

Three-dimensional structure of the zona pellucida

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The zona pellucida is the extracellular coat that surrounds the mammalian oocyte. It forms a spherical shell of remarkably uniform thickness (5–10 μm in eutherian mammals). The mouse is currently the largest source of data on the zona pellucida and this review is built largely on these data. The zona pellucida is composed of three proteins in both mice and humans: ZP1, ZP2 and ZP3. These proteins are glycosylated and, in mice, have mature relative molecular masses of 200 000, 120 000 and 83 000, respectively. ZP1 is a dimer of two apparently identical subunits. All three mouse proteins have been sequenced and possess transmembrane domains at their C-terminal ends coupled with furin cleavage sites immediately upstream. Sequence data have been used to provide an accurate assessment of the mole ratios of the three proteins. The ratio of ZP2:ZP3 is close to 1:1, whereas ZP1 is approximately 9% of the combined mole amounts of ZP2 and ZP3. Ultrastructural evidence suggests that the mouse zona pellucida is composed of filaments constructed by head-to-tail association of globular proteins. The coordinate synthesis of the three zona pellucida proteins coupled with the near 1:1 stoichiometry of ZP2 and ZP3 is consistent with a model in which ZP2–ZP3 heterodimers are the basic repeating units of the filament, with cross-linking of filaments by dimeric ZP1. This model is also consistent with data from ZP2 and ZP3 gene knockout and antisense experiments. However, the structure remains unproven. The small amount of ZP1 relative to ZP2 and ZP3 may have important implications for the distribution of ZP1 cross-links, since the number of cross-linking sites potentially exceeds the number of ZP1 dimer molecules by a considerable margin. The evidence that ZP1, ZP2 and ZP3 are all synthesized via a membrane-bound step is discussed and two models are proposed for the assembly of the zona pellucida. The cortical reaction and its effect on the zona pellucida are examined in detail. It is shown that the amount of material released by cortical granules could be of the order of 30% by mass of ZP1, and that if this material was distributed predominantly on the inner face of the zona pellucida, its local concentration could approach that of ZP1. A model in which the zona block to polyspermy is caused by direct titration of zona pellucida binding sites is suggested as an alternative to the explanation that relies on enzyme cleavage of ZP2 to ZP2_f. Finally, some of the major experimental and structural issues that remain to be addressed are identified.

The zona pellucida is the spherical glycoprotein shell that surrounds the mammalian oocyte at fertilization. Penetration of this shell by spermatozoa plays a crucial role in mammalian fertilization and any inability of spermatozoa to penetrate the zona pellucida inevitably leads to infertility. The purpose of this review is to summarize what we know or can infer about the three-dimensional structure of the zona pellucida, its construction and its properties as a polymer. Because the focus of the review is on three-dimensional structure, it relies heavily on data from mice. Features of the zona pellucida are discussed and two models for its construction are considered. The cortical reaction and its possible effects are discussed and avenues for future experimental work are identified.

Molecular composition of the zona pellucida

Most of the structural information about the mammalian zona pellucida is derived from mice (Wassarman, 1988, 1990; Wassarman and Mortillo, 1991; Dean, 1992). However, significant

progress has been made in elucidating the structure of zona pellucida proteins in other mammals (Sacco *et al.*, 1989; Kinloch *et al.*, 1990; Moller *et al.*, 1990; Schwoebel *et al.*, 1991; Lee *et al.*, 1993; Yurewicz *et al.*, 1993; van Duin *et al.*, 1993; Harris *et al.*, 1994; Hasegawa *et al.*, 1994; Taya *et al.*, 1995). The zona pellucida of mice is composed of three proteins: ZP1, ZP2 and ZP3, all of which are sulfated glycoproteins. Collectively, ZP1, ZP2 and ZP3 make up at least 95% of the total zona protein (Wassarman, 1988). The three proteins have relative molecular masses (M_r) of 200 000, 120 000 and 83 000, respectively (Bleil and Wassarman, 1980). ZP2 and ZP3 are monomers but, in mice, ZP1 is a dimer that can be cleaved into apparently identical subunits by elastase or disulfide bond reduction. Since all three proteins are sulfated and heavily glycosylated, the masses of the protein cores are considerably lower than their overall relative molecular masses. Genomic or cDNA clones have been sequenced for the three proteins (Kinloch *et al.*, 1988; Ringuette *et al.*, 1988; Liang *et al.*, 1990; Epifano *et al.*, 1995a) and these give M_r values for secreted protein cores (minus the signal

sequences) of 69 679, 76 373 and 43 943, respectively. All three proteins possess transmembrane domains at their C-terminal ends coupled with furin cleavage sites immediately upstream (Yurewicz *et al.*, 1993; Epifano *et al.*, 1995a). The precise significance of these two features is unclear but secretion of the zona pellucida proteins via a membrane route may be fundamental for its construction. Furin is a protease associated with the cleavage of constitutively secreted proteins (Hosaka *et al.*, 1991), of which all three zona pellucida proteins are examples (Epifano *et al.*, 1995a). The reduction in M_r on furin cleavage can be calculated from DNA sequence data because the furin cleavage site can be identified by inspection (Arg-X-Lys/Arg-Arg; see Hosaka *et al.*, 1991). Furin cleavage gives M_r values for the protein core of cleaved ZP1, ZP2 and ZP3 of 61 700, 68 134 and 36 069, respectively.

ZP1 has six potential N-linked glycosylation sites; ZP2 has seven, and ZP3 has six (Ringuette *et al.*, 1988; Liang *et al.*, 1990; Epifano *et al.*, 1995a). All three possess a large number of potential O-glycosylation sites. Although the sequences of ZP2 and ZP3 were originally considered to be unrelated, more recent analysis has indicated that they share a large common domain with each other and with a number of other proteins (Bork and Sander, 1992; discussed in Epifano *et al.*, 1995b). The C-terminal half of rabbit rc55 (ZP1) is similar to ZP2 (Schwoebel *et al.*, 1991; Epifano *et al.*, 1995b) and the rabbit homologue of ZP3 shares a carbohydrate recognition domain with a number of other proteins (Bork, 1993).

Stoichiometry of the zona pellucida components

The stoichiometry of the three constituents of the mouse zona pellucida is fundamental to any model of zona pellucida construction. There is approximately 5 ng of protein in the mouse zona pellucida, based on labelling with either dinitrofluorobenzene (4.8 ng protein) or dansyl chloride (5 ng protein) using bovine serum albumin as a standard (Bleil and Wassarman, 1980). Since both labelling reagents react with amino groups, the consistency is perhaps not surprising. The dry mass of the mouse zona pellucida is approximately 6 ng (Loewenstein and Cohen, 1964). There is a discrepancy here since approximately 5 ng of zona protein should give a dry mass of 7.9–9.5 ng when the carbohydrate is taken into account. Alternatively, for a dry mass of 6 ng, the protein content could be as little as 3.2 ng. These figures can be used to calculate the absolute quantities of the three zona pellucida proteins present in each oocyte, and are referred to later.

There are three sources of data that can be used to derive the mole ratios of ZP1, ZP2 and ZP3. The first source is data obtained from Coomassie brilliant blue staining of polyacrylamide gels (Bleil and Wassarman, 1980). The second is the published OD₂₈₀ profile of an HPLC eluate derived from 30 000 mouse zonae pellucidae (Wassarman *et al.*, 1989). Finally, the amount of mRNA associated with the synthesis of ZP1, ZP2 and ZP3 is known (Epifano *et al.*, 1995a) and by making the assumption that the zona pellucida proteins are synthesized in proportion to their mRNA, a mole ratio for the three proteins can be obtained. (The mole ratios of the three mRNAs is 1:4:4; see Epifano *et al.*, (1995a)). Of the three sets of data, the most reliable by far would be that derived from the HPLC data of Wassarman *et al.* (1989) because, in principle, there is no

distortion between measurement of the areas under the curves and the conversion to mole ratios (although there is some slight uncertainty associated with the areas under the curves because of their incomplete separation). The gel staining with Coomassie blue makes assumptions about the uniformity of dye uptake and the linearity of the relationship between staining density and the amount of protein. The mole ratios of protein derived from the amounts of mRNA make assumptions about rates of translation.

The HPLC data of Wassarman *et al.* (1989) can be used to give accurate mole ratios for ZP1, ZP2 and ZP3 using the extinction coefficients at 280 nm for tyrosine and tryptophan, since the amino acid sequences for the proteins and, therefore, their tryptophan and tyrosine contents, are known (Kinloch *et al.*, 1988; Ringuette *et al.*, 1988; Liang *et al.*, 1990; Epifano *et al.*, 1995a). The contents of tryptophan and tyrosine for secreted and subsequently furin-cleaved proteins are: ZP1, 8 and 20, and 5 and 19; ZP2, 7 and 26, and 7 and 24; and ZP3, 6 and 5, and 5 and 4, respectively. Taking the molar extinction coefficients of tryptophan and tyrosine at 280 nm as 5.00×10^3 and 1.08×10^3 , respectively, the mole ratios of ZP1:ZP2:ZP3 for secreted and furin-cleaved proteins are 1:4.68:4.03 and 1:5.51:6.11. The ratios of ZP2:ZP3 are 1.16:1 and 0.90:1, respectively. Note that the ratios for ZP2:ZP3 reverse on furin cleavage. This largely reflects the loss of a tryptophan on furin cleavage of ZP3. Note also that we do not know whether zona pellucida proteins in intact zonae pellucidae are actually furin-cleaved or not.

Two important points emerge immediately from these figures. The first is the near equimolar amounts of ZP2 and ZP3. The lack of complete 1:1 stoichiometry may reflect an actual departure or it may reflect the uncertainties of measuring the areas under the curve in the HPLC data of Wassarman *et al.* (1989). The second point of importance to emerge from these data is the relatively low proportion of ZP1. This is approximately 9% in mole terms of the combined amounts of ZP2 and ZP3. As discussed later, this may carry important consequences.

Assembly of the component parts of the zona pellucida

The current model of mouse zona pellucida structure is that proposed by Wassarman (1988; Fig. 1). It is derived from the conjunctive application of ultrastructural observation (Greve and Wassarman, 1985; Wassarman and Mortillo, 1991), which strongly suggests a filamentous structure for the zona pellucida, and quantification of ZP1, ZP2 and ZP3 by gel densitometry (Bleil and Wassarman, 1980).

There are two key features of the model. The first is the filament. Low power ultrastructural evidence suggests that the zona pellucida is filamentous in its basic construction (Dietl and Czuppon, 1984). Such filaments can be constructed either from extended polypeptide chains which interact with other extended chains along their lengths or globular proteins in head-to-tail attachment. Higher power ultrastructural data obtained by shadowing of isolated filaments suggest strongly that filaments are made up from head-to-tail attachment of globular proteins in single file (Greve and Wassarman, 1985). The micrographs in Greve and Wassarman (1985) were derived from filaments prepared from intact zonae pellucidae under conditions that cleave the ZP1 dimer into its constituent monomers. An

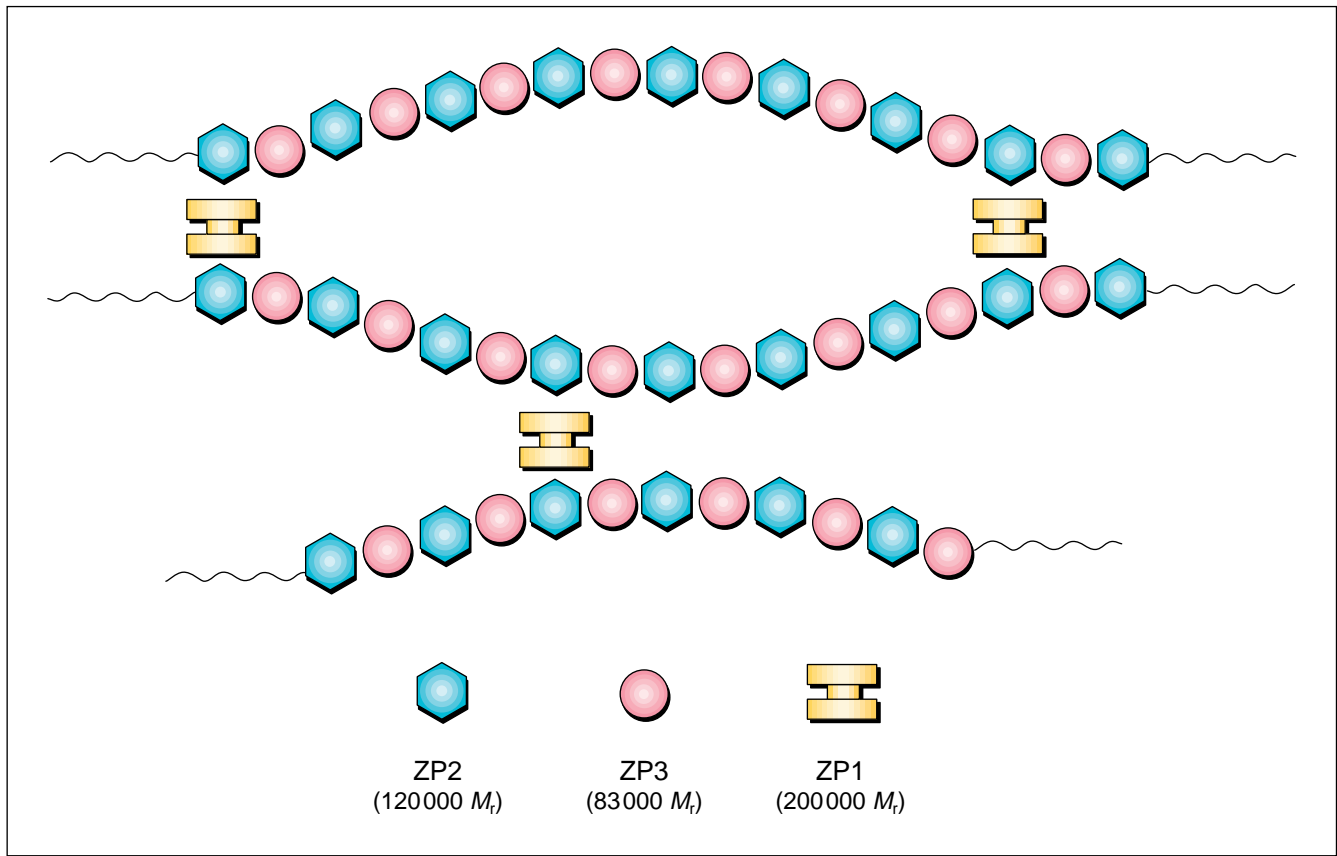


Fig. 1. The current model of the zona pellucida as proposed by Wassarman and redrawn from Wassarman (1988). Filaments are constructed of repeating ZP2–ZP3 units and are cross-linked by ZP1. Calculations of the ZP2:ZP3 ratio using recent sequence data confirm that it is close to 1:1. Although the model is consistent with experimental observations, formal proof that the zona pellucida is constructed in this way is still lacking. It is unclear whether ZP1 binding sites are present on every ZP2–ZP3 dimer and, if so, how ZP1 cross-links are actually distributed.

alternative method of filament preparation, in which ZP1 dimers are not cleaved, shows the apparent cross-linking of filament structures (Greve and Wassarman, 1985). The inference is that cross-linking of unbranched filaments is mediated by dimeric ZP1 units. This conclusion still seems justified.

The second key feature of Wassarman's model is that the filaments are made of repeating heterodimeric units of ZP2 and ZP3. There is no direct evidence that this is the case but the near equimolar amounts of ZP2 and ZP3, together with their co-ordinate synthesis (Epifano *et al.*, 1995a), is consistent with this view. The possible existence of the 1:1 stoichiometry for ZP2:ZP3 was not clear when Wassarman's model for the zona pellucida was first proposed, since the data then available derived from gel staining with Coomassie brilliant blue (Bleil and Wassarman, 1980). The possible 1:1 stoichiometry is supported strongly by the later data derived from protein sequences (Wassarman, 1988; Kinloch *et al.*, 1988; Ringuette *et al.*, 1988; Liang *et al.*, 1990; Epifano *et al.*, 1995a) when used in conjunction with the HPLC data. These two key features of Wassarman's original model have stood the test of time. Moreover, the model is consistent with recent experimental evidence. There is evidence that elimination of either ZP2 or ZP3 synthesis, either with antisense oligonucleotides or gene knockouts, prevents zona pellucida assembly (Tong *et al.*, 1995; Liu *et al.*, 1996;

Rankin *et al.*, 1996), indicating that both are required for synthesis of the complete three-dimensional zona pellucida structure. It is unknown at this stage where the failure of assembly occurs but if the basic filament unit is the ZP2–ZP3 heterodimer, then presumably the absence of either ZP2 or ZP3 aborts filament construction (Wassarman *et al.*, 1996).

It is important to consider the relatively small amount of ZP1 in mole terms (approximately 9%) when examining the stoichiometric ratios of ZP1, ZP2 and ZP3. At present, it is not known how ZP1 interacts with filaments of ZP2 and ZP3 but if ZP1 binding is based on precise recognition, as seems likely, then superficially there should be a considerable excess of ZP1 binding sites on the filaments. If ZP1 acts as a dimer joining two filaments of ZP2–ZP3 together in a 'clip', then the ratio of ZP1 to ZP2–ZP3 dimer should be about 1:5, leaving four vacant binding sites for every site occupied. Of course, it may not be as simple as this. It could be that ZP1 recognizes a carbohydrate complex on either ZP2 or ZP3 that is distributed more parsimoniously than ZP2 and ZP3 themselves. If this is the case, then the sites for ZP1 attachment might be no more numerous than the molecules of ZP1. However, if it is not the case, there is a potentially significant problem. If ZP1 binding sites on the filaments are more numerous than the number of ZP1 molecules (say, at the 5:1 ratio of ZP2–ZP3 dimer:ZP1

dimer that leaves four vacant ZP1 binding sites for every site occupied), then what determines the distribution of ZP1 cross-links along a filament? Are they distributed in an arrangement that reflects an equilibrium distribution? Or rather, are they 'frozen' into an otherwise, metastable distribution? At present, it is not known but, intuitively, it would seem likely that ZP1 molecules that are free to dissociate and reassociate will tend to redistribute and cluster in regions where there is already a cross-link. This would reflect the greater likelihood of finding adjacent filaments in close proximity to an existing ZP1-cross-linked site. Supposing that clustering of ZP1 cross-links is an outcome of allowing the zona pellucida to move toward an equilibrium structure, this means that longer tracts of uncross-linked filaments would also be present between clusters. Each ZP2–ZP3 unit has been estimated at 15–17 μm in length (Greve and Wassarman, 1985), and for a ratio of ZP1:ZP2–ZP3 dimer of 1:5, the mean length between attachment sites on each filament is approximately 75–85 nm. This would lengthen under clustering of ZP1 dimers to 100 nm or more. The zona pellucida may indeed be anisotropic at this level. However, if it is not, and if ZP1 cross-links have to be 'frozen in' to a non-clustered, metastable distribution, then there will be consequences. One is that the protein–protein or protein–carbohydrate interactions that stabilize the zona pellucida must possess long half-lives (days or weeks), otherwise dissociations will occur that would allow the zona pellucida to move from its metastable state to one closer to its equilibrium configuration. A second is that a 'frozen', metastable distribution of ZP1 cross-links in the zona pellucida might require careful assembly to avoid formation of ZP1 clusters.

The discussion of assembly has dealt with data on the mouse zona pellucida. Individual zona proteins are conserved in eutherian mammals and show evidence of common ancestry, both between the three basic classes of zona pellucida protein and also with related proteins in *Xenopus* and fish (Epifano *et al.*, 1995b; Hedrick, 1996). However, there are species differences between zona pellucida proteins. Two examples are the formation by human ZP2 of a disulfide bonded homodimer and the absence of disulfide bonding in the pig homologue of ZP1, ZP3a. These differences will ultimately require accommodation when more is known of zona pellucida structure.

Physical properties of the zona pellucida

The zona pellucida is essentially a sulfated glycoprotein gel of the order of 2–6% (w/v). Gels vary widely in their physical properties, in ways often reflecting their underlying chemical structure. One major structural difference between gels is between those that are covalently cross-linked, such as polyacrylamide, and those that are not, such as gelatin.

When covalently cross-linked polymers are stretched or deformed at relatively modest strains (that is, their extension is a few per cent of their total length), the simplest behaviour they show is elastic recoil. On removal of the load that is stretching them, they return to their original position. This is a direct consequence of the covalent structure of the polymer, in which, for small strains, no covalent bonds are broken. Their recovery may be time-dependent if there are additional viscous elements involved, but otherwise the polymer behaves as a spring and changes in strain are essentially instantaneous after application

of stress. A viscous element in series with the elastic element (a Maxwell body) allows the spring to extend instantaneously but also allows it to return slowly to its original extension. In doing so, the system as a whole loses its memory of its former position. This phenomenon is known as stress relaxation. A gelatin gel behaves as a Maxwell body. It is held together by non-covalent interactions with relatively long lifetimes. When it is lightly stretched and released immediately, it returns to its original position; that is, it behaves as a Hookean spring. When it is stretched and held for, say 20 min, before release, it does not return to its original position but only to some intermediate position. The longer the strain is applied, the smaller the relaxation when the stretching force is eliminated. At the molecular level, the non-covalent interactions in gelatin that hold the gel together persist over the lifetime of a short stretch, but when strain is applied for longer periods, the spontaneous dissociation of the non-covalent interactions allows the protein chains that carry the binding sites to recoil and establish new interactions nearer to themselves, eliminating the tension due to elastic extension.

When the zona pellucida is exposed to sodium dodecyl sulfate (SDS) denaturation it generates the three constituent proteins, ZP1, ZP2 and ZP3. In hamsters, denaturation of the zona pellucida with SDS causes complete dissolution of the zona pellucida without the use of reducing agents, and this dissociation apparently leaves no residual scaffolding, even after the cortical reaction (Green, 1989). Thus, the zona pellucida is, on the face of it, a wholly non-covalent gel like gelatin. The physical properties of the zona pellucida have not been studied extensively, but it behaves for practical purposes as an elastic solid over time periods of minutes and possibly much longer (Green, 1987). In addition, when oocytes with intact zonae pellucidae are subjected to vigorous pipetting, the zona pellucida can be induced to rupture and fragment without loss of spherical shape in the fragments. The shearing forces that cause rupture must involve considerable temporary distension of the zona pellucida but it is apparently elastic enough to recover. These considerations all suggest an elastic solid. It follows that the non-covalent interactions that hold the zona pellucida together probably have relatively long half-lives, and that the interactions are of high affinity. This is also suggested by observations that zonae pellucidae do not dissolve rapidly when free to do so (for example, when placed as cumulus-free oocytes in buffers). This does not demonstrate that zonae pellucidae are constructed in a metastable configuration; however, it does provide evidence that if they were constructed in such a configuration, then it could be maintained, possibly for a day or two.

This still leaves open the question of how stable the zona pellucida is in the long term and how much it could creep under load. Under normal conditions, is it under tensile load at all? The most likely source of load would appear to be an internal swelling pressure. Collectively, carbohydrate accounts for nearly half the zona pellucida by mass and this carbohydrate is either neutral or acidic (Mori *et al.*, 1991; Noguchi and Nakano, 1992; Noguchi *et al.*, 1992; Hirano *et al.*, 1993; Hokke *et al.*, 1993; Nakano *et al.*, 1996) and requires solvation. In addition, in any charged gel, there is the potential for mutual repulsion between charges. Mutual repulsion between charges can be masked by high ionic strength and, in a covalently

cross-linked gel, there is an equilibrium between any swelling pressure that the mutual repulsion generates and tension in the matrix. In a structure such as the zona pellucida, it might be predicted that low ionic strength would generate a swelling pressure by unmasking charge, particularly on carbohydrate, which could lead in time to irreversible swelling after an initial period of reversible stretching.

There is circumstantial evidence that a swelling pressure can be generated in zonae pellucidae at low ionic strength. Pig zonae pellucidae undergo spontaneous dissociation at 37°C at low ionic strength more rapidly than at high ionic strength (Takano *et al.*, 1989). When the dissociation at low ionic strength is prevented by lowering the temperature, the zona proteins undergo more extensive proteolysis on exposure to acrosin than zonae pellucidae in normal ionic strength solutions. This suggests that, at lower temperatures, tensioning of the zona pellucida filaments is occurring by gel swelling without actual dissociation of its constituent components, and that this tensioning is opening the gel and exposing sites for proteolytic cleavage. Both these observations are consistent with low ionic strength unmasking fixed charge and swelling the zona pellucida. Low ionic strength also reduces greatly the time needed for mouse zonae pellucidae to dissolve at 50°C considerably (Wassarman and Mortillo, 1991) and, again, this can be explained by an increased swelling pressure subjecting the non-covalent interactions of the filaments to greater tensile load, making it impossible for spontaneously dissociating interactions to recombine with their original partners during any re-association. The stability of zonae pellucidae in solutions of normal ionic strength suggests that any swelling pressure within the zona pellucida under these circumstances is either small or nonexistent, and long-term creep is likely to be small or nonexistent.

The conclusion from this part of the discussion is that the interactions between zona pellucida proteins have half-lives long enough to make the zona pellucida behave elastically. Whether the initial pattern of cross-linking established during zona pellucida synthesis persists for much, if not all, of the existence of the zona pellucida is still not clear.

Three-dimensional construction of the zona pellucida

There are two immediate challenges in seeking to describe the mechanism by which the three-dimensional structure of the zona pellucida is assembled: to understand why the zona pellucida is apparently produced from cleavage of the extracellular domains of membrane proteins and to explain the ultrastructural morphology of its synthesis.

There are three lines of evidence for a membrane-bound phase for the three zona pellucida proteins. First, it is clear from the DNA and inferred amino acid sequences of ZP1, ZP2 and ZP3 that all three carry hydrophobic domains that are potentially membrane spanning. These domains are all in the C-termini, leaving the bulk of each protein able to project from the membrane. Second, all three proteins carry potential furin cleavage sites adjacent to the hydrophobic C-terminal regions. This provides a possible mechanism for cleaving the extracellular domains of each zona pellucida protein away from its membrane stub. Furin cleavage would reduce each ZP protein M_r by about 8000. Third, one of the consequences of constructing *Zp3^{-/-}*

knockout mice (Rankin *et al.*, 1996) is that an extracellular orientation can be established for ZP1 and ZP2 by indirect immunofluorescence in the absence of zona pellucida assembly (Epifano *et al.*, 1995a).

Why should zona pellucida protein expression go through a membrane-bound route rather than secretion of soluble proteins? Any answer to this question is inevitably speculative. It is not clear where assembly of zona pellucida filaments (that is, ZP2 to ZP3 binding and ZP1 cross-linking) begins but there is the potential for it to start in the secretory pathway inside the oocyte (Wassarman *et al.*, 1996). If this is the case, then one problem the oocyte presumably has to avoid is extensive self-assembly of the zona pellucida within either the endoplasmic reticulum or Golgi apparatus, which could produce a polymerized matrix incapable of being packaged and exported. The oocyte also has to avoid the formation of granule cores of zona pellucida proteins which, when exported, are both condensed (through extensive protein cross-linking inside the granule) and incapable of fusing with other condensed cores of zona pellucida released from adjacent secretory granules. If they exist, these problems may be avoided if the zona pellucida begins its self-assembly during passage through the cell as two-dimensional, membrane-bound sheets. Another possibility is that the zona pellucida proteins are prevented from self-assembling at all when membrane-bound, delaying assembly until they are outside the cell.

The primordial follicle contains no zona pellucida and the oocyte is surrounded by thin granulosa cells. As the follicle develops, the surrounding granulosa cells multiply and establish extensive processes towards the oocyte. The zona pellucida is built in the cleft between the two. Despite considerable speculation about the origins of the zona pellucida, it has now been established unequivocally that all three zona pellucida proteins are synthesized exclusively by the oocyte in a co-ordinate manner (Epifano *et al.*, 1995a). There has been limited ultrastructural work on the synthesis of the zona pellucida and the clearest micrographs are those produced by Dietl (1989). Initially, the zona pellucida is assembled as isolated islands of material which presumably coalesce as they enlarge (Dietl, 1989). Epifano *et al.* (1995a) have shown that the oocyte is expanding during zona pellucida protein expression and continues to expand after expression has stopped. It appears that the oocyte both stretches the emerging zona pellucida after the individual islands of zona pellucida material have coalesced, and continues to deposit more material underneath the, by now, continuous layer of glycoprotein. The size of the oocyte when the coalescence of the zona pellucida occurs and, therefore, the degree of stretch that might subsequently be imposed, is unknown. The mature zona pellucida shows evidence of closer packing on its inner surface (Dietl, 1989), which may reflect the secretion of zona pellucida proteins into a mechanically compressed space under a stretched zona pellucida. However, the extent of any mechanical compression is unknown, and would depend upon the extent to which the already-formed zona pellucida can or cannot undergo stress relaxation as it is stretched.

Taken together, these observations suggest that the oocyte is the spherical mould upon which the zona pellucida is built and that it is the source of the remarkably uniform spherical shape of the zona pellucida. Once the zona pellucida is assembled, the oocyte shrinks to leave the perivitelline space between itself

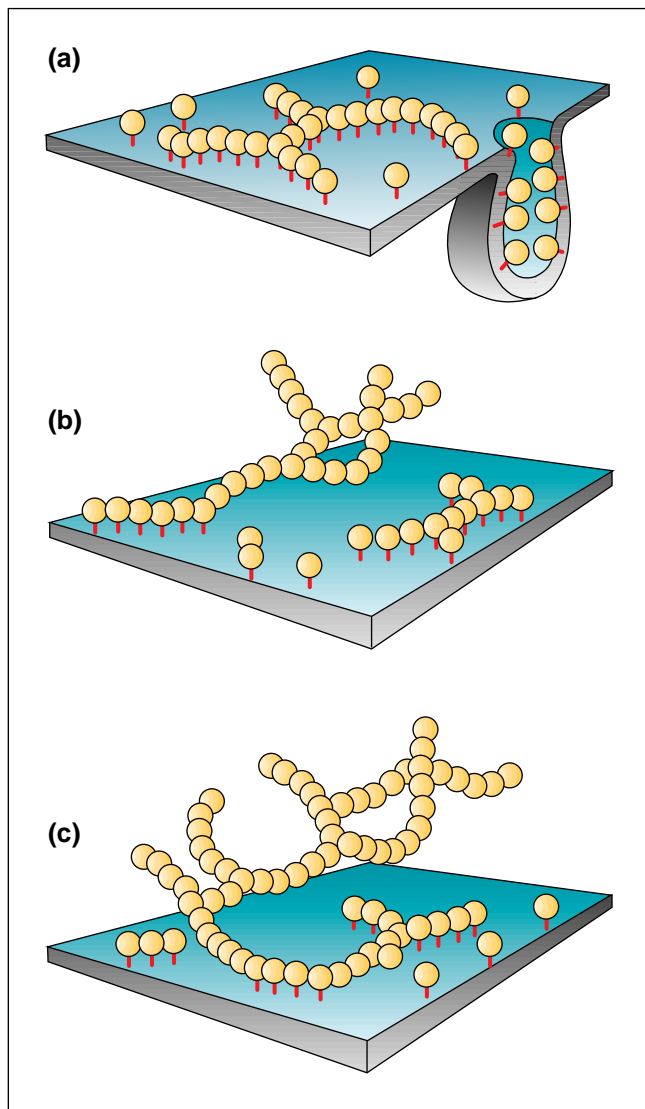


Fig. 2. A diagrammatic representation of one of two models proposed for the assembly of the zona pellucida. (a) Zona pellucida proteins are delivered to the plasma membrane of the oocyte by exocytosis. The bulk of each protein projects from the extracellular face of the plasma membrane on a short membrane stub. Synthesis of ZP1, ZP2 and ZP3 is co-ordinate and is assumed to pass through the same synthetic pathway. It is unclear to what extent prior assembly of zona pellucida takes place within the secretory granules but it is proposed that filament assembly takes place substantially on the surface of the oocyte. (b) Assembled filament is cleaved partially from the plasma membrane by the action of furin at the furin cleavage site adjacent to the membrane spanning region of each protein. Additional filament is being assembled. (c) Further filament has been cleaved away from the membrane. The model is seen as providing the basis for: (i) the emergence of 'islands' of zona pellucida early in its construction, (ii) the regulation of ZP1 distribution along filaments, and (iii) a membrane-bound intermediate in zona pellucida synthesis. In an alternative model, zona pellucida proteins arrive at the surface of the oocyte as in step (a), but are then cleaved off with little additional zona pellucida assembly, and condense into cross-linked filaments in the space between the oocyte and surrounding follicle cells.

and the newly formed zona pellucida and then undergoes first polar body emission. It is not known by how much the zona pellucida relaxes elastically as the oocyte shrinks, or even if it does so at all.

Two models for zona pellucida construction

Two models for zona pellucida construction will be considered. Both are inevitably speculative but will serve to focus attention on a number of key issues. These include the evidence for membrane-bound production of the three zona pellucida proteins, the apparent excess of ZP1 cross-linking sites on ZP2–ZP3 filaments (with its potential for clustering of ZP1 molecules), and the potential longevity of protein associations in the zona pellucida once it has formed. The two models propose different sites of assembly for the three-dimensional zona pellucida.

In the first model, the zona pellucida is constructed in its three-dimensional form in the cleft between oocyte and follicle cells. Soluble zona pellucida proteins are presented as membrane proteins on the extracellular face of the oocyte plasma membrane and deposited by proteolytic cleavage by furin (Rankin *et al.*, 1996). There may be some assembly of filaments in secretory granules before exocytosis (Wassarman *et al.*, 1996), but the three-dimensional cross-linking of the zona pellucida is established extracellularly, away from the oocyte surface. In this model, the reason for the membrane-bound expression of the three zona pellucida proteins may be that somehow it prevents premature, and potentially overly condensed, assembly of zona pellucida that cannot subsequently be assembled into a matrix. The model still has to explain the self-assembly of the islands of zona pellucida material seen in the early stages of extracellular assembly (Dietl, 1989), but it is possible that self-assembly occurs once nucleation of three-dimensional construction has started. The model has little difficulty in explaining how the gaps left by projections of the follicle cells onto the oocyte are filled, since soluble zona pellucida proteins could diffuse into the spaces and polymerize there. The model implies that the supernumerary ZP1 cross-linking sites on the ZP2–ZP3 filaments either do not exist or that, if they do, they are immaterial to the three-dimensional configuration of the zona pellucida.

In the second model (Fig. 2), the zona pellucida is assembled quite specifically on the surface of the oocyte. Sheets of two-dimensional zona pellucida are formed which are continuously but incompletely cleaved off before fresh zona pellucida membrane protein arrives. In this model, the membrane-bound expression is an integral part of zona pellucida construction. As in the first model, the membrane route may play a role in preventing premature self-assembly of three-dimensional zona pellucida but it may also play an important role in regulating the way in which ZP1 cross-links the filaments of ZP2–ZP3. This could be important if the zona pellucida was 'frozen' during construction into a metastable state. This second model has no difficulty in explaining the early appearance of islands of zona pellucida polymer, but has a greater problem in explaining the infilling required when the follicle cell projections onto the oocyte retreat. Clearly, considerably more work is needed to identify when and where zona pellucida assembly is initiated.

Features of these models have been discussed previously, notably the possibility of ZP2–ZP3 dimer formation within

secretory granules (Wassarman *et al.*, 1996) and the repetitive release of zona pellucida proteins from the oocyte surface (Rankin *et al.*, 1996). It should be noted that any cleavage of zona pellucida proteins from the membrane may not be mediated by furin, but rather a furin-like or hex-like endoprotease. Alternatively, since furin is normally associated with the trans-Golgi, it may be that detachment of the zona pellucida proteins from their membrane stubs begins intracellularly in the secretory pathway. The ultrastructural evidence is not extensive but the secretory granules that purportedly deliver zona pellucida proteins appear to contain fine filamentous material rather than condensed cores (Dietl, 1989).

One of the consequences of aborting zona pellucida construction with *Zp3*^{-/-} gene knockouts is a compromised relationship between follicle cells and the oocyte (Rankin *et al.*, 1996; Wassarman *et al.*, 1996). Although it has been speculated that the zona pellucida serves as a 'glue' to maintain the stability of gap junctions between the oocyte and follicle cell plasma membranes (Wassarman *et al.*, 1996), the secretion of unpolymerized or poorly polymerized ZP1 and ZP2 molecules in *Zp3*^{-/-} knockout mice may have osmotic effects similar to those responsible for lifting the vitelline membrane in marine invertebrate fertilization. It may be that osmotic forces lift the follicle cell layer away from the oocyte surface in these mice.

The cortical reaction

The cortical reaction is a secretory discharge from the oocyte that occurs after sperm fusion and activation, and that acts as a block to polyspermy in many species. One way in which it functions as a block is to remove surface receptors for spermatozoa on ZP3, thereby preventing attachment and triggering of the acrosome reaction on the outer surface of the zona pellucida (Florman and Wassarman, 1985; Litscher and Wassarman, 1996). The molecular basis for this removal is poorly understood. There is evidence that the cortical reaction modifies the body of the zona pellucida to prevent sperm penetration through a process of 'zona hardening' (DeFelici *et al.*, 1985; Kurawawa *et al.*, 1989; Ducibella *et al.*, 1990; Vincent *et al.*, 1990, 1991).

It is important to be clear about what is meant by 'zona hardening' because superficially the term appears to indicate an alteration to the physical properties of the zona pellucida, that is, a stiffening of the zona pellucida matrix. However, what is normally meant by 'zona hardening' is not increased stiffness but rather an increased resistance of the zona pellucida to proteolytic digestion, normally tested with α -chymotrypsin. These two factors need not be coupled since there is some evidence that increased resistance to chymotrypsin digestion is not always accompanied by an increase in mechanical stiffness (Drobnis *et al.*, 1988). Increases in stiffness require an increase in the force needed to produce a given strain. This could be achieved by increasing the number of inter-filament cross-links within the zona pellucida, by making the filaments or their junctions more resistant to bending, by increasing their resistance to extension or by increasing the swelling pressure. However, changes that increase the resistance of the zona pellucida to proteolysis require alterations to access by proteolytic enzymes. These could occur, for example, by masking the sites of attack or by cross-linking the zona pellucida to prevent proteins unfolding.

Some estimate of the amount of protein released during the cortical reaction can be made as follows. The surface area of a sphere with the diameter of a mouse oocyte is approximately 16 890 μm^2 , of which only 60% contains cortical granules (Ducibella *et al.*, 1988). The density of cortical granules is 43 (100 μm)⁻² of granule-occupied cortex, giving a total of 4127 mature granules per oocyte (Ducibella *et al.*, 1988). Ca^{2+} -activated oocytes retain 1235 granules. This appears to reflect a pool of granules that is unresponsive to increases in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) and which corresponds to the pool of dark granules in the cortex (Nicosia *et al.*, 1977). Mouse cortical granules are 0.2–0.6 μm in diameter (Nicosia *et al.*, 1977) but the distribution of sizes is unknown. If we assume that they are all 0.6 μm in diameter, the total cortical granule volume that can be released cannot exceed 327 μm^3 . Protein (for example, haemoglobin) has a density of approximately 1.08 g cm^{-2} , which gives a total possible cortical granule protein content of 350 pg, or 7% of total zona protein. In practice, it is difficult for protein to pack at densities much higher than 35% of granule volume (for example, haemoglobin in erythrocytes is about 33%), and not all the granules are 0.6 μm in diameter. Therefore, the amount of protein released may be nearer 100 pg, or 2% of zona protein. The figures would be expected to be very similar for hamsters, in which crude estimates of cortical granule density in oocytes have been put at somewhere between 16 μm^{-2} (inferred from Cran *et al.* (1988) who found four granules per 10 μm of cell perimeter) and 100 μm^{-2} (Green, 1989). At most there appear to be about 16 000 cortical granules of which approximately 25% are not releasable by increased $[\text{Ca}^{2+}]_i$. The largest of these granules have diameters of 0.3 μm , giving a total releasable cortical granule volume of 169 μm^3 . This value is somewhat less reliable than that for mice but it is clearly of the same order.

The question arises as to how this relatively small amount of protein causes 'zona hardening', either through increased stiffness or increased resistance to α -chymotrypsin digestion. However, the first matter that needs to be dealt with is whether we are looking for a change in the zona pellucida that is caused catalytically, that is, by an enzyme, or whether we are looking for the titration of specific sites on a stoichiometric basis by cortical granule material. Enzyme-induced changes are attractive in view of the relatively small amount of material released by the cortical reaction. However, it is possible that the cortical granule material does contain enough material to increase zona stiffness by simply cross-linking the zona pellucida through multiple ligand interactions, analogous to ZP1 cross-linking. As we have seen, the cortical granules probably release 100–350 pg of protein, or 20–30% of the mass of ZP1, which is itself 8–10% (approximately 300–500 pg) of zona protein by mass, depending on whether the zona pellucida proteins are furin-cleaved or not. There is no requirement in establishing any zona block to polyspermy that cortical granule material be distributed evenly throughout the zona pellucida and there is some evidence of uneven distribution (Dandekar and Talbot, 1992). A layer of modified zona pellucida adjacent to its inner surface may be all that is required to block sperm penetration. If this is the case, the local concentration of cortical granule material could approach that of ZP1. There is at least one cortical granule protein that binds to the zona pellucida and which is still detectable at the two-cell stage (Pierce *et al.*,

1990), so the persistence of the zona reaction is not currently an obstacle to the model.

The possibility of enzyme-produced changes to the zona pellucida after the cortical reaction cannot be ruled out, particularly in the light of evidence that protease inhibitors, such as fetuin, prevent the establishment of the zona block to polyspermy (Schroeder *et al.*, 1990; Kalab *et al.*, 1991). There are two enzymes that may play a role: a cortical granule protease which converts ZP2 to ZP2_f (Moller and Wassarman, 1989), and a peroxidase which may cross-link tyrosine residues (Gulyas and Schmell, 1980; Schmell and Gulyas, 1980). In support of the involvement of a protease, the cortical reaction results in the conversion of 60% of ZP2 to ZP2_f. However, the protease inhibitor fetuin blocks conversion of ZP2 to ZP2_f and, simultaneously, prevents zona hardening (as tested with α -chymotrypsin) (Schroeder *et al.*, 1990; Kalab *et al.*, 1991). At first sight, zona pellucida hardening is completely contingent on conversion of ZP2 to ZP2_f. However, there is a problem here which has not been adequately addressed. The cortical granule contents, like those of many other secretory granules, are in the form of a condensed matrix which is dispersed after exocytosis. In many cases, matrix dispersal requires the activation of proteolysis. Therefore, there is a possibility that a cortical granule protease is needed to solubilize the cortical granule material after exocytosis and that, in turn, this solubilization is a prerequisite for its penetration of the zona pellucida. At present, we have no information on whether fetuin inhibits the dispersal of this cortical material (possibly including the ovoperoxidase). If it does, then this could explain why fetuin inhibits both ZP2 to ZP2_f conversion and zona hardening in activated oocytes and why these two processes appear to be connected. The inhibition of dispersal would be analogous to the prevention by protease inhibitors of the dispersal of the acrosomal matrix in spermatozoa (Green, 1978). This objection could be overcome by demonstrating that a purified preparation of the cortical granule protease that converts ZP2 to ZP2_f, freed from other cortical granule constituents, could produce zona hardening. This experiment has yet to be undertaken. However, there is currently no satisfactory molecular explanation as to how any zona block to polyspermy can be accounted for by ZP2 to ZP2_f conversion, and it is also unclear how resistance to chymotrypsin digestion could arise at the molecular level through the conversion. Increases in stiffness of the zona pellucida after the cortical reaction are particularly difficult to account for by enzymic cleavage, which would be expected to reduce stiffness.

Finally, there is evidence that ovoperoxidase, released by the cortical reaction, can produce hardening by itself (Gulyas and Schmell, 1980; Schmell and Gulyas, 1980). It is conceivable that ovoperoxidase could produce physical stiffening by chemically cross-linking tyrosines and preventing their elastic uncoiling or bending, but this has yet to be tested. Whatever cross-linking does occur, it clearly falls short of polymerizing the zona pellucida into a covalently coherent structure since SDS remains capable of dissolving the zona pellucida after the cortical reaction (Green, 1989).

There is little doubt that physical cross-linking provides the simplest explanation for the changes to the zona pellucida that follow the cortical reaction. The amount of protein released from the cortical granules, although small, appears to be

approximately commensurate to the task of binding filaments together non-covalently, particularly if localized mainly to the inner part of the zona pellucida, but much more work needs to be done.

Outstanding issues

It should be clear by now that, although considerable progress has been made in our understanding of the primary structure of the zona pellucida proteins, a large number of major questions remain about zona pellucida structure and the effect of the cortical reaction on it. These range from the detailed composition of the carbohydrate attached to each zona pellucida protein to the secondary, tertiary and quaternary structure of the zona pellucida itself. A number of major tasks remain ahead of us. First, it needs to be established whether the filaments are constructed exclusively of ZP2 and ZP3 and whether the ZP2–ZP3 heterodimer is indeed the basic building block. It would be of great value if the three-dimensional shapes of ZP1, ZP2 and ZP3 could be established since this would help identify potential protein–protein and protein–carbohydrate binding sites. Progress in this direction may be possible by pursuing the structural similarity of zona pellucida proteins to other proteins and focusing on common domains within the zona pellucida proteins themselves. There is a need to establish the number of potential ZP1 binding sites on each filament so that the question of distribution of ZP1 molecules and the ratio of ZP1 to ZP2–ZP3 can be tackled. It needs to be established how the zona pellucida proteins are secreted and whether the zona pellucida is made up wholly of furin-cleaved material. If this is the case, it needs to be established whether furin or a furin-like protease is available at the appropriate place at the appropriate time for cleavage of the proteins from their membrane stubs. Larger amounts of zona pellucida proteins are needed for study and, if possible, these proteins need to be capable of self-assembly. It may prove possible to synthesize active glycosylated and sulfated zona pellucida proteins in quantity using eukaryotic expression vectors. It might then become possible to build parts or all of the zona pellucida artificially. However, if the three-dimensional assembly of the zona pellucida requires an obligatory membrane step, as the second of the two models discussed in this review suggests, then assembly or re-assembly of three-dimensional zona pellucida polymer from soluble components cannot be expected to be straightforward.

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