanism would apply as well to mZP3 synthesized in the presence of DPC. Whether a shuttle protein binds to mZP3 remains to be determined.

In conclusion, evidence presented here strongly suggests that secretion of nascent mZP3 from transfected cells is dependent on cleavage of its polypeptide at the CFCS. Failure to cleave nascent mZP3, due either to mutation of critical

residues at or after the CFCS, or to the presence of a furin-like enzyme inhibitor, results in its accumulation in ER. It has been well documented that proteolytic cleavage of secreted glycoproteins may be essential for biological activity. It seems likely that the intimate relationship between proteolysis by PCs and secretion demonstrated here for mZP3 will apply as well to other glycoproteins secreted by cells.

ACKNOWLEDGMENT

We are very grateful to Eveline Litscher, Luca Jovine, and other members of our laboratory for advice and constructive criticism. We also thank Scott Henderson, Gillian Small, and Carol Bodian for instruction, advice, and discussion, and Jonathan Licht for a gift of Flag-tagged Sprouty.

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BI002275X