Tracking Down the ZP Domain: From the Mammalian Zona Pellucida to the Molluscan Vitelline Envelope

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ABSTRACT

Oocytes from virtually all organisms are surrounded by at least one coat. This specialized extracellular matrix, called the zona pellucida (ZP) in mammals and the vitelline envelope (VE) in nonmammals, has a structural function and plays essential roles in oogenesis, fertilization, and early development. During the last 15 years, compelling evidence has accumulated that all ZP/VE subunits polymerize using a conserved sequence, the ZP domain, so that the basic structural features of egg coat matrices have been maintained through evolution. Moreover, ZP domains have been identified in many other polymeric extracellular proteins from eukaryotes. This review compares the ultrastructure and molecular composition of egg coats from mollusc to human, suggests a common mechanism for assembly of ZP/VE proteins, and discusses alternative models of how these could be arranged within filaments.

KEYWORDS: Zona pellucida, vitelline envelope, extracellular matrix, fertilization, sperm-egg recognition

STRUCTURE AND COMPOSITION OF EGG COATS

The mouse zona pellucida (ZP) is undoubtedly the most studied of all egg coats, and mammalian ZP subunits have become the accepted reference system for classifying ZP/vitelline envelope (VE) proteins from other organisms.[1,2] However, alternative nomenclature schemes have been proposed[3,4]; to avoid confusion, in this article we adopt the original convention (ZP1 to ZP3),[5] so that, for example, the recently identified ZPB subunit of rat and human ZP[6,7] will be referred to as ZP4. To facilitate comparisons, we also start this section with the mammalian ZP, the genes of which were the first to be cloned and characterized,[2] then move down the evolutionary tree of deuterostomes, and finally describe the VE of molluscs (of which a recent analysis revealed that ZP domain proteins are also found in the egg coat of protostomes).[8]

Mammalian ZP

The mammalian ZP (Fig. 1A) is responsible for the species specificity of egg–sperm recognition, participates in the slow block to polyspermy that is established following fertilization, and protects the developing embryo until this hatches and implants into the wall of the uterus.[1,5,9] In the mouse, the ZP is synthesized exclusively by growing oocytes in the course of 2 to 3 weeks. During this period, oocytes increase in diameter from ~15 to ~80 μm and are surrounded by multiple layers of granulosa cells, giving rise to progressively larger

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follicles. Bidirectional communication is essential for the development of both granulosa cells and the oocyte, and involves formation of gap junctions between granulosa cell processes and egg microvilli that traverse the ZP.[5] Containing ~3.5 ng of protein (>10% of total egg protein), the mouse ZP consists of ~2- to 3-μm-long filaments that have a structural repeat of ~150 Å and are cross-linked into a highly porous, 6.5-μm-thick elastic network.[2,5] This is composed of three highly glycosylated proteins: ZP1 (~200 kd; disulfide-bonded homodimer), ZP2 (~120 kd; monomer), and ZP3 (~83 kd; monomer). The three subunits are coordinately synthesized as precursors that share a common molecular organization with four main features (Fig. 2A): (1) an N-terminal secretory signal peptide (SP); (2) the ZP domain, a conserved sequence of ~260 amino acids, including 8 or 10 invariant Cys residues that adopt two alternative disulfide bond connectivities (Fig. 2B)[10–13]; (3) a recognition site for members of the proprotein convertase family of proteolytic enzymes (consensus furin cleavage site [CFCS]); and (4) a C-terminal propeptide (CTP) including a single-spanning transmembrane (TM) domain.

Recent studies suggest that these elements play crucial roles in secretion and assembly of ZP subunits.[14–18] Following cleavage of the SP, ZP precursors are transported through the endoplasmic reticulum and the Golgi, while remaining bound to the membrane of these cellular compartments via the TM domain in the CTP. In the endoplasmic reticulum/Golgi, ZP proteins form intramolecular and (in the case of mouse ZP1) intermolecular disulfide bonds, and they are modified with both N- and O-linked oligosaccharides. The membrane-anchored precursors are then packaged into very large (~2 μm diameter) vesicles, which fuse with the plasma membrane of the oocyte. At this stage, or possibly already within the trans-Golgi, ZP precursors are cleaved at the CFCS. This C-terminal processing depends on the TM domain and releases mature ZP proteins into the perivitelline space, where they incorporate into the inner layer of the growing ZP via their ZP domain. Other sequences appear to be responsible for the specific function of each subunit: cross-links between ZP filaments are believed to be established by the trefoil domain-containing N-terminal part of ZP1 (Fig. 2A), whereas regions N- and C-terminal to the ZP domain of ZP2 and ZP3, respectively, have been involved in egg–sperm interaction.[1,2,5]

According to the most widely accepted model of mammalian fertilization,[1,5,9,19–21] initial species-specific binding of sperm to the ZP—an event that takes place in the ampulla of the oviduct—relies on direct recognition of O-linked sugars located within a so-called sperm-combining site on ZP3 (Fig. 2A). ZP3 would then induce the acrosome reaction, a form of cellular exocytosis that allows sperm to penetrate the ZP while binding to the secondary receptor ZP2. Following fusion between gametes, modification of ZP3 carbohydrates and site-specific cleavage of the N-terminal region of ZP2 (Fig. 2A) would inactivate both receptors and cause structural alterations of the ZP, thus preventing the binding of additional sperm to the fertilized egg. Although this model continues to be supported by substantial experimental evidence,[9,22] recent work[12,23] has led to the proposal of an alternative hypothesis.[24] In this second model, sperm binding would be determined by the overall three-dimensional structure of the ZP, dependent on the cleavage status of ZP2. Clearly, additional studies are needed to establish firmly the molecular basis of egg–sperm interaction in mammals; nevertheless, it has been pointed out that at least part of the apparently contrasting data could be reconciled by postulating a different location of the essential O-linked sugars on ZP3.[25]

Most of our current knowledge on ZP glycoproteins is based on research with mice, but studies on other eutherian mammals suggest that their ZP, although variable in thickness (~2 to 20 μm, depending on the species), is highly similar to the mouse egg coat.[1,9,26] Moreover, marsupial oocytes are also surrounded by a thin ZP, the components of which are highly related to their eutherian counterparts.[27] Although the basic molecular features of the mouse ZP clearly are applicable to all mammals, the recent finding that rat and human ZP contain a fourth ZP1-like subunit (ZP4), which is not expressed in mice, suggests that the presence of additional subunits could introduce structural differences in the ZP of certain species.[4,6,7]

Avian VE

The VE of bird eggs consists of two layers that differ in both composition and structure, and are separated by a thin continuous membrane. The inner or perivitelline layer is a 1- to 3.5-μm-thick network of fibers that corresponds to the mammalian ZP. The perivitelline layer is responsible for species-specific binding of sperm and starts assembling around ovarian oocytes about a week before ovulation (Fig. 1B).[28,29] Newly ovulated eggs are fertilized within the infundibulum of the oviduct; subsequently, they acquire the continuous membrane and outer VE layer (0.1 to 0.5 and 3 to 8 μm thick, respectively), which are thought to be involved in the block to polyspermy that follows the acrosome reaction.[30] The albumen, as well as inner and outer shell membranes, are then added to fertilized oocytes as they progress further down the oviduct.

The avian perivitelline layer consists of several glycoproteins: homologues of ZP3 and ZP1 have been characterized in both hen and quail, and genes for

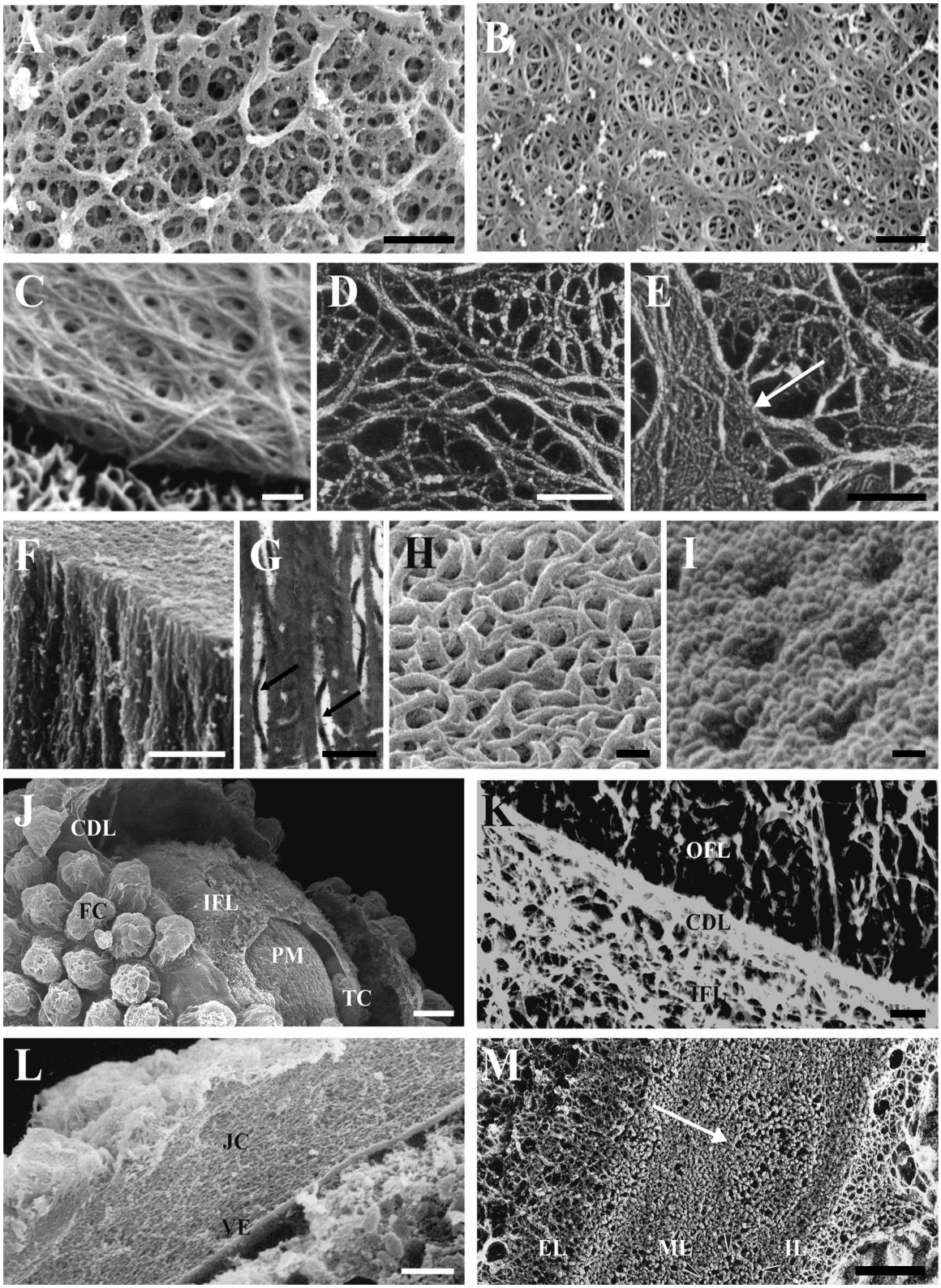

Figure 1 Ultrastructural analysis of egg coats from representative organisms by scanning and transmission electron microscopy (SEM/TEM). (A) Mammalian zona pellucida (ZP; human, SEM). (B) Avian perivitelline layer (chicken, SEM). (From Takeuchi Y, Cho R, Iwata Y, et al. Morphological and biochemical changes of isolated chicken egg-envelope during sperm penetration: degradation of the 97-kilodalton glycoprotein is involved in sperm-driven hole formation on the egg-envelope. Biol Reprod 2001;64:822–830.) (C to E) Amphibian coelomic envelope, vitelline envelope (VE), and fertilization envelope (*Xenopus*, SEM). The oocyte extracellular matrix is modified from coelomic envelope (C) to VE (D) and, finally, to fertilization envelope (E) (D and E from Larabell CA, Chandler DE. Stepwise transformation of the vitelline envelope of *Xenopus* eggs at activation: a quick-freeze, deep-etch analysis. Dev Biol 1990;139:263–268).

additional putative subunits have also been identified within the chicken genome.[3] Like its mammalian counterpart, mature avian ZP3 (~34 to 42 kd) originates from cleavage of a membrane-bound precursor at the CFCS; however, in birds the protein is secreted by the apical surface of testosterone-stimulated granulosa cells that surround the oocyte within the ovary.[31–34] Bird ZP1 exists both as a ~97-kd monomer and a homodimer held together by disulfide bonds. The protein includes a glutenin-like Pro/Gln-rich repeat region N-terminal to the trefoil domain, and its precursor, which is secreted by the liver in response to estrogens, is characterized by a short CTP lacking a TM domain.[35,36] A third subunit of chicken VE, called ZPD, has been described recently that consists of a SP, an epidermal growth factor (EGF) domain, a type II ZP domain, a CFCS, and a TM domain. This ~42-kd protein is synthesized by granulosa cells, appears to be loosely associated with the perivitelline layer and, together with dimeric ZP1, has been implicated in sperm activation.[37] Upon binding of sperm, avian ZP1 is degraded into ~35- to 50-kd fragments and hole-like structures appear on the perivitelline layer[38]; subsequently, during oviductal transport of fertilized eggs, a trypsin activity is believed to hydrolyze a short sequence at the N-terminus of ZP3, the carbohydrate components of which also appear to be modified.[39]

Amphibian VE

Amphibians are divided into anurans (with external fertilization in water) and urodels (with internal fertilization in the female cloaca).[40] The egg of *Xenopus laevis* is the most investigated model system for anurans, but *Xenopus tropicalis*, *Discoglossus pictus*, and *Bufo arenarum* have also been studied to some extent and found to be similar.

From ovulation until after fertilization, the amphibian egg coat undergoes a series of complex physicochemical and functional transformations (Figs. 1C to 1E).[41,42] Newly ovulated oocytes are surrounded by a coelomic envelope that consists of interconnected fascicular bundles; this structure has no sperm-binding activity and is linked to microvillar projections of the plasma membrane through a dense network of fibers that span the perivitelline space (Fig. 1C). The egg becomes competent to bind sperm when it reaches the oviduct, where it acquires an outer ~1-mm-thick jelly coat and the coelomic envelope is converted into a ~1-μm-thick VE (Fig. 1D). This appears to contain two populations of loosely woven cable-like fibers of ~19 and ~12 nm diameters, which are randomly cross-linked by short 5-nm-diameter filaments. Upon fusion of the gametes, the whole egg coat is modified dramatically to prevent polyspermy; as part of this process, the VE is converted into a hardened fertilization envelope made up of concentric fibrous sheets (Fig. 1E).

In *Xenopus*, the VE consists of five main glycoproteins that are synthesized by the oocyte: ZP1 (gp37),[43] ZP2 (gp69/64),[44] ZP3 (gp43/41),[45] and two additional subunits called ZPAX (gp120/112)[46] and ZPD (gp80).[47] ZPAX has a ~600-amino-acid-long N-terminal domain, a short CTP without predicted TM domain, and is related to ZP2 because it has a type II ZP domain but no trefoil domain.[46] ZPD, on the other hand, has a minimal architecture (SP-ZP domain-CFCS-TM domain) and appears to constitute a subfamily of its own, given that its ZP domain sequence is most similar to type II ZP domains but lacks conserved Cys 6 and 8.[47] As a result of specific proteolytic events, some VE components have two apparent molecular weights. Conversion from coelomic envelope to VE involves both N- and C-terminal processing of ZP3 by the trypsin-like protease oviductin in the *pars recta* of the oviduct.[45,48] Thereby, carbohydrate chains on either ZP3 itself[49] or ZP2[50] that are responsible for the species-specific binding of sperm to the egg would become accessible. A secreted cortical granule metalloprotease then truncates the N-terminal 27 amino acids of ZP2, abolishing its sperm-binding activity and leading to the conversion of the VE into hardened fertilization envelope.[44,51,52] Finally, hatching of the embryo from the VE is dependent on a Zn^{2+} metalloprotease that specifically cleaves ZPAX.[46]

Little is known about fertilization in urodels other than the newt *Cynops pyrrhogaster*, the eggs of which can undergo polyspermy because they lack cortical granules, so that no fertilization envelope is formed during fertilization.[40] So far, only a homologue of ZP3 has been identified in this species.[53]

Perivitelline space fibers connect the coelomic envelope to oocyte microvilli, which can be seen at the bottom of (C). Upon activation, VE fibrils (D) merge into large sheets, indicated by the arrow in (E). (F to I) Fish VE (rainbow trout, SEM/TEM). Cross-sections of chorion are visualized by SEM (F) and TEM (G); note how VE protein bundles are perforated by microvilli projecting from the oocyte (G, arrows). Fine details of the inner and outer surface of the VE are shown by SEM in (H) and (I), respectively. (J, K) Ascidian VE (*Phallusia mammillata*, SEM/TEM). SEM overview of oocyte investments (J) and quick-freeze/deep-etch/rotary-shadow (QF/DE/RS) cross-section, highlighting the tripartite structure of the VE (K). CDL, central dense layer; FC, follicle cell; IFL, inner fibrous layer; OFL, outer fibrous layer; PM, plasma membrane; TC, test cell. (L, M) Molluscan VE (abalone, SEM/TEM). (From Mozingo NM, Vacquier VD, Chandler DE. Structural features of the abalone egg extracellular matrix and its role in gamete interaction during fertilization. Mol Repro Dev 1995;41:493–502). QF/DE/RS analysis reveals that the VE consists of three sublayers, with ovoid structures embedded within the thicker medial sublayer (M, arrow). EL, external sublayer; ML, medial sublayer; IL, internal sublayer. Scale bars are 0.2 μm (D, E, M); 0.25 μm (I); 0.5 μm (K); 1 μm (G, H); 2 μm (L); 5 μm (A, B, F); 10 μm (J); and 1 mm (C).

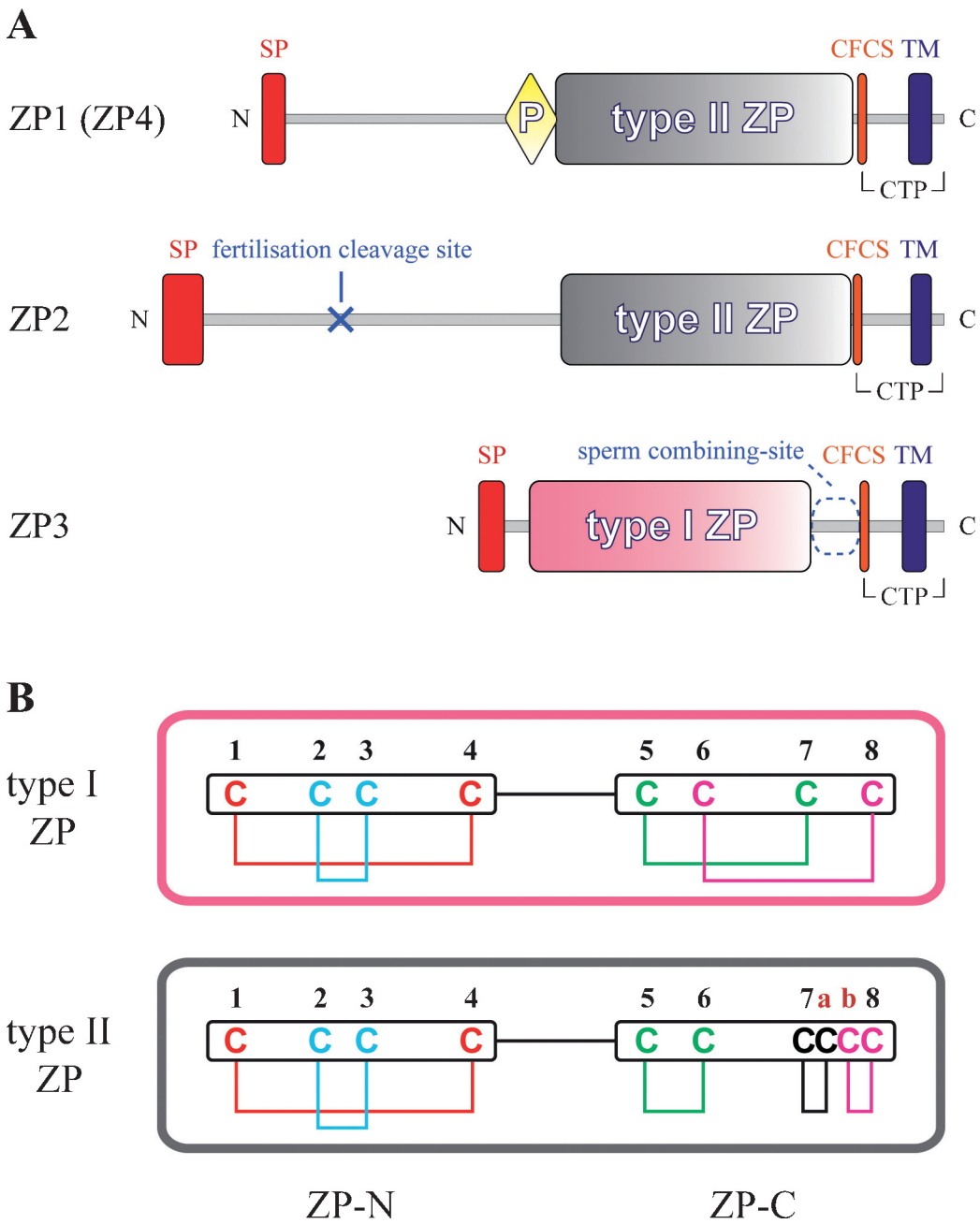

Figure 2 Architecture of mammalian zona pellucida (ZP) glycoproteins. (A) Domain structure of ZP1 to ZP3 precursors. Polypeptide sequences are represented by gray bars, drawn to scale, with N- and C-termini indicated. Signal peptide (SP), type I and type II ZP domains, consensus furin cleavage site (CFCS), and transmembrane (TM) domain are depicted as red, pink, gray, orange, and blue rectangles, respectively; the trefoil (P) domain of ZP1 is shown as a yellow rhombus, and the C-terminal propeptide (CTP) is marked by brackets. Regions of ZP2 and ZP3 important for fertilization are indicated in light blue. Subunit ZP4, which is not expressed in all mammals and has the same domain organization as ZP1, has been omitted. (B) The ZP domain consists of two subdomains, ZP-N and ZP-C. ZP-N contains conserved Cys 1 to 4, which have invariant 1-4, 2-3 disulfide connectivity in all ZP proteins. On the other hand, ZP-C can contain either 4 or 6 Cys, resulting in two different connectivities in type I (ZP3) and type II (ZP1/ZP2/ZP4) ZP domains.

Fish VE

The extreme diversity of teleost fish, which constitute almost half of the total number of vertebrates, is reflected in the morphology of the egg coat surrounding their oocytes. The fish VE (also referred to as chorion or zona radiata) varies in thickness, structure, and number of layers not only between, but also within species; however, in all cases a wave-shaped fibrillar component embedded within an amorphous matrix can be recognized.[54] Growing fish oocytes are surrounded by a single

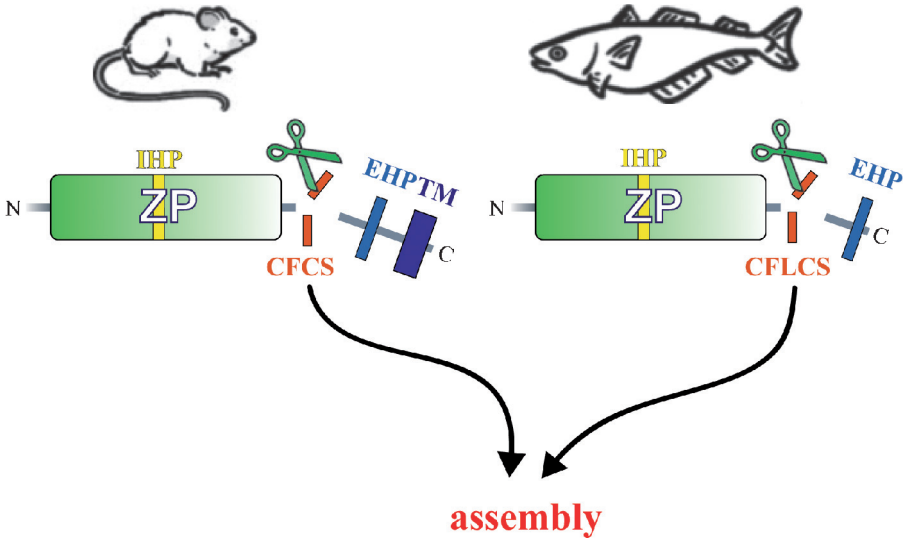

Figure 3 A common mechanism regulates the assembly of zona pellucida (ZP) domain egg coat proteins. Regardless of whether they contain a transmembrane (TM) domain or not, ZP/vitelline envelope (VE) precursors are always cleaved at the consensus furin cleavage site (CFCS)/consensus furin-like cleavage site (CFLCS), dissociating from the external hydrophobic patch (EHP) either immediately before or while they incorporate into the inner layer of the coat. Assembly is dependent on the ZP domain and involves the internal hydrophobic patch (IHP) motif located between its two subdomains.

layer of follicle cells that are separated from a thick outer layer of thecal cells by a basal lamina. In response to several external signals, follicle cells induce the synthesis of both egg yolk precursor (vitellogenin) and VE precursors by producing 17β-estradiol.[55] In the majority of teleosts, soluble VE precursors are secreted by the hepatocytes and travel in the blood to the oocyte, where they are incorporated into the chorion.[56,57] Deposition of electron-dense precursor material can first be observed in the perivitelline space, at the base of long microvilli that stretch from the plasma membrane of the oocyte to the follicle cells. Assembly of the VE then continues throughout the vitellogenic growth of the oocyte, the volume of which expands by accumulating considerable amounts of egg yolk, and eventually gives rise to a radially striated structure in which large helicoidal glycoprotein bundles are separated by extended microvilli (Figs. 1F and 1G).

The VE of the salmonid *Oncorhynchus mykiss* (rainbow trout) is one of the simplest so far described, consisting of a very thin outer layer and a ~50-μm-thick inner layer made up of only three major subunits (Figs. 1F to 1I).[58,59] Like avian ZP1, its components VEα (~58 kd), VEβ (~52 kd), and VEγ (~47 kd) are characterized by an N-terminal Pro/Gln-rich repeat region of variable length and a short CTP lacking a predicted TM domain. VEα and VEβ are highly similar in sequence and contain a trefoil domain immediately before of a type II ZP domain, whereas VEγ contains a sperm-combining site-like sequence C-terminal to its type I ZP domain. Thus, VEα and VEβ are related to mammalian ZP1/ZP4, whereas VEγ is a homologue of ZP3.[13,59,60] Analysis of the VE from other fish species has revealed a highly variable number of components (up to ~20 in the goldfish[61] and zebrafish[62]), that have been assigned to several different classes.[63,64] Nevertheless, all of these subunits appear to have the same basic structure as that of trout VE proteins, with the notable exception that in species such as carp, goldfish, and zebrafish, they are synthesized by the ovary, can lack a Pro/Gln-rich repeat region, and in a few cases also contain a predicted TM domain within their CTP.[65–67] Interestingly, a mixed situation is found in medaka, in which some subunits are synthesized by the liver and others by the oocyte.[63]

Recent studies in trout and medaka have shown that VE precursors are cleaved at a consensus furin-like cleavage site (CFLCS) upon reaching the egg, and that this event triggers the incorporation of the resulting mature proteins into the inner layer of the growing VE.[15,57,68–70] Furthermore, there is evidence suggesting that the chorion of trout eggs is assembled from VEα/γ and VEβ/γ heterodimers, although it remains to be established when these complexes are formed.[13,59,68] Teleost fish sperm generally lack an acrosome and reach the egg plasma membrane through the micropyle, a narrow channel that traverses the VE. The micropylar region is responsible for attracting sperm to the egg and its precise diameter prevents polyspermy by allowing the passage of only a single gamete from the same species; thus, fish VE proteins seem to have a purely structural function. After fertilization, the egg undergoes a cortical reaction and secretes a transglutaminase that hardens the chorion by introducing isopeptide cross-links between

VE subunits, most likely involving the Pro/Gln-rich repeats.[57,71] The hardened VE has a different morphology and plays a crucial role in protecting the developing embryo against environmental hazards as well as bacterial and fungal infections. As in amphibians, the hardened VE is eventually proteolyzed by choriolytic metalloproteases during hatching.[71]

Ascidian VE

With few exceptions, ascidians (familiarly called sea squirts) are hermaphroditic urochordates that produce self-sterile gametes because of a self-incompatibility system.[72,73] The plasma membrane of their 130- to 150-μm-diameter oocytes is in contact with groups of accessory test cells, surrounded by an acellular VE, the outer surface of which, in turn, is covered by a layer of follicle cells (Fig. 1J).[74] The ascidian VE is responsible for species-specific binding of sperm to the egg, plays a role in the slow block to polyspermy, and prevents self-fertilization in self-sterile species.[75–78] Assembly of the envelope starts shortly after the release of oocytes from the ovarian germinal strand, where they are associated with nongerminal cells. Initially, fluffy fibrillar patches of VE precursor material are found at the surface of the oocyte; subsequently, enlargement and merging of the patches give rise to a loose meshwork of 3-nm fibrils that completely surround the growing gamete.[79] The 0.4- to 1.2-μm-thick VE of mature ascidian oocytes consists of three layers: an outer dense or fibrous layer (up to 200 nm thick) made up of fibrils 15 to 30 nm in diameter arranged into a porous matrix; a compact, central dense layer (40 to 60 nm); and an inner fibrous layer (400 to 900 nm), the 15- to 85-nm diameter fibers of which are mostly oriented parallel to the plasma membrane (Fig. 1K).[74] No inner fibrous layer is found in the VE of *Ciona intestinalis*, the outer fibrous layer and central dense layer of which are penetrated by microvilli originating from the follicle cells and the egg, respectively.[79] Nevertheless, freeze-substitution studies showed that test cells within the perivitelline space of eggs from all ascidians are embedded into a system of interconnected fibers, denominated the perivitelline fibrous matrix.[74]

Partial solubilization of ascidian VEs revealed that they contain multiple glycoproteins,[80–82] but the nature of these subunits remained obscure until a gonad-specific gene encoding the precursor of the major component of *Halocynthia roretzi* VE was cloned.[83,84] HrVC120 consists of a SP, 12 EGF-like repeats and a type II-like ZP domain, followed by a CFCS and a TM domain. After removal of the SP and, presumably, processing at CFCS, the secreted protein is further cleaved C-terminal to the last repeat by a trypsin-like protease. The resulting 70-kd fragment, containing the 12 EGF repeats, has sperm receptor activity and appears to be specifically degraded by the ubiquitin/proteasome system of *Halocynthia* sperm during fertilization, allowing penetration of the VE.[83,85] Sequence analysis of HrVC70 from different individuals,[72] as well as cloning of the VC120 homologue from *Halocynthia aurantium*,[12] strongly suggest that the protein is a highly polymorphic allorecognition molecule responsible for the self-sterility of mature ascidian oocytes.

Molluscan VE

Marine invertebrates have long been used as a model system for the study of fertilization. Most of the work has focused on the large gastropod abalone (genus *Haliotis*), the eggs of which are fertilized externally in a species-specific manner. The extracellular coat of abalone eggs consists of three layers: an outer 5-μm-thick spongy jelly coat matrix made up of fibers with variable diameter, a central 0.6-μm-thick compact VE, and a thin fibrous egg surface coat, the filaments of which appear to contact directly the plasma membrane (Fig. 1L).[86] The VE, in turn, has a tripartite structure: a 0.4-μm-thick medial sublayer, consisting of uniform fibers that are tightly woven and appear to contain ovoid inclusions, is flanked on both sides by thin internal and external sublayers (Fig. 1M). The acid-liable jelly coat is believed to play a role in attracting sperm to the egg, and the VE is responsible for the species-specificity of gamete interaction and triggers the sperm acrosome reaction. The exocytic event releases lysin, a dimeric 16-kd basic protein that becomes monomeric upon binding to a $\sim 10^6$ d acidic glycoprotein constituting at least 30% of VE. This receptor for lysin (VERL) forms polymers that have a diameter of 13 nm and are held together primarily by hydrogen bonds. High-affinity, species-specific binding of ~60 lysin molecules per receptor causes VERL filaments to unravel nonenzymatically, creating a hole that allows sperm to penetrate the VE and reach the oocyte plasma membrane.[87]

Cloning of a full-length cDNA for VERL revealed that the protein consists of a SP, an array of 22 tandem repeats of a ~153 amino acid sequence, a type II ZP domain, a CFCS, and a TM domain.[8] As a result of strong adaptive evolution, the sequence of lysin homologues from different abalone species is extremely divergent[88]; similarly, it was recently shown that (unlike repeats 3 to 22, which evolve neutrally and are homogenized by concerted evolution[89]) VERL repeats 1 and 2 are significantly different and subjected to positive Darwinian selection.[90] At least five additional glycoprotein subunits are found within the VE of abalone,[87,91] and molecular characterization of two ~40-kd glycosylated VE components of the genus *Tegula* (vcp41/2) indicated that they both contain a SP, followed by a type II ZP domain, a CFCS, and a short CTP with no predicted TM domain. Moreover, two distinct acid polysaccharide complexes have also been identified in *Tegula* VE.[92]

BUILDING THE EGG COAT: ZP DOMAIN-MEDIATED ASSEMBLY OF ZP/VE PROTEINS

As emphasized by the finding that recombinant mouse ZP subunits can incorporate into the VE of *Xenopus* oocytes,[93] the basic molecular structure of egg coat proteins has been conserved through millions of years of evolution. Furthermore, the ZP domain signature shared by all ZP/VE subunits has also been recognized in hundreds of other extracellular proteins from nematodes to human, with a very broad range of biological functions.[1,10,11] Although most of these systems have not been studied in the same detail as the mammalian ZP, a common overall picture has started to emerge from the combination of these diverse sources of information.[11]

As first suggested on the basis of its conservation within different proteins that formed filaments or matrices,[94] the ZP domain has been shown to function as a polymerization module.[14] Furthermore, recent data indicate that the ZP domain consists of two subdomains (Fig. 2B),[11,15] the N-terminal of which (ZP-N) appears to be mainly responsible for the polymerization activity.[95] ZP domain proteins have a highly modular architecture, with a variable number and different combinations of additional domains in front of a single ZP domain close to the C-terminus[1,10,11]; nevertheless, filaments formed by different ZP domain proteins appear to have a similar overall three-dimensional structure.[14] Thus, it is believed that ZP domain polymers constitute a common molecular framework onto which additional domains are grafted that give to each protein its unique biological function.[11] As should be clear from the previous section, such a modular organization is particularly evident within VE/ZP subunits, which evolved very different architectures to carry out their specific roles in fertilization. Furthermore, fusion of reporter proteins N-terminal to the ZP domain of mouse ZP components was found not to hinder their incorporation into the ZP[96] (L.J. and P.M. Wassarman, unpublished data, 2002).

Proteins that assemble in the extracellular space must develop mechanisms to prevent their premature association within the cell. Precursors of ZP domain proteins are locked into a soluble, nonpolymerization competent state by two short conserved motifs: an external hydrophobic patch (EHP), located in the CTP between the CFCS/CFCLS and (if present) the TM domain; and an internal hydrophobic patch (IHP), positioned between ZP-N and ZP-C (Fig. 3).[15] Cleavage of the precursors at the CFCS/CFLCS, an event that is required for secretion of mammalian ZP proteins[18] and incorporation of both fish and mammalian subunits into the inner layer of the growing VE/ZP,[16,17,70] dissociates mature polypeptides from the EHP, thus activating them for polymerization (Fig. 3).[15] Therefore, although fish VE and mammalian ZP proteins differ in their site of synthesis and CTP architecture, they share a common assembly mechanism. Indeed, because it relies on highly conserved features (presence of EHP/IHP and cleavage at the CFCS/CFLCS), this mechanism most likely applies to ZP domain proteins in general.[11,15]

Ultrastructural and biophysical analyses of VE/ZP from different organisms suggest that they all consist of filaments of comparable dimensions that are rich in β-structure[11,97,98]; however, the way in which subunits are organized within these polymers is unclear. Biochemical, electron microscopy, and gene knockout studies point to a model in which filaments are constituted by a linear repetition of ZP2/ZP3 heterodimers, with the interface between ZP2 and ZP3 running perpendicular to the axis of the filaments (Fig. 4A).[2,5,24] However, an alternative arrangement could in principle also be possible, in which filaments are generated by association of heterodimers with the interface between subunits running parallel to the axis of the filaments (Figs. 4B and 4C). In both models, filament formation would be dependent strictly on the interaction between type I and type II ZP domain proteins, possibly through their different ZP-C subdomains (Fig. 2B). Although this clearly is the case for the mammalian ZP and related egg coats, it raises the question of how different ZP domain filaments, which consist of single type II subunits (such as, for example, Tamm-Horsfall protein[11]), are assembled. Perhaps, in such cases, the connectivity of ZP domain disulfides is altered within a fraction of the molecules, which can then mimic the missing type I subunit.[11,13] This would be consistent with the idea that type I ZP domain proteins evolved, by gene duplication and loss of Cys a and b (Fig. 2B), from a 10-Cys ZP domain ancestor that also originated the more ancient type II proteins.[11] Alternatively, it is possible that each type of ZP domain protein is able to homopolymerize into protofilaments, but that, in the case of the ZP, stable filaments are only formed upon side-by-side association of type I and type II protofilaments (Fig. 4D). Interestingly, a similar final arrangement (Fig. 4C) would eventually result from the different pathways depicted in Figs. 4B and 4D.

Whatever is the architecture of individual filaments, several lines of evidence indicate that subunit ZP1 is responsible for cross-linking them into a three-dimensional matrix.[99,100] As mentioned, this is believed to be mediated by the N-terminal region of the protein, which most likely assembles into the filaments through its type II ZP domain. In the mammalian ZP, where it is expressed at much lower levels than the other subunits, ZP1 (and, if present, ZP4) would be incorporated only occasionally, in place of a ZP2 subunit (Fig. 4A). On the other hand, lack of a ZP2 homologue and presence of two or more ZP1-like molecules, at least one of which is always highly expressed (such as VEβ in *O. mykiss*[59]),

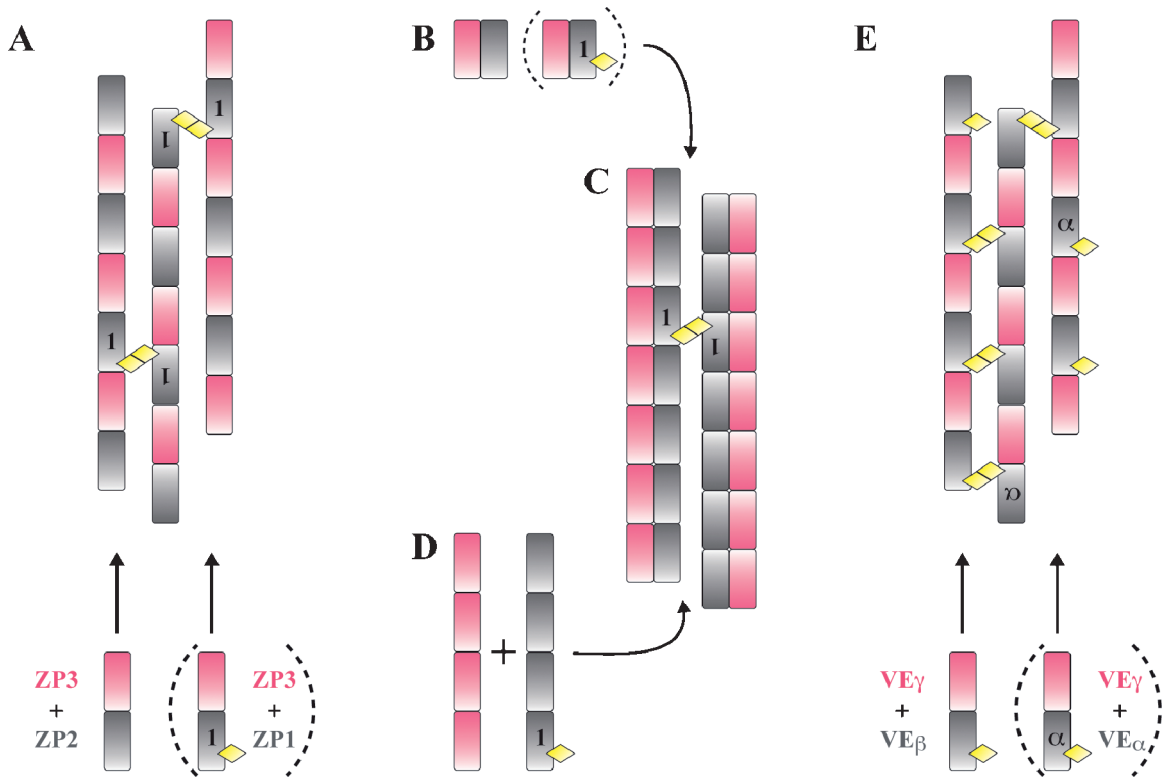

Figure 4 Possible models of egg coat assembly. (A) In the classical model of the mammalian zona pellucida (ZP), filaments are constituted by alternating ZP2 and ZP3 subunits, which are believed to be incorporated as heterodimers. (B to D) An alternative subunit arrangement (C) could result from a different interface within ZP2/ZP3 heterodimers (B), or if filaments were generated by lateral interaction between protofilaments consisting of only ZP2 or ZP3 (D). In both cases, cross-linking of individual filaments is mediated by pairs of the low expressed ZP1 subunit, incorporated stochastically under the form of ZP1/ZP3 heterodimers (A, C). (E) Model of trout vitelline envelope (VE), with ZP1-like subunits VEβ and VEα establishing a large number of interconnections between filaments. The relative orientation of filaments is entirely hypothetical. ZP proteins are depicted using the same color code as their respective ZP domains in Fig. 2A, with yellow rhombi representing the N-terminal region of mammalian ZP1 and fish VEα and VEβ. Low abundant ZP3/ZP1 and VEγ/VEα heterodimers are enclosed by brackets; ZP1 and VEα subunits within filaments are labeled 1 and α, respectively.

would result in the introduction of a much larger number of cross-links in the VE of fish (Fig. 4E). This could explain the remarkable resistance to mechanical and chemical stress displayed by fish egg coats, even before they harden. These considerations suggest that transcriptional control of ZP1-like proteins plays an important role in determining the specific properties of egg coats, by varying the number of cross-links that they contain.[11] In agreement with this view, formation of additional intra- and intermolecular disulfide bonds has been implicated in hardening of the mammalian ZP.[101]

FINAL COMMENTS

Mutations affecting the interface between gametes can generate barriers to fertilization that ultimately lead to speciation, and egg coat proteins from mollusc to human have been shown to diverge rapidly in response to adaptive evolution.[90,102] At the same time, anomalies in the structure and the thickness of the ZP have long been implicated in reduced reproductive fitness,[103–105] and a high degree of sequence variation was found recently in the ZP1 and ZP3 genes of infertile women.[106] It is hoped that the studies summarized in this review, together with parallel research on other ZP domain proteins,[11] will constitute a solid foundation on which to build to increase our understanding of the role played by the ZP in both health and disease.

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