MOLECULAR AND CELLULAR BIOLOGY, May 2002, p. 3111–3120 Vol. 22, No. 9
0270-7306/02/$04.00+0 DOI: 10.1128/MCB.22.9.3111–3120.2002

Conserved Furin Cleavage Site Not Essential for Secretion and Integration of ZP3 into the Extracellular Egg Coat of Transgenic Mice

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Received 27 September 2001/Returned for modification 13 December 2001/Accepted 11 January 2002

The extracellular zona pellucida surrounding mammalian eggs is formed by interactions of the ZP1, ZP2, and ZP3 glycoproteins. Female mice lacking ZP2 or ZP3 do not form a stable zona matrix and are sterile. The three zona proteins are synthesized in growing oocytes and secreted prior to incorporation into the zona pellucida. A well-conserved furin site upstream of a transmembrane domain near the carboxyl terminus of each has been implicated in the release of the zona ectodomains from oocytes. However, mutation of the furin site (RNRR → ANAA) does not affect the intracellular trafficking or secretion of an enhanced green fluorescent protein (EGFP)-ZP3 fusion protein in heterologous somatic cells. After transient expression in growing oocytes, normal EGFP-ZP3 and mutant EGFP-ZP3 associate with the inner aspect of the zona pellucida, which is distinct from the plasma membrane. These in vitro results are confirmed in transgenic mice expressing EGFP-ZP3 with or without the mutant furin site. In each case, EGFP-ZP3 is incorporated throughout the width of the zona pellucida and the transgenic mice are fertile. These results indicate that the zona matrix accrues from the inside out and, unexpectedly, suggest that cleavage at the furin site is not required for formation of the extracellular zona pellucida surrounding mouse eggs.

Extracellular matrices provide assorted roles in biology, and the mechanisms of their formation appear quite disparate. The matrix that surrounds vertebrate eggs and early embryos is variously known as the vitelline envelope, the perivitelline membrane, or the zona pellucida. The component parts are most commonly synthesized and secreted from female germ cells, but in some vertebrates they are produced in the somatic compartment of the ovary or in the liver, which requires both transport to the egg and assembly on its surface. Although the molecular biology of the mouse egg coat has been well studied, little is known about the intracellular protein trafficking of individual components or the mechanisms by which they are secreted to form the insoluble extracellular matrix required for fertilization and embryonic development.

The mouse zona pellucida consists of three major sulfated glycoproteins (ZP1, ZP2, and ZP3), each of which is encoded by a single-copy gene in the mouse genome. Although the primary structures of ZP1 (623 amino acids, 68 kDa), ZP2 (713 amino acids, 80 kDa), and ZP3 (424 amino acids, 46 kDa) are distinct, they share certain motifs among themselves, as well as with homologues in other mammals (29). Each zona protein has an N-terminal signal peptide, a signature zona box (260 amino acids with eight conserved cysteine residues, (3), and a transmembrane domain near its carboxyl terminus. The conservation of zona proteins among mammals suggests that the three-dimensional structures of the proteins in each class of zona proteins are similar and that the interactions among classes that effect the supramolecular structure of the zona pellucida may be preserved as well. This hypothesis has been substantiated by the ability of transgenic mice expressing human ZP3 to restore the zona matrix and fertility in *Zp3*-null mice that are otherwise unable to form a zona pellucida (33). Additionally, although *Xenopus laevis* and mammals evolutionarily diverged 350 to 370 million years ago (16), microinjected mRNAs encoding mouse ZP1, ZP2, and ZP3 are translated and incorporated into the extracellular vitelline envelop surrounding *Xenopus* eggs (7).

The zona pellucida matrix is assembled during oogenesis as the oocyte grows from ~15 to 80 μm prior to ovulation into the oviduct. Targeted mutagenesis of each mouse zona gene indicates that either ZP1 and ZP3 or ZP2 and ZP3 are sufficient for zona matrix formation, although the ZP1-and-ZP3 matrix is not sustained through the latter stages of oogenesis (18, 30–32). The detection of zona proteins (ZP1and ZP2) at the zona-free oocyte surface from *Zp3*-null mice (30) is consistent with tethering of the zona proteins to a plasma membrane. However, the presence of the perivitelline space between the plasma membrane of the mature eggs and the extracellular zona matrix indicates that the zona proteins are subsequently released from the membrane for incorporation into the zona pellucida. A conserved furin cleavage site (RXK/RR) upstream of the carboxyl-terminal transmembrane domain in most, but not all, mammalian zona proteins has been suggested as the processing site for cleavage and release of zona proteins (17, 42, 44). In this study, by using site-specific mutagenesis, we examined the involvement of the furin recognition site in the secretion of mouse ZP3 in mouse embryonic fibroblasts and its incorporation into the zona pellucida surrounding mouse oocytes. Unexpectedly, our data indicate that cleavage at the conserved furin site is not required for ZP3 secretion or its participation in the extracellular egg coat.

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MATERIALS AND METHODS

Immunocytochemical analysis of zona-free oocytes and eggs. Growing oocytes and eggs were isolated from NIH Swiss mice, and after their zonae were removed by exposure to acidified Tyrode's medium (12), they were processed for imaging by Nomarski optics and confocal microscopy (30) with monoclonal antibodies specific to mouse ZP1 (33), ZP2 (8), and ZP3 (9) as primary antibodies and rhodamine-lissamine-conjugated goat anti-rat immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) as a secondary antibody.

EGFP-mouse ZP3 (MoZP3) expression vectors. The 5′ region (bp 1 to 149) of pZP3.5 (34), including the N-terminal signal peptide sequence (amino acids 1 to 40), was amplified by PCR with two primers, each containing either an *Nhe*I or an *Age*I recognition site, respectively, i.e., 5′-GGCTAGCTGAGCCCAGCTGT ACTCCA-3′ and 5′-AACCGGTGATGAGGACCCCACTGGGG-3′. The resultant PCR product was cloned into the *Nhe*I-*Age*I site of pEGFP-C2 (Clontech Laboratories, Palo Alto, Calif.) upstream and in frame with enhanced green fluorescent protein (EGFP). The remainder of ZP3 was assembled from a second PCR product (bp 93 to 435) by using primers 5′-TCAGATCTCCCAGAC TCTGTGGCTT-3′ and 5′-CAATGGGTACCTCCACAC-3′, each with a *Bgl*II or a *Kpn*I recognition site, respectively. This PCR fragment was cloned into the *Bgl*II and *Kpn*I sites downstream of and in frame with EGFP. The rest of ZP3 (bp 179 to 1189) was isolated from pZP3.5 after digestion with *Spe*I and *Sac*II and cloned into the corresponding sites in ZP3 and the multicloning site of pEGFP-C2. The resultant plasmid was designated pSEGFP-MoZP3.

PCR mutagenesis was used to change the furin recognition site (amino acids 350 to 353) in mouse pSEGFP-MoZP3 from Arg-Asn-Arg-Arg-COOH to Ala-Asn-Ala-Ala-COOH. With pZP3.5 as a template, fragments from PCR amplifications with either primer set 5′-CCAAGCTAGTTTCTGCCAACGCAGCTC ACGTGACCG-3′ and 5′-TAATACGACTCACTATAGGG-3′ or primer set 5′-TACATCACCTGCCATCTCAA-3′ and 5′-CGGTCACGTGAGCTGCGTTG GCAGAAACTAGCTTGG-3′ were isolated by agarose gel electrophoresis and reamplified without additional primers or templates. The *Sap*I-*Apa*I fragment of the resultant PCR product (bp 1031 to 1123) containing the mutated ZP3 furin site was substituted for the corresponding region of pSEGFP-MoZP3, and the result was designated pSEGFP-FurX. *Taq* DNA polymerase was used for amplification, and all PCRs were performed for 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s with a Perkin-Elmer GeneAmp PCR System (Perkin-Elmer, Norwalk, Conn.). The sequences of PCR fragments were confirmed by dideoxy sequencing (36).

Transient expression of pSEGFP-MoZP3 and pSEGFP-FurX. Transient-transfection assays were performed with Lipofectamine (Life Technologies, Rockville, Md.) in accordance with the manufacturer's protocol. On the day before transfection, 1.5×10^5 mouse 10T[1/2] embryonic fibroblast cells in 2 ml of Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) were seeded in each well of six-well (35-mm-diameter) tissue culture plates. The cells were incubated overnight until they were 50 to 80% confluent. For each transfection, 6 μl of PLUS reagents was added to 100 μl of DMEM (without FBS) predissolved with 1 μg of either pSEGFP-MoZP3 or pSEGFP-FurX and incubated at room temperature (RT) for 15 min. Lipofectamine (4 μl in 100 μl of DMEM) was then added and mixed. After 15 min of incubation at RT, the mixture was diluted with 0.8 ml of DMEM and overlaid on the growing cells. After 3 h at 37°C, the medium was replaced and incubated for an additional 48 h with one refeeding prior to harvest. Alternatively, 1 day after transfection, the cells were changed to the same medium containing 0.2 μg of brefeldin A (BFA) per ml and cultured for an additional 12 h. The cells were then washed with phosphate-buffered saline (PBS) and changed to DMEM with 10% FBS (lacking BFA). After 30 min in culture, the cells were fixed with 2% paraformaldehyde for 40 min at RT.

The fixed 10T[1/2] cells were blocked and permeabilized with blocking solution (PBS supplemented with 1.5 mg of glycine per ml, 1 mg of saponin [S-4521; Sigma, St. Louis, Mo.] per ml, 10% donkey serum [Jackson ImmunoResearch Laboratories]) at RT for 1 h. Rabbit anti-bovine protein disulfide isomerase (PDI; 1:200; StressGen Biotechnologies, Victoria, British Columbia, Canada) or rabbit anti-rat α-mannosidase II (1:100; gift of Kelley Moremen [University of Geogia] and Marilyn G. Farquhar [University of California, San Diego]) diluted with blocking solution was added, and the mixture was incubated at RT for 1 h. After being washed with the blocking solution, the cells were further incubated with Cy5-labeled donkey anti-rabbit antibodies (1:500; Jackson ImmunoResearch Laboratories) at RT for 1 h. Following additional washes with the blocking solution and then PBS, the slides were plated with mounting medium (ProLong Antifade Kit; Molecular Probes, Eugene, Oreg.) and examined on a 510 LSM confocal microscope (Carl Zeiss, Thornwood, N.Y.). EGFP was excited at 488 nm with an argon laser light, and fluorescence images were collected at 515 to 530 nm. Cy5 was excited with a HeNe laser light at 633 nm, and fluorescence images were collected at ≥670 nm.

Western blot analyses. Cell supernatants from transient transfections were concentrated by Microcon 10 (Millipore, Bedford, Mass.), and cell pellets were washed twice with PBS and lysed with 50 mM Tris-HCl (pH 7.5)–1% NP-40–10 mM EDTA containing 4-(2-aminoethyl) benzene sulfonyl fluoride hydrochloride (1 mM), aprotinin (5 μg/ml), leupeptin (1 μg/ml), benzamimidine hydrochloride (0.1 mM), and pepstatin (1 μg/ml). Protein samples (pellets or supernatants from 10^4 cells) were treated with or without endoglycosidase H (endo-H) or peptide-*N*-glycosidase F (PNGase F), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and assayed by Western blotting (4). For endo-H or PNGase F treatment, samples were denatured at 100°C for 10 min and then incubated with 100 U of enzyme per sample at 37°C for 1 h in accordance with the manufacturer's (New England Biolabs, Beverly, Mass.) instructions.

Expression of EGFP-MoZP3 and EGFP-FurX in microinjected oocytes. Oocytes isolated from 11- to 13-day-old mouse ovaries (23) were incubated in M199 medium supplemented with 0.28 mM sodium pyruvate, 25 mM HEPES (pH 7.4), and 2 mg of BSA per ml at 37°C prior to microinjection. About 10 pl of solution, containing 50 ng of plasmid DNA per ml, was injected into the nucleus of each oocyte. The injected oocytes were cultured (37°C, 5% CO_2) for 24 h in the same supplemented M199 medium. The oocytes were then stained with 1 μg of the lipophilic dye PM-R18 (octadecyl rhodamine B Cl^-; Molecular Probes) per ml for 20 min and separated into two groups. One group was fixed with 2% paraformaldehyde for 1 h at RT. The other group was transferred into 20 mM Tris-HCl, pH 7.4, containing 1% NP-40 and 0.5 M NaCl and freeze-thawed 10 times on ethanol-dry ice to isolate zona ghosts (37). The fixed oocytes and treated zona ghosts were washed three times with PBS and put on a slide chambered with Gene-Frame (20-μl cavity volume; Advanced Biotechnologies, Leatherhead, United Kingdom). Images were obtained by confocal microscopy with an argon laser light (488 nm) to visualize EGFP at 515 to 530 nm and a HeNe 543 laser light to detect PM-R18 at 570 nm.

EGFP-MoZP3 and EGFP-FurX transgenic mice. *Nhe*I-*Eco*RV fragments isolated from pSEGFP-MoZP3 and pSEGFP-FurX were inserted into the *Spe*I-*Eco*RV sites of a plasmid containing 6 kbp of the mouse *Zp3* promoter, which was previously shown to direct oocyte-specific expression in transgenic mice (33). A bovine growth hormone polyadenylation signal was cloned into *Eco*RV-*Not*I sites downstream of the ZP3-EGFP sequences, and the 8.1-kbp *Zp3*-MoZP3-EGFP fragment was purified by agarose electrophoresis after digestion with I-*Sce*I meganuclease and *Not*I. Following pronuclear injection, founders were identified and transmission of the transgene in their progeny was followed by PCR and Southern analyses (33). The primers used for the PCR were specific to EGFP (5′-GTCGCCACCATGGTGAGCAA-3′ and 5′-ACTTGTACAGCTCG TCCATG-3′), and the conditions are described above. The presence of the normal and mutant furin sites was confirmed by sequencing of PCR products of the transgenes with genomic tail DNA as a template, flanking primers (5′-GTC GCCACCATGGTGAGCAA-3′ at the 5′ end of EGFP and 5′-CCTCGAGTT TCTTCTTTTATTGCGG-3′ at the 3′ end of ZP3), and the above-described PCR conditions. The nucleic acid sequence was obtained with ABI PRISM Fluorescent DNA Analysis Kits and an ABI Prism 310 Genetic Analyzer in accordance with the manufacturer's instructions. All experiments with mice were conducted under protocols approved by the National Institute of Arthritis and Musculoskeletal and Skin Diseases-National Institute of Diabetes and Digestive and Kidney Diseases Animal Care and Use Committee.

RESULTS

Detection of zona proteins at the plasma membrane. To confirm biochemically the presence of the zona proteins at the growing-oocyte surface, zonae pellucidae were removed from growing oocytes or eggs by brief exposure to acidified Tyrode's medium (Fig. 1A and B). After being washed, the germ cells were incubated with monoclonal antibodies to either ZP1, ZP2, or ZP3. With a rhodamine secondary antibody and confocal microscopy, each zona protein was detected on the growing-oocyte surface (Fig. 1C, E, and G). All of the zona proteins were also detected within growing oocytes, where they are actively synthesized, but were not present (or were present in very diminished amounts) in ovulated eggs, in which zona protein synthesis has stopped (Fig. 1D, F, and H).

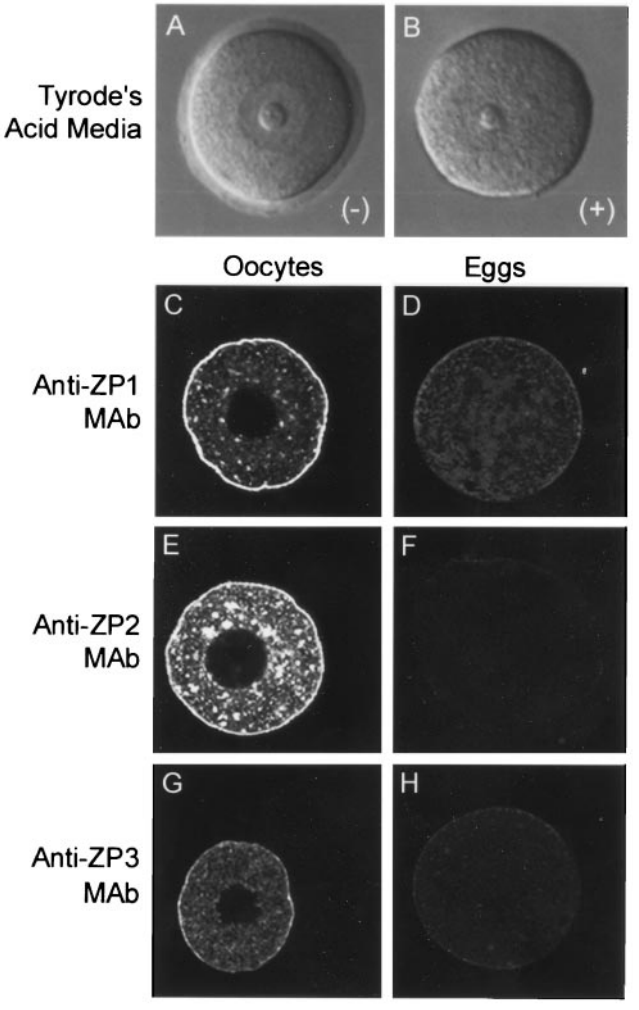

FIG. 1. Immunocytochemical analysis of oocytes and eggs. Nomarski images of a growing oocyte from a 3-week-old NIH Swiss mouse before (A) and after (B) treatment with acidified Tyrode's medium to remove the zona pellucida. Confocal microscopy of zona-free oocytes (C, E, and G) and eggs (D, F, and H) stained with antibodies specific to ZP1 (C and D), ZP2 (E and F), and ZP3 (G and H) after permeabilization with saponin. Each zona protein was present within and at the cell surface of growing oocytes (C, E, and G) but not of ovulated eggs (D, F, and H). Bar, 25 μm. MAb, monoclonal antibody.

ZP1, ZP2, and ZP3 each have a signal peptide that directs them into a secretory pathway, a 260-amino-acid zona box of unknown function, and a transmembrane domain near the carboxyl terminus. At 31 to 47 amino acids upstream of the transmembrane domain are short regions with basic residues implicated as the furin cleavage site involved in zona protein secretion (Fig. 2A). The 424-amino-acid ZP3 protein was selected as a model with which to determine if the furin recognition site is required for zona protein incorporation into the egg coat. The identification of a monoclonal antibody binding site (22) and its ability to bind to the extracellular zona pellucida (9) suggested that cleavage occurs downstream of the antibody binding site (amino acids 336 to 342) and presumably upstream of the transmembrane domain (amino acids 385 to 410), in a region that includes the potential furin cleavage site (amino acids 350 to 353).

Expression of EGFP-ZP3 fusion proteins in 10T[1/2] cells. ZP3 cDNA was cloned into the expression vector pEGFP-C2, which encodes EGFP and is well suited for analysis of intracellular trafficking by confocal microscopy. The 5′ region of the ZP3 cDNA that included the N-terminal signal sequence (amino acids 1 to 22) was cloned upstream, and the reminder of the ZP3 cDNA was cloned downstream of EGFP to establish an EGFP-ZP3 fusion protein driven by the cytomegalovirus promoter (Fig. 2B). PCR mutagenesis was used to change the potential furin recognition site, RNRR, to ANAA, and the *Sap*I-*Apa*I fragment from a correctly mutated clone was substituted for the same region of EGFP-MoZP3. The fidelity of the normal (pSEGFP-MoZP3) and mutant (pSEGFP-FurX) constructs was verified by DNA sequencing.

Because of variable levels of expression in clonal cell lines after stable transfection, transient-expression assays with populations of mouse 10T[1/2] embryonic fibroblasts were used to examine the expression, processing, and secretion of the normal (ZP3) and mutant (FurX) proteins. Both constructs were expressed in 10T[1/2] embryonic fibroblast cells, and EGFP could be detected by confocal microscopy (data not shown). BFA dissociates Golgi structures, mixes them with the endoplasmic reticulum, and blocks protein translocation to the trans-Golgi. To investigate the processing of the EGFP-fused proteins, transfected 10T[1/2] cells were cultured in the presence of 0.2 μg of BFA per ml for 12 h, at which time the Golgi structures were disrupted and the synthesized EGFP fusion proteins were distributed in endoplasmic reticulum networks (data not shown). The cells were then incubated in the absence of BFA. Enhancement of the EGFP-ZP3 signal occurs concomitantly with reassembly of the Golgi structure (near the nucleus, often in contact with the nuclear membrane). After 30 min, the EGFP signals were localized in the Golgi region (Fig. 3) and no significant differences in synthesis and translocation were observed between the normal ZP3 (Fig. 3B and H) and FurX (Fig. 3E and K) proteins.

To identify whether the EGFP-fused proteins correctly traffic from the endoplasmic reticulum to the Golgi, two antibodies, one to an endoplasmic reticulum residential enzyme, PDI, and the other to a Golgi marker, α-mannosidase II, were used to colocalize the expressed EGFP-ZP3 fused proteins. Although some EGFP-ZP3 signal was observed in the endoplasmic reticulum at 30 min after release from the BFA block, most had progressed to the Golgi (Fig. 3B, E, H, and K). In some cells, the EGFP signal in the endoplasmic reticulum seemed more pronounced with the furin mutant ZP3 (Fig. 3B and E) but this observation was inconsistent (Fig. 3H and K). More consistent was the colocalization of the EGFP signal with antibodies specific to α-mannosidase II in the Golgi (Fig. 3I and L) and the absence of colocalization with antibodies to PDI in the endoplasmic reticulum (Fig. 3C and F). This progression to the Golgi occurred in the presence (Fig. 3J through L) or absence (Fig. 3G through I) of the mutated furin site. These observations suggested that processing of the recombinant ZP3 protein in the Golgi apparatus did not require the integrity of the furin site, although differences in the efficiency of transport of normal and mutant ZP3 in heterologous cells would not be detected in these studies.

Secretion of EGFP-MoZP3 fusion proteins from 10T[1/2]

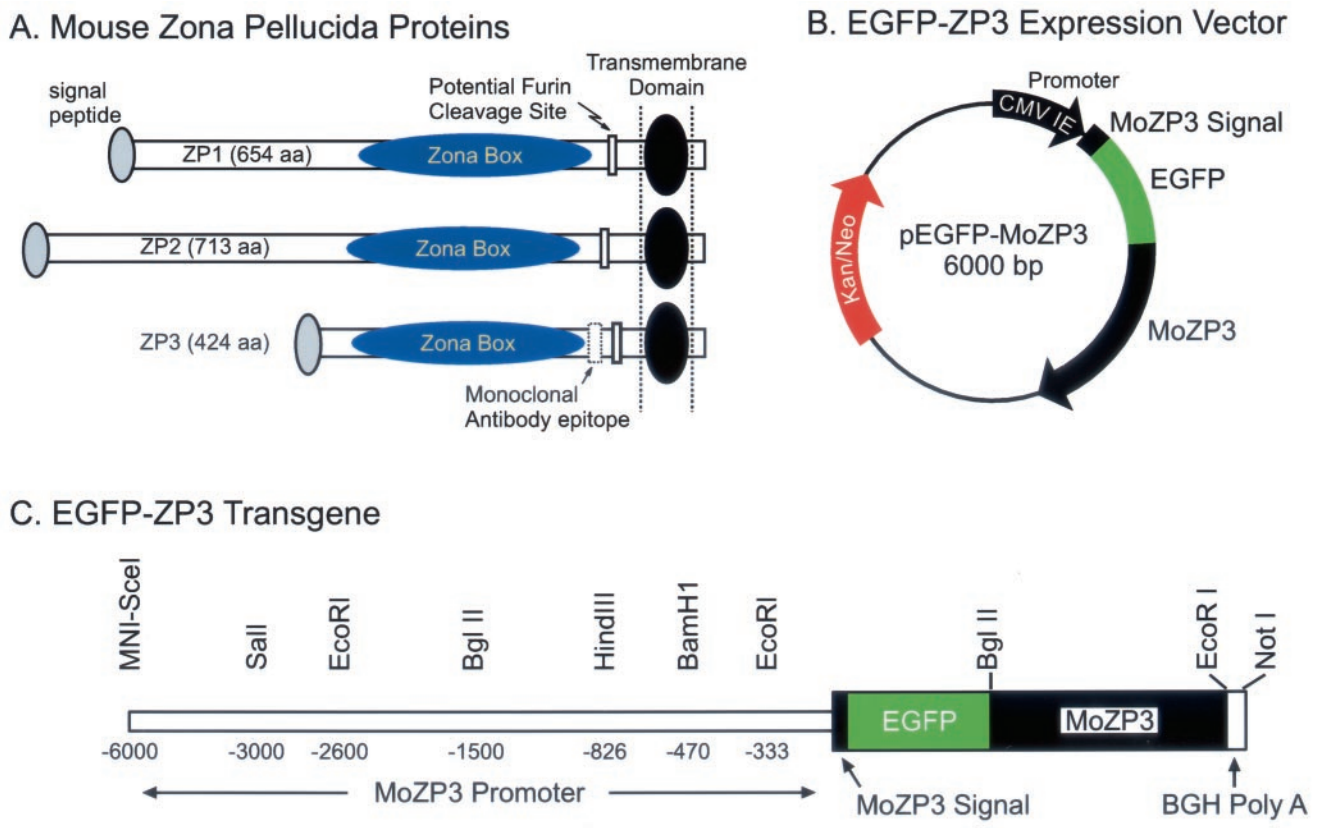

FIG. 2. Conserved zona pellucida protein motifs and construct design. (A) Each of the three mouse zona pellucida proteins, ZP1, ZP2, and ZP3, has a signal peptide that directs it into a secretory pathway, a signature zona box, and a transmembrane domain near the carboxyl terminus, upstream of which (30 to 47 residues) is a potential furin cleavage site. A monoclonal antibody binds a linear epitope on ZP3 (amino acids [aa] 336 to 342). (B) cDNA encoding ZP3 with a normal (RNRR) or a mutant (ANAA) furin site was cloned in frame with EGFP into expression plasmids under the control of a cytomegalovirus immediate-early (CMV IE) promoter. Constructs were transfected into 10T[1/2] cells or injected into the nuclei of growing mouse oocytes for expression studies. (C) The normal and furin site mutant EGFP-ZP3 cDNAs were inserted downstream of ~6.0 kbp of the mouse ZP3 promoter and just upstream of a bovine growth hormone (BGH) poly(A) signal. After isolation, the DNA fragment was injected into the nuclei of one-cell zygotes to establish transgenic mouse lines expressing normal and furin site mutant EGFP-ZP3 proteins.

cells. The transiently transfected 10T[1/2] cells and the culture supernatants were analyzed by Western blotting for expression and secretion of the EGFP-ZP3 proteins. Because bovine serum in the culture medium distorted the electrophoretic separation, the transfected cells were cultured in the absence of serum. NP-40 extracts of the cell pellets were separated by SDS-PAGE and probed with a monoclonal antibody specific to ZP3. Both normal ZP3 and FurX proteins were detected (Fig. 4A) as major bands migrating with an apparent molecular mass of 75 kDa. After treatment with endo-H, the bands shifted to 70 kDa, which is consistent with the removal of three or four N-linked, high-mannose oligosaccharides normally attached to the core protein during transit through the endoplasmic reticulum (35). The remaining 70 kDa is accounted for by EGFP (27 kDa) and mouse ZP3 polypeptide lacking the signal peptide (44 kDa). The faint higher-molecular-weight bands in Fig. 4A may represent further posttranslational modification in the Golgi (~100 kDa) or aggregation (>200 kDa).

Supernatants (0.6 ml) from transfected cells were collected and concentrated prior to Western blot analysis. Both normal ZP3 and FurX proteins were secreted with apparent molecular masses of ~105 kDa and were indistinguishable from one another (Fig. 4B). The mature form of native mouse ZP3 has a polypeptide chain of ~37 kDa and migrates at ~83 kDa on SDS-PAGE after posttranslational modifications. The addition of EGFP (27 kDa) would account for the observed molecular masses of the secreted recombinant proteins. Normally, high-mannose, N-linked oligosaccharides attached in the endoplasmic reticulum are further matured into complex side chains as a glycoprotein passes through the Golgi apparatus. Consistent with such modifications, neither normal ZP3 nor FurX secreted protein was appreciably affected by treatment with endo-H, which does not cleave complex oligosaccharides (data not shown). However, after treatment with PNGase F, which cleaves complex mannose N-linked oligosaccharides, the molecular mass of both normal ZP3 and FurX decreased to ~80 kDa (consistent with the removal of three or four N-linked sugar side chains). Thus, mutation of the furin site did not appear to affect the intracellular trafficking, the posttranslational modification, or the secretion of EGFP-ZP3 protein from 10T[1/2] cells. Similar results were obtained when the blots were probed with antibodies to EGFP (data not shown).

Expression of EGFP-ZP3 in growing oocytes and incorporation into the zona pellucida. To confirm these unexpected results with cells that normally express zona proteins, pSEGFP-MoZP3 and pSEGFP-FurX were microinjected into the nuclei

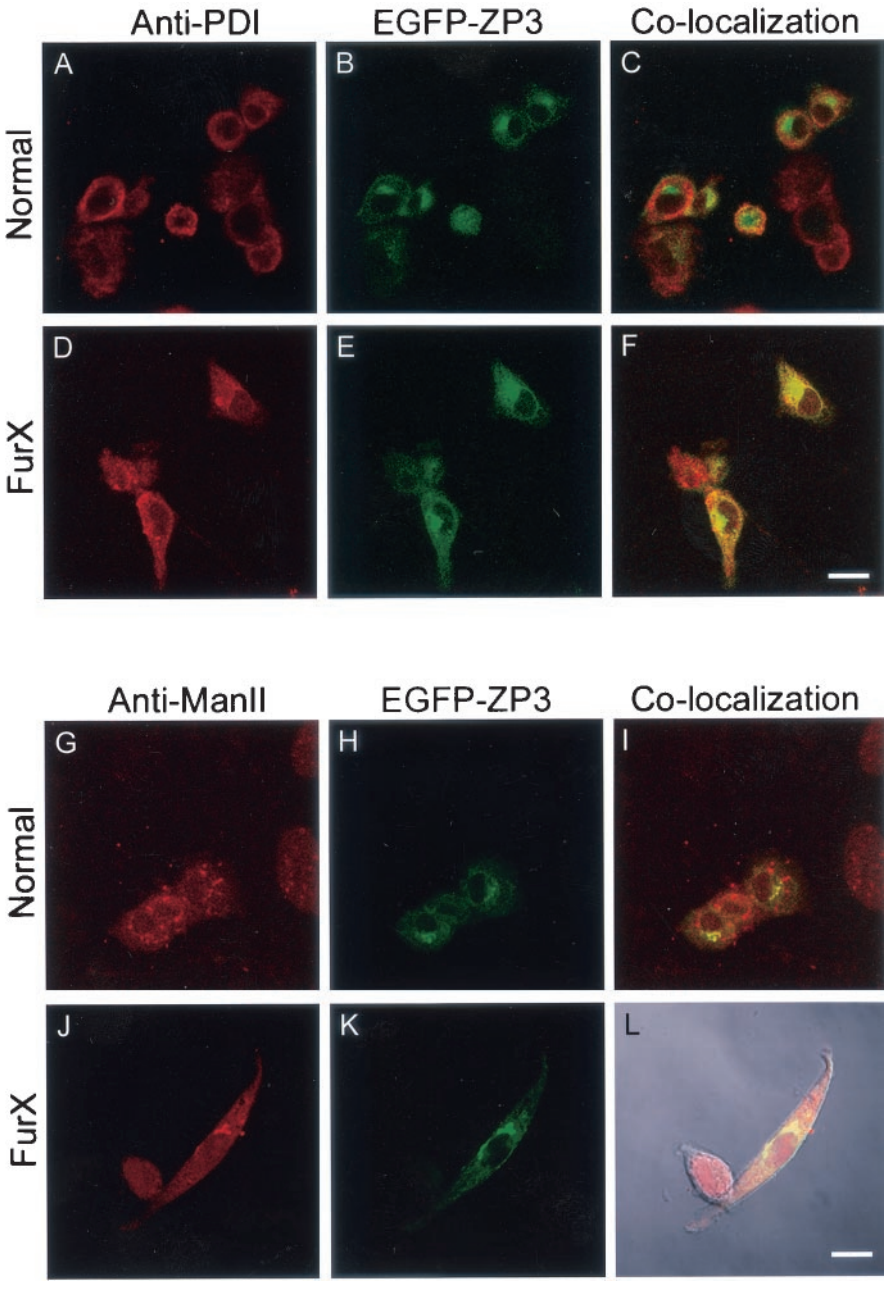

FIG. 3. Confocal microscopy of EGFP-ZP3 in 10T[1/2] cells. Cells transfected with plasmid expression vectors were incubated with BFA overnight, allowed to recover for 30 min, and fixed for imaging. Cells expressing normal EGFP-ZP3 (A to C and G to I) or EGFP-ZP3 mutated at the furin site, FurX (D to F and J to L), were incubated with antibodies to PDI (A and D) or α-mannosidase II (ManII) (G and J) and imaged to detect antibody binding with a Cy-5-conjugated secondary antibody (A, D, G, and J), EGFP-ZP3 (B, E, H, and K), or both (C, F, I, and L). Bar, 20 μm.

of growing oocytes isolated from 11- to 13-day-old mice (Fig. 5). Signals obtained by confocal microscopy were optimized by injecting ~0.5 fg of plasmid/oocyte and incubating the oocytes for 20 to 40 h. Under the experimental conditions described, the lipophilic dye PM-R18 primarily stained the plasma membrane of the oocytes although weak staining of the microvilli between oocytes and cumulus cells was observed in some instances (Fig. 5A and D).

Both constructs, pSEGFP-MoZP3 and pSEGFP-FurX, were successfully transcribed by the oocytes, and recombinant fusion proteins were directed into secretory pathways prior to detection at the periphery (Fig. 5B and E). Because there is close contact between the zona pellucida and the plasma membrane in growing oocytes, it was difficult to determine if the colocalization at the periphery was limited to the membrane or also reflected incorporation into the extracellular zona pellucida (Fig. 5C and F). Therefore, zona ghosts were prepared by freeze-thawing in the presence of detergent (1% NP-40) and salt (0.5 M NaCl) to remove the plasma membrane. Solubilization of the plasma membrane was confirmed by absent stain-

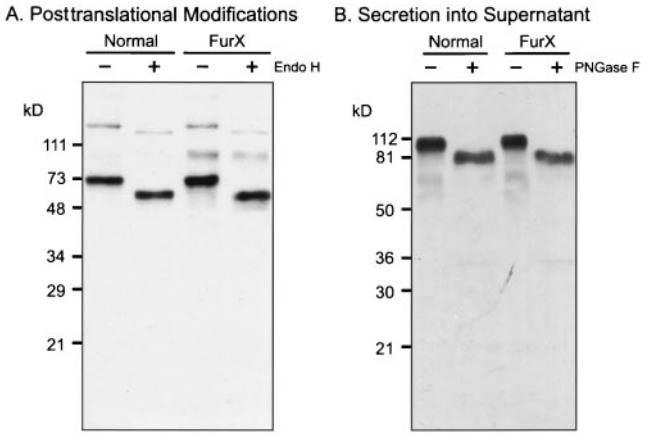

FIG. 4. Western blot assay of EGFP-ZP3. Three days after transfection with plasmid vectors encoding either EGFP-ZP3 (normal) or EGFP-ZP3 mutated at the furin site (FurX), 10T[1/2] cell pellets (A) and supernatants (B) were harvested. Pellets were lysed with detergent and incubated with (+) or without (−) endo-H, which cleaves high-mannose, but not complex, N-linked oligosaccharide side chains. Cell supernatants were incubated with (+) or without (−) PNGase F, which cleaves high-mannose, hybrid, and complex N-linked oligosaccharides. Proteins in the pellets and supernatants were separated by SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed with a monoclonal antibody specific to mouse ZP3.

ing with PM-R18 (Fig. 5G and J), and the persistence of an EGFP signal at the periphery (Fig. 5H, I, K, and L) indicated incorporation of ZP3 into the inner aspect of the zona pellucida matrix. No significant difference was observed between normal ZP3 and ZP3 with the mutated furin site.

EGFP-MoZP3 is incorporated into the zona pellucida of transgenic mice. These in vitro tissue culture studies were extended in transgenic mice. An 8.0-kbp fragment containing the 6.0-kbp oocyte-specific mouse ZP3 promoter (33) upstream of EGFP-ZP3 with the normal or the mutated furin site, followed by the bovine growth hormone poly(A) signal, was assembled (Fig. 2C). After its injection into the male pronuclei of one-cell zygotes, mouse lines with normal EGFP-ZP3 or with EGFP-FurX were established and they passed the transgene through their germ line. Mice expressing either EGFP-ZP3 or EGFP-FurX protein had normal-appearing zonae pellucidae (Fig. 6H, K, M, and Q). Hemizygous EGFP-ZP3 and EGFP-FurX females were fertile when mated with normal males and produced litters (11.4 ± 0.8 and 10.6 ± 0.7 [standard error of the mean] pups, respectively), the sizes of which were not statistically significantly different (unpaired *t* test, $P > 0.05$) from those of the litters of normal mice (8.8 ± 0.6 [standard error of the mean] pups) or from one another. Thus, the presence of EGFP (27 kDa) at the N terminus of mouse ZP3 did not adversely affect formation of the zona pellucida matrix or fecundity.

Oocytes and eggs were collected from transgenic animals, fixed in 2% paraformaldehyde, and examined by confocal microscopy. The EGFP signal was not observed in nontransgenic oocytes and eggs (Fig. 6A through F) but was readily detected in the zona pellucida surrounding growing oocytes (Fig. 6G, I, M, and O) and ovulated eggs (Fig. 6J, L, P, and R) isolated from transgenic mice expressing either EGFP-ZP3 or EGFP-FurX. As anticipated, no EGFP expression was observed in surrounding somatic cells (Fig. 6I and O). The EGFP-ZP3 fusion proteins were present throughout the width of the zona matrix in mice expressing either the normal or the mutant form of FurX. The mutation of the furin site in the genome of the transgenic mice was confirmed by nucleic acid sequencing of PCR products specific to the transgene with tail DNA as the template (Fig. 7), and the phenotype was the same in three independently derived ZP3-FurX mutant lines. Thus, the integrity of the furin site does not appear to be required for ZP3 synthesis, intracellular trafficking, secretion, or incorporation into the extracellular zona pellucida matrix of transgenic mice.

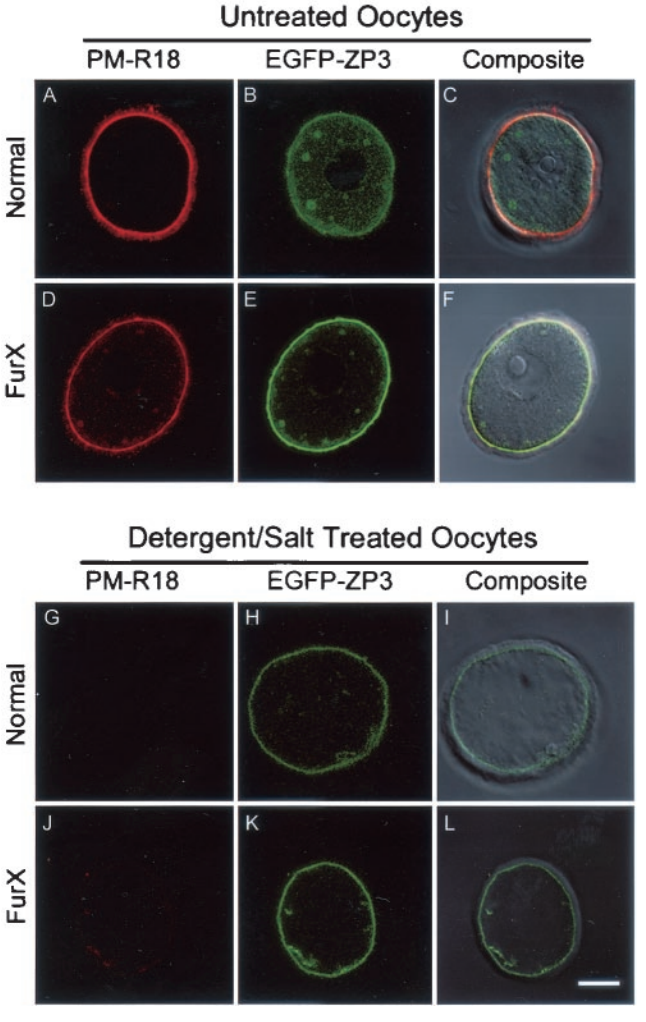

FIG. 5. Localization of EGFP-ZP3 within the growing-oocyte zona pellucida. Plasmid vectors expressing either normal EGFP-ZP3 or EGFP-ZP3 mutated at the furin site (FurX) were injected into the nuclei of growing oocytes, which were cultured for 20 to 40 h. Oocytes were incubated with a lipid membrane stain (PM-R18) before (A and D) or after (G and J) freeze-thawing in the presence of 0.5 M NaCl and 1% NP-40. PM-18 (A, D, G, and J) and EGFP-ZP3 (B, E, H, and K) were viewed individually and together (C, F, I, and L) after superimposition on a light microscopic image. Bar, 20 μm.

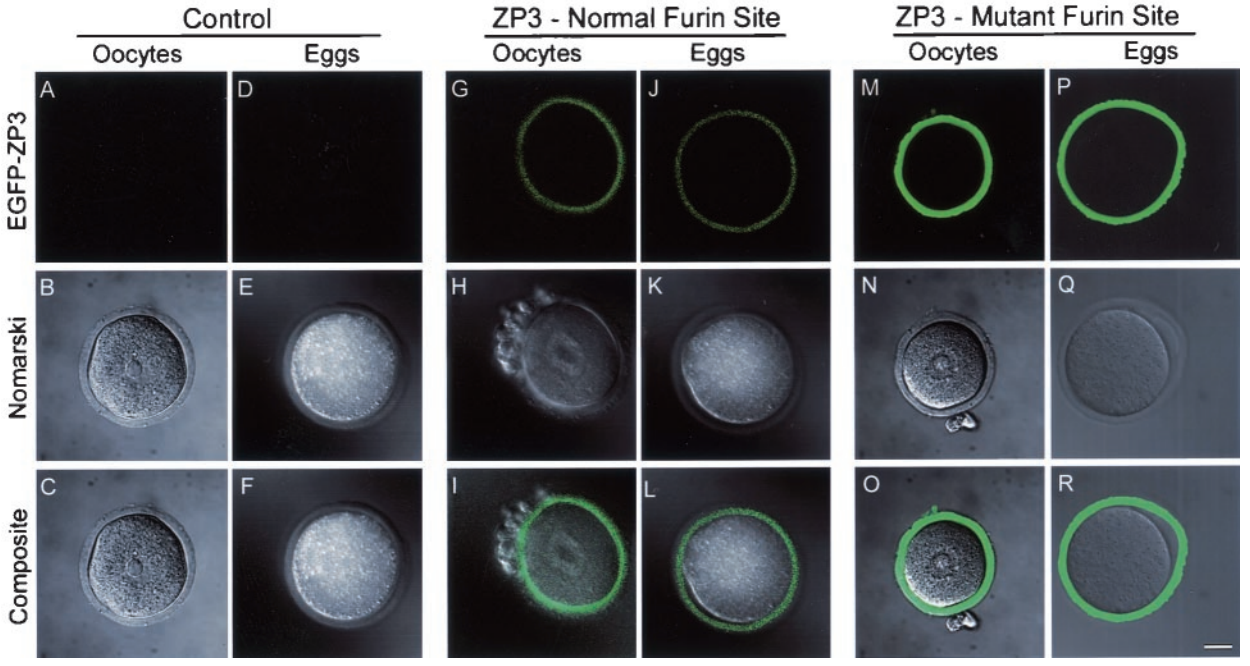

FIG. 6. Transgenic mice incorporate EGFP-ZP3 into the zona pellucida. Mouse lines with transgenes expressing either EGFP-ZP3 or EGFP-ZP3 mutated at the furin site were established. The morphology of growing oocytes (H and N) and ovulated eggs (K and Q) from normal (H and K) and furin mutant (N and Q) EGFP-ZP3 transgenic mice was indistinguishable from that of oocytes (B) and eggs (E) isolated from nontransgenic mice. EGFP signals were observed throughout the width of the zona pellucida surrounding oocytes (G, I, M, and O) and eggs (J, L, P, and R) from normal (G to L) and furin mutant (M to R) EGFP-ZP3 transgenic mice. No signal was observed in oocytes (A to C) and eggs (D to F) isolated from nontransgenic mice. Bar, 20 μm.

DISCUSSION

During much of oogenesis, the mouse zona pellucida is closely apposed to the plasma membrane of the germ cell but a perivitelline space eventually develops between the extracellular zona matrix and the plasma membrane. Although the three zona proteins detected on the growing-oocyte surface must be released prior to incorporation into the zona pellucida, the molecular mechanism of matrix formation is unknown. Individual mouse zona proteins, when expressed in heterologous cells, are secreted as soluble macromolecules (2, 15). The primary structure of each zona protein predicts a transmembrane domain near the carboxyl terminus, and the presence of a potential furin cleavage site 30 to 50 amino acids N terminal to this domain suggests that release of zona ectodomains occurs prior to zona matrix formation (44). This hypothesis is supported by the conservation of this potential proteolytic cleavage site in more than 25 zona proteins across 12 mammalian species, as well as more recent biochemical analyses of mouse zona proteins (17, 42). However, the present results indicate that the integrity of the furin site is not required for secretion of ZP3 from mouse embryonic fibroblasts or for its incorporation into the extracellular zona pellucida of transgenic mice.

In the present experiments, mouse ZP3 was expressed as a recombinant fusion protein with EGFP. In heterologous somatic cells, the EGFP-ZP3 fusion protein enters into a secretory pathway and is posttranslationally modified as it passes through the endoplasmic reticulum and the Golgi apparatus prior to secretion as a soluble ~105-kDa protein. The 70-kDa molecular mass of the fusion protein in the cell pellet after digestion with endo-H correlates well with the combined mass of EGFP (27 kDa) and ZP3 (46.3 kDa) after removal of the signal peptide (2 kDa) but with an intact carboxyl-terminal tail. Thus, these data are consistent with cleavage of the transmembrane domain in a compartment subsequent to the trans-Golgi or at the plasma membrane. Detection of the zona proteins on the zona-free oocyte surface suggests the latter. The region of cleavage can be delimited by an epitope (amino acids 336 to 342) recognized by a monoclonal antibody that binds to the zona pellucida (9, 22) and an antibody raised to a C-terminal peptide (amino acids 370 to 389) that does not (17). The potential furin cleavage site (amino acids 350 to 353) is ideally positioned between these two limits.

Furin, present in multiple cell types but in low abundance, is a member of the proprotein convertases that include Kex2, PC1/3, PC2, PC4, PACE4, PC5/6, and PC7 (24, 26, 45). It is translated in the endoplasmic reticulum as an integral membrane precursor prior to activation. Autoproteolysis cleaves an N-terminal peptide that binds, in part, to the catalytic domain and inhibits enzymatic activity. However, once translocated to the Golgi stack, the peptide itself is cut and released to produce fully active furin. Although, at steady state, furin is primarily (>80%) localized to the trans-Golgi by determinants on its carboxyl cytoplasmic tail, a significant amount of furin cycles between the Golgi and the plasma membrane and small amounts have been detected in the extracellular space (24, 43). Thus, even though furin is available to cleave the zona proteins, mutation of the furin site (RNRR → ANAA) in EGFP-

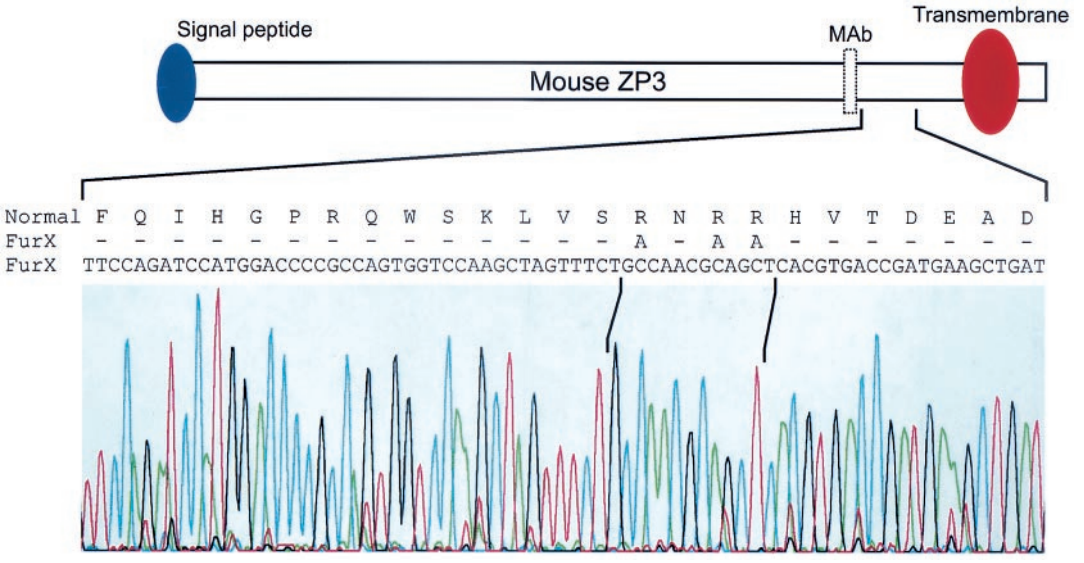

FIG. 7. The furin site in transgenic mice. A schematic representation of mouse ZP3 (424 amino acids) with the N-terminal signal peptide, the monoclonal antibody (MAb) binding site, and the transmembrane domain is at the top. The nucleic acid sequence of a PCR product obtained with primers that flank the potential furin cleavage site in the transgene confirmed the mutation (RNRR → ANAA) in genomic tail DNA isolated from EGFP-FurX transgenic mice. The nucleic acid sequence of FurX is color coded (A, green; C, blue; G, black; T, red) and shown directly above the traces. The single-letter amino acid sequences (amino acids 336 to 360) of normal ZP3 and FurX are at the top, and dashes indicate identical residues.

ZP3 does not prevent the intracellular trafficking, the post-translational processing, or the secretion of the fusion protein from heterologous 10T[1/2] fibroblasts. These unexpected results are consistent with our unpublished observation that mouse ZP3 can be secreted, albeit in diminished amounts, from stably transformed FD11 cells that lack furin (10), although they differ from the observed inability of epitope-tagged, furin mutant ZP3 to be secreted by embryonic carcinoma cells (42). This discrepancy may result from using transient transfections that analyze a population of cells versus stable transfections in which the analysis is restricted to clonal lines or differences in the tissue culture lines themselves (embryonic fibroblast versus embryonic carcinoma cells).

Under more physiologic conditions, the secretion and incorporation of EGFP-ZP3 into the zona pellucida of growing oocytes and transgenic mice is also unaffected by mutation of the furin site. The presence of an intact furin site in ZP3 isolated from normal zonae pellucidae additionally confirms that cleavage at the furin consensus sequence is not required for incorporation of ZP3 into the extracellular zona matrix. Normally, the zona pellucida matrix is first detected as non-contiguous clumps of electron-dense material near the plasma membrane of oocytes within the ovary. Upon additional oocyte growth, the zona matrix becomes a continuous band that eventually reaches a width of 7 μm (6, 11). Detection of EGFP-ZP3 on the inner surface of the zona matrix in the transient oocyte assay, but throughout the width of the zona pellucida in transgenic mice, suggests that the matrix grows by addition of zona proteins at the inner aspect closest to the plasma membrane. Earlier pulse-chase studies indicated that zona proteins, once incorporated into the zona pellucida matrix, are quite stable (37). Thus, it is likely that the zona proteins forming the initial zona matrix are gradually displaced from the inner aspect of the zona toward the periphery as the zona pellucida reaches its maximal width. Laser-bleaching studies do not detect zona protein mobility in the zona matrix (8), but these results do not preclude stretching of the zona filaments to accommodate the ~30-fold increase in the surface area of the oocyte as its diameter increases from 15 to 80 μm. The observation that the mesh-like network detected by scanning electron microscopy is more tightly packed in the inner than on the outer aspect of the zona pellucida (27) is consistent with this formulation.

These results raise two interrelated questions. (i) If the furin site is not required, how is the ZP3 ectodomain released from the membrane for incorporation into the zona matrix, and (ii) why are the furin cleavage site and its location upstream of the transmembrane domain so well conserved among zona proteins? Glycosylphosphatidylinositol (GPI)-anchored proteins can be released at the plasma membrane by phospholipase D (19). This pathway involves cleavage from a carboxyl-terminal transmembrane domain and transfer to the membrane-anchored GPI moiety in the endoplasmic reticulum (39, 40). However, the presence of a cytoplasmic tail (present in all zona proteins) is not usually observed in GPI-anchored proteins and our inability to release ZP3 from the zona-free oocyte surface with phospholipase (data not shown) makes GPI anchorage unlikely. Alternatively, there is considerable evidence on the role of sheddases or secretases in the release of ectodomains from membrane-spanning domains in a variety of proteins (13, 28). The sorting of proteins for entry into a regulated secretase pathway occurs in the trans-Golgi, and many elements of the pathway appear to be present in diverse cell types. We speculate that zona proteins traffic the trans-Golgi and progress to the plasma membrane via a secretase-regulated pathway, either separately or complexed with other zona proteins while still tethered to the lipid bilayer. Exposure to the extracellular

environment could result in conformational changes that trigger cleavage of the ectodomains of zona proteins by a membrane-anchored secretase and facilitate their subsequent incorporation into an insoluble zona matrix. Identification of the putative cleavage site and the hypothetical secretase is necessary to test these possibilities. Presumably, this site lies between amino acid residues 342 and 385 in the region bounded by the monoclonal antibody binding site and the transmembrane domain (Fig. 7).

The conservation of the furin site (RXK/RR) upstream of the transmembrane domain in most mammalian zona proteins is quite striking. Even when it is absent (as in human ZP1 and cat ZPB), there are dibasic residues in the same region that provide comparable potential cleavage sites. Either higher-order folding constraints, interactions with other proteins, or specialized compartmentalization could prevent cleavage at the furin site during normal intracellular trafficking. Although the present results indicate that the furin site is not necessary for secretion or incorporation of ZP3 into the zona pellucida, the site could be used to dispose of zona proteins that are misdirected and fail to enter the secretase-regulated pathway. If these errant zona proteins are cleaved from their transmembrane domain by furin in the trans-Golgi, they could be released after they traverse the constitutive secretory pathway and avoid cluttering the plasma membrane. If this scenario is operative, the integrity of the secretase-regulated pathway must be quite high as no increase in EGFP-ZP3 was observed at the plasma membrane of cells expressing the mutant furin. Of note is the presence of unutilized furin cleavage sites in several other secreted proteins, including the metalloprotease meprin A, human immunodeficiency virus type 1 envelope protein gp160, and the thyrotropin receptor (5, 14, 21, 38). A further complication to our understanding of the formation of the zona pellucida is the observation that nonmammalian vertebrates have similar extracellular matrices that surround their eggs and are composed of proteins homologous to ZP1, ZP2, and ZP3. However, some of these proteins are expressed in the liver (1, 20, 25) or in granulosa cells within the ovary (41) and it remains unclear how they are transported to the oocyte and assembled to form the insoluble extracellular egg envelope.

ACKNOWLEDGMENTS

We appreciate the initial help with confocal microscopy by Nancy Dwyer and critical reading of the manuscript by John Hanover and Gary Thomas.

REFERENCES

1. **Bausek, N., M. Waclawek, W. J. Schneider, and F. Wohlrab.** 2000. The major chicken egg envelope protein ZP1 is different from ZPB and is synthesized in the liver. J. Biol. Chem. **275:**28866–28872.
2. **Beebe, S. J., L. Leyton, D. Burks, T. Fuerst, J. Dean, and P. M. Saling.** 1992. Recombinant ZP3 inhibits sperm binding and induces the acrosome reaction. Dev. Biol. **151:**48–54.
3. **Bork, P., and C. Sander.** 1992. A large domain common to sperm receptors (Zp2 and Zp3) and TGF-beta type III receptor. FEBS Lett. **300:**237–240.
4. **Burnette, W. N.** 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112:**195–203.
5. **Chazenbalk, G. D., and B. Rapoport.** 1994. Cleavage of the thyrotropin receptor does not occur at a classical subtilisin-related proprotein convertase endoproteolytic site. J. Biol. Chem. **269:**32209–32213.
6. **Chiquoine, A. D.** 1960. The development of the zona pellucida of the mammalian ovum. Am. J. Anat. **106:**149–169.
7. **Doren, S., N. Landsberger, N. Dwyer, L. Gold, J. Blanchette-Mackie, and J. Dean.** 1999. Incorporation of mouse zona pellucida proteins into the envelope of Xenopus laevis oocytes. Dev. Genes E **209:**330–339.
8. **East, I. J., and J. Dean.** 1984. Monoclonal antibodies as probes of the distribution of ZP-2, the major sulfated glycoprotein of the murine zona pellucida. J. Cell Biol. **98:**795–800.
9. **East, I. J., B. J. Gulyas, and J. Dean.** 1985. Monoclonal antibodies to the murine zona pellucida protein with sperm receptor activity: effects on fertilization and early development. Dev. Biol. **109:**268–273.
10. **Gordon, V. M., K. R. Klimpel, N. Arora, M. A. Henderson, and S. H. Leppla.** 1995. Proteolytic activation of bacterial toxins by eukaryotic cells is performed by furin and by additional cellular proteases. Infect. Immun. **63:**82–87.
11. **Hadek, R.** 1965. The structure of the mammalian egg. Int. Rev. Cytol. **18:**29–68.
12. **Hogan, B., R. Beddington, F. Costantini, and E. Lacy.** 1994. Manipulating the mouse embryo. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
13. **Hooper, N. M., E. H. Karran, and A. J. Turner.** 1997. Membrane protein secretases. Biochem. J. **321:**265–279.
14. **Inocencio, N. M., J. F. Sucic, J. M. Moehring, M. J. Spence, and T. J. Moehring.** 1997. Endoprotease activities other than furin and PACE4 with a role in processing of HIV-I gp160 glycoproteins in CHO-K1 cells. J. Biol. Chem. **272:**1344–1348.
15. **Kinloch, R. A., S. Mortillo, C. L. Stewart, and P. M. Wassarman.** 1991. Embryonal carcinoma cells transfected with ZP3 genes differentially glycosylate similar polypeptides and secrete active mouse sperm receptor. J. Cell Biol. **115:**655–664.
16. **Kumar, S., and S. B. Hedges.** 1998. A molecular timescale for vertebrate evolution. Nature **392:**917–920.
17. **Litscher, E. S., H. Qi, and P. M. Wassarman.** 1999. Mouse zona pellucida glycoproteins mZP2 and mZP3 undergo carboxy-terminal proteolytic processing in growing oocytes. Biochemistry **38:**12280–12287.
18. **Liu, C., E. S. Litscher, S. Mortillo, Y. Sakai, R. A. Kinloch, C. L. Stewart, and P. M. Wassarman.** 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. USA **93:**5431–5436.
19. **Low, M. G., and K. S. Huang.** 1993. Phosphatidic acid, lysophosphatidic acid, and lipid A are inhibitors of glycosylphosphatidylinositol-specific phospholipase D. Specific inhibition of a phospholipase by product analogues? J. Biol. Chem. **268:**8480–8490.
20. **Lyons, C. E., K. L. Payette, J. L. Price, and R. C. C. Huang.** 1993. Expression and structural analysis of a teleost homolog of a mammalian zona pellucida gene. J. Biol. Chem. **268:**21351–21358.
21. **Marchand, P., M. Volkmann, and J. S. Bond.** 1996. Cysteine mutations in the MAM domain result in monomeric meprin and alter stability and activity of the proteinase. J. Biol. Chem. **271:**24236–24241.
22. **Millar, S. E., S. M. Chamow, A. W. Baur, C. Oliver, F. Robey, and J. Dean.** 1989. Vaccination with a synthetic zona pellucida peptide produces long-term contraception in female mice. Science **246:**935–938.
23. **Millar, S. E., E. Lader, L.-F. Liang, and J. Dean.** 1991. Oocyte-specific factors bind a conserved upstream sequence required for mouse zona pellucida promoter activity. Mol. Cell. Biol. **12:**6197–6204.
24. **Molloy, S. S., E. D. Anderson, F. Jean, and G. Thomas.** 1999. Bi-cycling the furin pathway: from TGN localization to pathogen activation and embryogenesis. Trends Cell Biol. **9:**28–35.
25. **Murata, K., T. Sasaki, S. Yasumasu, I. Iuchi, I. Yasumasu, and K. Yamagami.** 1995. Cloning of cDNA for precursor protein of a low molecular weight subunit of the inner layer of the egg envelope (chorion) of the fish Oryzias latipes. Dev. Biol. **167:**9–17.
26. **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.
27. **Phillips, D. M., and R. Shalgi.** 1980. Surface architecture of the mouse and hamster zona pellucida and oocyte. J. Ultrastruct. Res. **72:**1–12.
28. **Primakoff, P., and D. G. Myles.** 2000. The ADAM gene family: surface proteins with adhesion and protease activity. Trends Genet. **16:**83–87.
29. **Rankin, T., and J. Dean.** 2000. The zona pellucida: using molecular genetics to study the mammalian egg coat. Rev. Reprod. **5:**114–121.
30. **Rankin, T., M. Familari, E. Lee, A. M. Ginsberg, N. Dwyer, J. Blanchette-Mackie, J. Drago, H. Westphal, and J. Dean.** 1996. Mice homozygous for an insertional mutation in the *Zp3* gene lack a zona pellucida and are infertile. Development **122:**2903–2910.
31. **Rankin, T., M. O'Brien, E. Lee, K. E. J. J. Wigglesworth, and J. Dean.** 2001. Defective zonae pellucidae in *Zp2* null mice disrupt folliculogenesis, fertility and development. Development **128:**1119–1126.
32. **Rankin, T., P. Talbot, E. Lee, and J. Dean.** 1999. Abnormal zonae pellucidae in mice lacking ZP1 result in early embryonic loss. Development **126:**3847–3855.
33. **Rankin, T., Z.-B. Tong, P. E. Castle, E. Lee, R. Gore-Langton, L. M. Nelson, and J. Dean.** 1998. Human *ZP3* restores fertility in *Zp3* null mice without affecting order-specific sperm binding. Development **125:**2415–2424.
34. **Ringuette, M. J., M. E. Chamberlin, A. W. Baur, D. A. Sobieski, and J. Dean.**

1988. Molecular analysis of cDNA coding for ZP3, a sperm binding protein of the mouse zona pellucida. Dev. Biol. **127:**287–295.

35. **Roller, R. J., and P. M. Wassarman.** 1983. Role of asparagine-linked oligosaccharides in secretion of glycoproteins of the mouse egg's extracellular coat. J. Biol. Chem. **258:**13243–13249.
36. **Seto, D.** 1990. An improved method for sequencing double stranded plasmid DNA from minipreps using DMSO and modified template preparation. Nucleic Acids Res. **18:**5905–5906.
37. **Shimizu, S., M. Tsuji, and J. Dean.** 1983. In vitro biosynthesis of three sulfated glycoproteins of murine zonae pellucidae by oocytes grown in follicle culture. J. Biol. Chem. **258:**5858–5863.
38. **Tang, J., and J. S. Bond.** 1998. Maturation of secreted meprin alpha during biosynthesis: role of the furin site and identification of the COOH-terminal amino acids of the mouse kidney metalloprotease subunit. Arch. Biochem. Biophys. **349:**192–200.
39. **Udenfriend, S., and K. Kodukula.** 1995. How glycosylphosphatidylinositol-anchored membrane proteins are made. Annu. Rev. Biochem. **64:**563–591.
40. **Vidugiriene, J., and A. K. Menon.** 1994. The GPI anchor of cell-surface proteins is synthesized on the cytoplasmic face of the endoplasmic reticulum. J. Cell Biol. **127:**333–341.
41. **Waclawek, M., R. Foisner, J. Nimpf, and W. J. Schneider.** 1998. The chicken homologue of zona pellucida protein-3 is synthesized by granulosa cells. Biol. Reprod. **59:**1230–1239.
42. **Williams, Z., and P. M. Wassarman.** 2001. Secretion of mouse ZP3, the sperm receptor, requires cleavage of its polypeptide at a consensus furin cleavage-site. Biochemistry **40:**929–937.
43. **Wouters, S., M. Leruth, E. Decroly, M. Vandenbranden, J. W. Creemers, J. W. van de Loo, J. M. Ruysschaert, and P. J. Courtoy.** 1998. Furin and proprotein convertase 7 (PC7)/lymphoma PC endogenously expressed in rat liver can be resolved into distinct post-Golgi compartments. Biochem. J. **336:**311–316.
44. **Yurewicz, E. C., D. Hibler, G. K. Fontenot, A. G. Sacco, and J. Harris.** 1993. Nucleotide sequence of cDNA encoding ZP3 alpha, a sperm-binding glycoprotein from zona pellucida of pig oocyte. Biochim. Biophys. Acta **1174:**211–214.
45. **Zhou, A., G. Webb, X. Zhu, and D. F. Steiner.** 1999. Proteolytic processing in the secretory pathway. J. Biol. Chem. **274:**20745–20748.