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REVIEW

Acrosome reaction: relevance of zona pellucida glycoproteins

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During mammalian fertilisation, the zona pellucida (ZP) matrix surrounding the oocyte is responsible for the binding of the spermatozoa to the oocyte and induction of the acrosome reaction (AR) in the ZP-bound spermatozoon. The AR is crucial for the penetration of the ZP matrix by spermatozoa. The ZP matrix in mice is composed of three glycoproteins designated ZP1, ZP2 and ZP3, whereas in humans, it is composed of four (ZP1, ZP2, ZP3 and ZP4). ZP3 acts as the putative primary sperm receptor and is responsible for AR induction in mice, whereas in humans (in addition to ZP3), ZP1 and ZP4 also induce the AR. The ability of ZP3 to induce the AR resides in its C-terminal fragment. *O*-linked glycans are critical for the murine ZP3-mediated AR. However, *N*-linked glycans of human ZP1, ZP3 and ZP4 have important roles in the induction of the AR. Studies with pharmacological inhibitors showed that the ZP3-induced AR involves the activation of the G_i-coupled receptor pathway, whereas ZP1- and ZP4-mediated ARs are independent of this pathway. The ZP3-induced AR involves the activation of T-type voltage-operated calcium channels (VOCCs), whereas ZP1- and ZP4-induced ARs involve both T- and L-type VOCCs. To conclude, in mice, ZP3 is primarily responsible for the binding of capacitated spermatozoa to the ZP matrix and induction of the AR, whereas in humans (in addition to ZP3), ZP1 and ZP4 also participate in these stages of fertilisation.
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INTRODUCTION

Mammalian fertilisation is a highly synchronized process that involves a complex series of interactions between the spermatozoon and the egg, culminating in their unison. The initial steps in fertilisation involves the binding of the spermatozoon to the zona pellucida (ZP) matrix surrounding the egg, followed by induction of the acrosome reaction (AR) in the zona-bound spermatozoon, a pre-requisite for penetration of the ZP matrix by the spermatozoon. The spermatozoon acrosome is a Golgi-derived organelle that forms a cap over the anterior two-thirds of its nucleus. The AR involves the fusion of the sperm membrane with the outer acrosomal membrane, resulting in release of the acrosomal contents and exposure of the inner acrosomal membrane on the anterior head of the spermatozoon. Various physiological agents, such as progesterone, serum albumin, follicular fluid, hormones (including biogenic amines), hydrolytic enzymes (particularly proteases), hyaluronic acid and ZP glycoproteins, have been implicated in the induction of the AR.[1–3] In the present paper, we review the role of the ZP matrix and its constituents in AR induction. Various downstream signalling pathways involved in the ZP glycoprotein-induced AR will also be discussed. On the basis of the current literature and studies from our group, the salient differences in the ZP glycoprotein-mediated induction of the AR in mouse versus human will be highlighted.

INDUCTION OF AR BY THE ZP MATRIX

Composition of the ZP matrix

The mammalian ZP is composed of either three or four glycoproteins (Figure 1). The murine ZP matrix is composed of three glycoproteins designated ZP1 (623 amino acids (aa)), ZP2 (713 aa) and ZP3 (424 aa).[4] Pig,[5] cow[6] and dog[7] also have three glycoproteins, but instead of ZP1, ZP4 is present (Figure 1). However, the ZP matrices of rats, hamsters, bonnet monkeys and humans are composed of four glycoproteins: ZP1, ZP2, ZP3 and ZP4.[8–13] In humans, ZP1 has a 638-aa polypeptide backbone; ZP2 has 745 aa; ZP3 has 424 aa and ZP4 has 540 aa. The ZP glycoproteins are heavily glycosylated and have *N*- as well as *O*-linked glycans, which have crucial roles in the spermatozoon–ZP interaction and AR induction.[14,15] The orthologue of the human *Zp4* gene is present in the mouse genome as a pseudogene and is, therefore, not expressed in the murine ZP matrix.[7] In non-mammalian species, more than four ZP genes have been detected; for example, the chicken genome contains six genes (*Zp1*, *Zp2*, *Zp3*, *Zp4*, *ZpAX* and *ZpD*),[16] and the *Xenopus* genome contains five genes (*Zp2*, *Zp3*, *Zp4*, *ZpD* and *ZpAX*).[7]

Induction of the AR by the ZP matrix

Pioneering work by Paul Wassarman's group established that the binding of mouse sperm to the egg ZP is followed by AR induction.[17] Solubilized ZPs isolated from unfertilized mouse eggs induce the AR, whereas those isolated from embryos fail to do so.[17] As observed in murine models, incubation of capacitated human sperm with intact zonae or acid-disaggregated zonae also leads to a significant increase in the AR.[18,19] Progesterone and follicular fluid have priming effects on the ZP-induced AR.[20] In contrast to the mouse ZP, the human ZP of fertilized oocytes retains its ability to bind sperm and also induce the AR.[21] However, the rate of penetration of the human ZP matrix by such acrosome-reacted sperm is much lower than that of human sperm that

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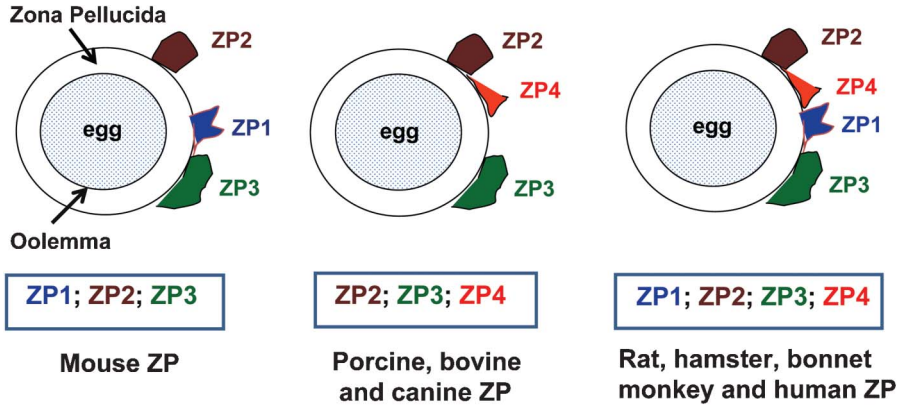

Figure 1 Schematic representation of the composition of the ZP in various mammals: The ZP matrix of the mammalian oocyte is composed of either three or four glycoproteins. The mouse ZP matrix is composed of three glycoproteins, namely, ZP1 (blue), ZP2 (brown) and ZP3 (green). The rat, hamster, bonnet monkey and human ZP matrices are composed of four glycoproteins: ZP1, ZP2, ZP3 and ZP4 (red). The bovine, porcine and canine ZP matrices contain three glycoproteins, with ZP4 replacing ZP1. ZP, zona pellucida.

has reacted with the ZP of unfertilized oocytes. These observations suggest that during fertilisation in humans, the block in polyspermy may also occur at the level of sperm penetration through the ZP matrix.[21]

SIGNALLING EVENTS DURING ZP-MEDIATED ACROSOMAL EXOCYTOSIS

Binding of a capacitated spermatozoon to the ZP matrix activates transmembrane signals that trigger cellular cascades resulting in the AR in the zona-bound sperm (Figure 2). At least two different receptor-mediated signalling pathways in the sperm plasma membrane are responsible for ZP-induced acrosomal exocytosis. One is a G_i protein-coupled receptor that activates the phospholipase $C\beta_1$ ($PLC\beta_1$)-mediated signalling pathway, and the other is a tyrosine kinase receptor coupled to PLCγ (Figure 2).[22] Incubation of mouse sperm membrane preparations with heat solubilized ZP prepared from unfertilized mouse eggs leads to a dose-dependent increase in guanosine triphosphate γ-S binding, as well as GTPase activity, suggesting that the G_i-coupled receptor pathway is involved in the ZP-mediated induction of the AR.[23] The ZP may selectively activate G_{i1} and G_{i2} subtypes of G_i in the sperm.[24] The participation of a second G protein, $G\alpha_{q/11}$, has also been suggested.[25]

G_i acts as a signal transducing element downstream of the ZP3–receptor interactions and couples receptor occupancy to changes in the ionic conductance and/or a variety of intracellular second messenger system cascades whose activation in turn results in the release of acrosomal contents.[26] One such cascade is likely to be the activation of sodium/proton (Na^+/H^+) exchange pumps, resulting in intracellular alkalinisation.[26,27] Second messengers include the adenylate cyclase–cyclic adenosine monophosphate system, which activates protein kinase A (PKA), leading to the phosphorylation of specific putative proteins involved in acrosomal exocytosis. In addition, the activation of $PLC\beta_1$ and/or PLCγ leads to an increase in the levels of 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). DAG may stimulate protein phosphorylation through PKC, whereas IP_3 may activate intracellular calcium ($[Ca^{2+}]_i$) release through the modulation of IP_3-sensitive intracellular calcium stores.[25–27] Studies with the mouse ZP solubilized by acid disaggregation have shown that the ZP-induced AR is a Ca^{2+}-dependent exocytotic event involving an increase in $[Ca^{2+}]_i$ mediated primarily by T-type voltage-operated calcium channels (VOCCs).[28–30] A role for L-type VOCCs has also been proposed during induction of the AR.[31,32] Inhibition of solubilized ZP-mediated AR induction by 3-quinuclidinyl benzilate (an antagonist of muscarinic receptors), tyrphostin A-48 (a tyrosine kinase inhibitor) and pertussis toxin (an inhibitor of G_i protein signalling) suggests that the binding of the ZP to sperm plasma membrane receptors involves several downstream signalling pathways.[28]

Spermatozoa maintain an inwardly negative membrane potential and conductance through cation channels, producing a depolarizing current. Binding of mouse ZP3 to sperm activates a cation channel (impermeable to anions) that conducts monovalent and divalent cations and leads to sperm membrane depolarisation from about −60 to −30 mV. Depolarisation of the sperm membrane potential opens the T-type VOCCs. However, the voltage-dependent inactivation of T currents occurs within 50–100 ms during depolarisation,[29,33,34] thereby terminating the ZP3-induced calcium influx. The T-type channels may also be modulated by their state of tyrosine phosphorylation during capacitation and ZP3 stimulation.[35] However, a sustained release of calcium is an absolute requirement for an induction of the AR.

After depletion of calcium from internal stores, store-operated channels, which are voltage-insensitive calcium channels in the plasma membrane, are activated and mediate the second phase of calcium entry, referred to as capacitative calcium entry.[36] Mammalian transient receptor potential proteins, which are homologues of the *Drosophila melanogaster* photoreceptor cell transient receptor potential protein, are involved in the ZP3-mediated capacitative calcium entry in mice.[36] Transient receptor potential homologues have also been located in human sperm.[37,38] In addition, members of soluble *N*-ethyl maleimide-sensitive factor attachment protein receptor proteins present in the acrosome region of mammalian sperm may also facilitate calcium entry, thereby leading to the AR.[39,40] The high intracellular free calcium concentration together with DAG leads to membrane fusion and finally acrosomal exocytosis.[2,41]

Induction of the AR by the solubilized human ZP depends on extracellular Ca^{2+}[20,42] and involves activation of G_i protein-coupled receptor pathway signalling,[20,42–45] tyrosine kinases,[42] PKA, PKC, phosphoinositide-3 kinase,[42,46] T-type VOCCs and gamma aminobutyric acid-A receptor-associated chloride channels.[42]

ROLES OF ZP CONSTITUENT GLYCOPROTEINS IN INDUCTION OF THE AR

It seems that the composition and, consequently, the structure of the mammalian ZP is more complicated than expected because, depend-

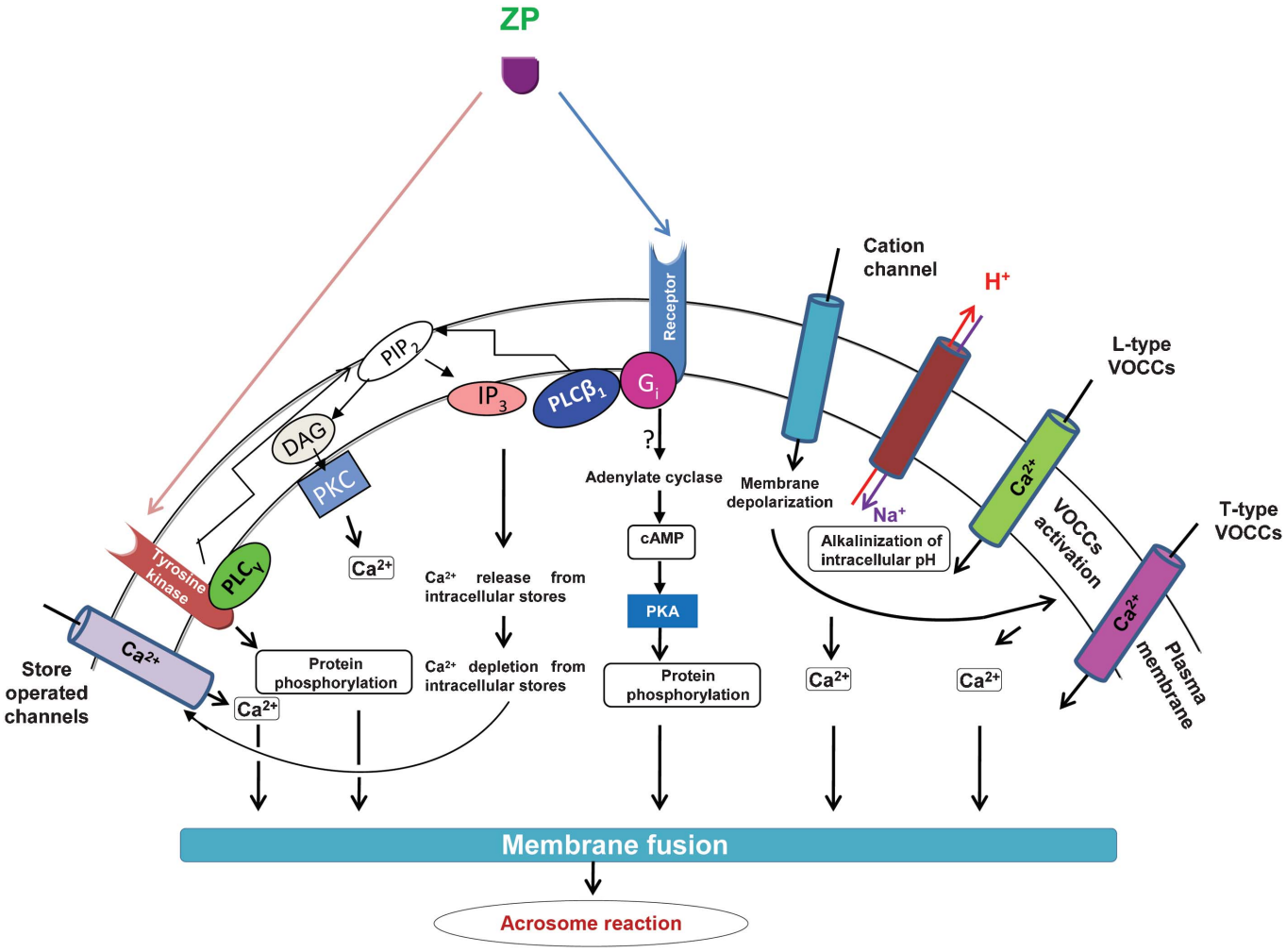

Figure 2 Schematic representation of various signalling pathways involved in ZP-mediated acrosomal exocytosis. Two prominent signalling pathways are known to operate in the sperm membrane upon ZP binding. One is the pertussis toxin sensitive G_i protein-coupled receptor linked to PLCβ_1. The other is a putative tyrosine kinase receptor coupled to PLCγ. Receptor activation also induces adenylate cyclase activation, leading to the generation of cAMP and activation of PKA, which phosphorylates and activates downstream effector proteins. Agonist binding also activates cation channels present on the sperm plasma membrane, leading to membrane depolarisation and activation of L- and T-type VOCCs. An increase in intracellular alkalinisation also occurs due to activation of sodium/proton exchange pump that probably increases or amplifies calcium signals. PLCβ_1 and PLCγ hydrolyse PIP_2 in the membrane, leading to the generation of IP_3 and DAG. DAG mediates PKC translocation to the plasma membrane and its activation, whereas IP_3 mediates calcium entry into the sperm cytosol from intracellular stores. Depletion of calcium from internal stores leads to activation of voltage insensitive SOCs on the sperm cell surface by an undefined mechanism. This mediates another round of calcium entry, which leads to activation of components involved in the fusion of the outer acrosomal membrane with the sperm plasma membrane resulting in the AR. AR, acrosome reaction; cAMP, cyclic adenosine monophosphate; DAG, 1,2-diacylglycerol; PKA, protein kinase A; IP_3, 1,4,5-inositol triphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; SOC, store-operated channel; VOCC, voltage-operated calcium channel; ZP, zona pellucida.

ing on the species: (i) it is formed by three or four ZP glycoproteins (Figure 1); (ii) in the three-glycoprotein model, it can be formed by ZP1, ZP2 and ZP3 or ZP2, ZP3 and ZP4 (Figure 1); and (iii) the protein responsible for sperm binding and AR induction may vary across species. To delineate the roles of individual zona proteins, various groups have either used the purified protein from a native source (which makes it difficult to rule out minor contamination from other egg-associated or zona proteins) or the recombinant protein. Using recombinant protein ensures that it is not contaminated by other zona proteins. However, the recombinant proteins may not have the conformation and glycosylation of its native counterpart. Nonetheless, both approaches have been used to delineate the role of individual zona proteins in binding sperm and inducing the AR. Table 1 summarizes the role of individual zona proteins in sperm binding and AR induction.

ZP1

In a murine model, ZP1 purified from ZPs of unfertilized eggs does not interfere significantly with the binding of sperm to eggs *in vitro*, suggesting that ZP1 does not bind to sperm.[47] Furthermore, ZP1 purified from mouse eggs has no significant effect on AR compared with the respective control.[17] However, it has been postulated that cross-linking by ZP1, the filaments formed by ZP2–ZP3 heterodimers, may provide stability and structural integrity to the ZP matrix.[48] Studies in quail and chicken have shown that ZP1 (dimeric in chicken) is capable of inducing the AR.[49,50] Recent studies from our group have

Table 1 Zona pellucida glycoproteins involved in sperm binding and induction of the acrosome reaction in mice and humans

		Function		
Species	*ZP protein*	*Binding to capacitated spermatozoa*	*Induction of acrosome reaction*	*Reference*
Mice	Native ZP3	Yes	Yes	17, 47
	Recombinant ZP3	Yes	Yes	58
Humans	ZP1			
	Baculovirus-expressed recombinant protein	Yes	Yes	31
	ZP3			
	Escherichia coli-expressed recombinant ZP3	ND	Yes	65
		Yes	No	53
	Baculovirus-expressed recombinant ZP3	Yes	Yes	52, 53
		ND	Yes	61
		ND	Yes	32
	Mammalian-expressed recombinant ZP3	ND	Yes	62
		Yes	Yes	63
		ND	Yes	64
	Native ZP3	Yes	Yes	55,60
	ZP4			
	E. coli-expressed recombinant ZP4	Yes	No	53
	Baculovirus-expressed recombinant ZP4	Yes	Yes	52,53
		ND	Yes	61
		ND	Yes	32
	Native ZP4	Yes	Yes	55, 60

Abbreviations: ND, not done; ZP, zona pellucida.

shown that both *Escherichia coli*- and baculovirus-expressed recombinant human ZP1 conjugated to fluorescein isothiocyanate bind to the anterior head of capacitated human spermatozoa (Table 1).[31] Baculovirus-expressed recombinant ZP1 also generates a dose-dependent increase in acrosomal exocytosis, which involves activation of both T- and L-type VOCCs. The failure of *E. coli*-expressed recombinant human ZP1 to induce the AR suggests that glycosylation of ZP1 is critical for its ability to induce the AR. Induction of the AR by ZP1 does not depend on activation of the G_i protein-coupled receptor pathway, whereas human solubilized ZP- as well as ZP3- (described below) mediated ARs involve activation of the G_i protein. Inhibition of PKA and PKC significantly reduces the ZP1-mediated induction of the AR.[31]

ZP2

Mouse ZP2 purified from ZPs of unfertilized eggs does not interfere with sperm–egg binding or with induction of the AR.[17,51] Monoclonal and polyclonal antibodies against mouse ZP2 do not affect the initial binding of the sperm to the egg but do significantly inhibit the binding of acrosome-reacted sperm to the ZP, suggesting that ZP2 serves as a secondary receptor for sperm during fertilisation.[51] In humans, neither *E. coli*- nor baculovirus-expressed recombinant ZP2 binds to the capacitated acrosome-intact human spermatozoa or induces any significant increase in AR.[52,53] The fluorescein isothiocyanate-coupled recombinant human ZP2 has shown binding to the equatorial region of acrosome-reacted spermatozoa, suggesting that as in mice, human ZP2 is not involved in the induction of the AR and primarily acts as a secondary sperm receptor.[53] Employing a highly specific monoclonal antibody (MA-1615) generated against baculovirus-expressed recombinant human ZP2 that is devoid of reactivity in ELISA and western blots with recombinant human ZP3 and ZP4,[54] purification of human ZP2 from ZPs of unfertilized human oocytes from the assisted reproduction program has been reported.[55] Purified native human ZP2 binds to the acrosomal region of only acrosome-reacted human spermatozoa, corroborating the findings observed using recombinant ZP2.[55]

ZP3

The initial adhesion event between the mouse sperm and the ZP is a high affinity event involving about 30 000 binding sites (300 molecules/μm^2) ascribed to ZP3, which are sufficient to tether a spermatozoon to the extracellular matrix prior to AR induction.[56] The contact subsequently becomes more tenacious, and the bound spermatozoon undergoes the AR. Among the various physiological and pharmacological inducers of the AR, ZP3 has been accepted as the natural agonist (except in guinea pig) that initiates the AR upon binding of the acrosome-intact mammalian spermatozoa to the ZP.[57] Purified mouse ZP3 binds to the anterior head region of the capacitated acrosome-intact spermatozoon, thus acting as a putative primary sperm receptor.[47] Further, recombinant mouse ZP3 expressed in mammalian cells also decreases sperm–ZP binding and triggers acrosomal exocytosis in capacitated mouse sperm.[58] In hamsters and humans, ZP3 performs the function of primary sperm receptor.[52,53,55,59] Studies employing purified native human ZP3,[60] as well as baculovirus-expressed recombinant ZP3,[32,52,53,61] have shown dose-dependent increases in acrosomal exocytosis. Moreover, human ZP3 expressed in mammalian cells also leads to an increase in the AR.[62–64] However, there are conflicting observations with respect to the efficacy of *E. coli*-expressed recombinant human ZP3 in inducing the AR. According to one report, *E. coli*-expressed recombinant ZP3 induces the AR, but a significant increase in the AR is observed only after 18 h of incubation of the capacitated sperm with the recombinant protein.[65] Our group has shown that *E. coli*-expressed recombinant human ZP3, though binding to the anterior head of the capacitated spermatozoon, fails to induce the AR, suggesting that glycosylation of ZP3 is critical for AR induction (Table 1).[52,53]

Delineation of the domain of ZP3 involved in induction of the AR. To understand the role of ZP3 during fertilisation, it is imperative to delineate the region(s) responsible for its functional activity. Studies with insoluble pronase-digested mouse ZP3 revealed that small glycopeptides (about 1.5–6.0 kDa) are capable of inhibiting the binding of

sperm to eggs; however, they did not induce the sperm to complete acrosomal exocytosis.[66] Further, mouse ZP3 was digested with either papain or V8 protease to yield a 55-kDa glycoprotein.[67,68] The ~55 kDa glycopeptide was derived from the carboxy-terminal half of ZP3 and possessed four or five potential *N*-linked glycosylation sites, and after removal of *N*-linked oligosaccharides by treating with *N*-glycanase, a 25-kDa glycopeptide was generated. Both untreated and *N*-glycanase treated glycopeptides inhibited the binding of sperm to eggs and induced sperm to complete the AR *in vitro* to about the same extent as intact ZP3. These findings suggest that the sperm-binding site of mouse ZP3 is located in the carboxy-terminal half of ZP3 and does not involve *N*-linked oligosaccharides. In addition to the biochemical approaches, several molecular genetic approaches have been used to identify the location of the sperm-binding site of ZP3. These approaches were made possible by the successful cloning and sequencing of the mouse *Zp3* gene and polypeptide in the late 1980s.[69–71] Exon swapping and site-directed mutagenesis studies with recombinant mouse ZP3 expressed in an embryonal carcinoma (EC) cell line revealed that the sperm combining site is located in the carboxy-terminal region of ZP3, encoded by exon 7 of the *Zp3* gene,[72–74] which corroborates the biochemical approaches described above.

Recombinant hamster ZP3 expressed in EC cells failed to inhibit *in vitro* binding of mouse sperm to eggs. However, substitution of the hamster *Zp3* exon 7 with mouse *Zp3* exon 7 of the recombinant hamster ZP3 led to inhibition of the binding of mouse gametes.[75] In this context, a fusion construct consisting of human IgG (F*c*) and either exon 7 or 8 of mouse *Zp3* were prepared. An EC cell line carrying the recombinant gene was produced that secreted chimeric glycoproteins designated either EC-huIgG (F*c*)/mouse ZP3 (7) or EC-huIgG (F*c*)/mouse ZP3 (8). It was observed that only EC-huIgG (F*c*)/mouse ZP3 (7) bound specifically to the plasma membrane overlying the sperm head to a similar extent as mouse ZP3 isolated from eggs, and at nanomolar concentrations EC-huIgG (F*c*)/mouse ZP3 (7) inhibited the binding of mouse sperm to eggs *in vitro*. Collectively, these observations provide evidence that sperm recognize and bind to a region of mouse ZP3 that is encoded by exon 7 and is immediately downstream of its 'ZP domain'. This conclusion is supported by another recent report on the inhibitory effects of the carboxy-terminal region of recombinant mouse ZP3 *in vitro*.[76] It is of interest that ZP3 is among the 10% most different proteins between rodents and humans.[77] The region of ZP3 encoded by exon 7 has undergone a relatively large number of changes during evolution compared with the remainder of the polypeptide and is a proposed site of positive Darwinian selection.[78,79]

Human ZP3 has a polypeptide backbone of 424 aa, with a signal peptide (SP) at 1–22 aa that facilitates its secretion (Figure 2a). A tetra basic consensus furin cleavage site (349–352 aa) is present upstream of a hydrophobic transmembrane-like domain (387–409 aa). In mature human ZP3, both the SP and the transmembrane-like domain are cleaved off. Of 12 cysteine (Cys) residues, eight are conserved in various species. The disulphide linkages of the first four Cys residues form a loop-within-loop motif (Cys^{46}/Cys^{140} and Cys^{78}/Cys^{99}), and the second four form a crossover motif (Cys^{217}/Cys^{282} and Cys^{239}/Cys^{300}).[80] The remaining four Cys residues (Cys^{319}, Cys^{321}, Cys^{322} and Cys^{327}) lying within a tight cluster towards the C-terminus are linked by two unassigned disulfide linkages.[80] Human ZP3 has a conserved domain designated the 'ZP domain' (45–304 aa), which is also present in other zona proteins and several extracellular proteins, such as Tamm–Horsefall protein and α- and β-tectorin.[81,82] The human ZP3 'ZP domain' consists of two conserved subdomains, the N-terminal (45–175 aa) and C-terminal (214–304 aa), separated by a short protease sensitive hinge (Figure 3a). To delineate the functional domain of human ZP3, cDNAs encoding various fragments of human ZP3 were cloned and expressed using a baculovirus expression system (Figure 3b). Significant induction of the AR was observed when capacitated human sperm were incubated with recombinant human ZP3 fragments corresponding to 214–348 and 214–305 aa.[83] A recombinant ZP3 N-terminal fragment (23–175 aa) failed to induce any significant increase in the AR, suggesting that the functional activity of human ZP3 also resides in its C-terminal domain (Figure 3b).

ZP4

The mouse ZP matrix is composed of ZP1, ZP2 and ZP3, but lacks a ZP4. Our group, along with others, has investigated the role of ZP4 in induction of the AR in humans. *E. coli*-expressed recombinant human ZP4 binds to the anterior head of capacitated acrosome-intact human spermatozoa but does not induce the AR.[53] On the other hand, baculovirus-expressed recombinant human ZP4 not only binds to the anterior head of capacitated acrosome-intact spermatozoa, but also induces a dose-dependent increase in the AR[32,52,53,61] (Table 1). These observations were further confirmed by employing immunoaffinity purified native human ZP4 from solubilized human ZP.[55,60] However, it may be noted that the purified human ZP4 fractions from eggs were contaminated with ZP1, and thus its ability to induce the AR may have been due to the combined effect of both ZP1 and ZP4. The importance of ZP4 either alone or as a hetero-oligomer complex with ZP3 during sperm binding and subsequent induction of the AR has also been demonstrated in *Xenopus*,[84] rabbits,[85] pigs[86] and non-human primates.[87] Hence, in humans, ZP4 also acts in conjunction with ZP1 and ZP3 to induce the AR.

DO DIFFERENT HUMAN ZONA PROTEINS USE THE SAME DOWNSTREAM SIGNALLING PATHWAY?

As discussed above, in humans (in addition to ZP3), ZP1 and ZP4 also mediate the induction of the AR.[31,32,52,53,60,61] Using pharmacological inhibitors, subtle differences in the downstream signalling pathways used by the ZP glycoproteins were observed, which are summarized in Table 2. ZP3-mediated induction of the AR in humans is inhibited by pertussis toxin, whereas pertussis toxin does not inhibit ZP1- or ZP4-mediated acrosomal exocytosis, which indicates that ZP1/ZP4 act through a G_i protein-independent pathway.[31,52,60]

T-type VOCC inhibitors (mibefradil and pimozide) inhibit acrosomal exocytosis mediated by ZP3 and a C-terminal fragment of recombinant ZP3, whereas L-type VOCC inhibitors do not.[60,83] However, ZP1- and ZP4-mediated increases in the AR involve both L- and T-type VOCCs.[31,60] (Table 2). Though ZP3 involves activation of adenylate cyclase, PKA is not critical in ZP3 downstream signalling, suggesting redundancy of PKA and supplementation by parallel signalling pathways. Activation of PKA, however, is crucial for ZP1-/ZP4-mediated signalling, as its pharmacological inhibitor, H-89, specifically inhibits the ZP1-/ZP4-induced AR.[31,60] These studies suggest that the downstream signalling pathways involved in the ZP1- and ZP4-induced ARs are very similar but are different from that employed by ZP3 (Table 2). Human ZP1 and ZP4 are paralogues that may have arisen from a common ancestral gene either by gene duplication or exon swapping.[11,78,79] The aa sequence identity of human ZP1 and ZP4 is 47%, which further supports the notion that AR induction mediated by ZP1/ZP4 is likely to follow similar downstream signalling events.

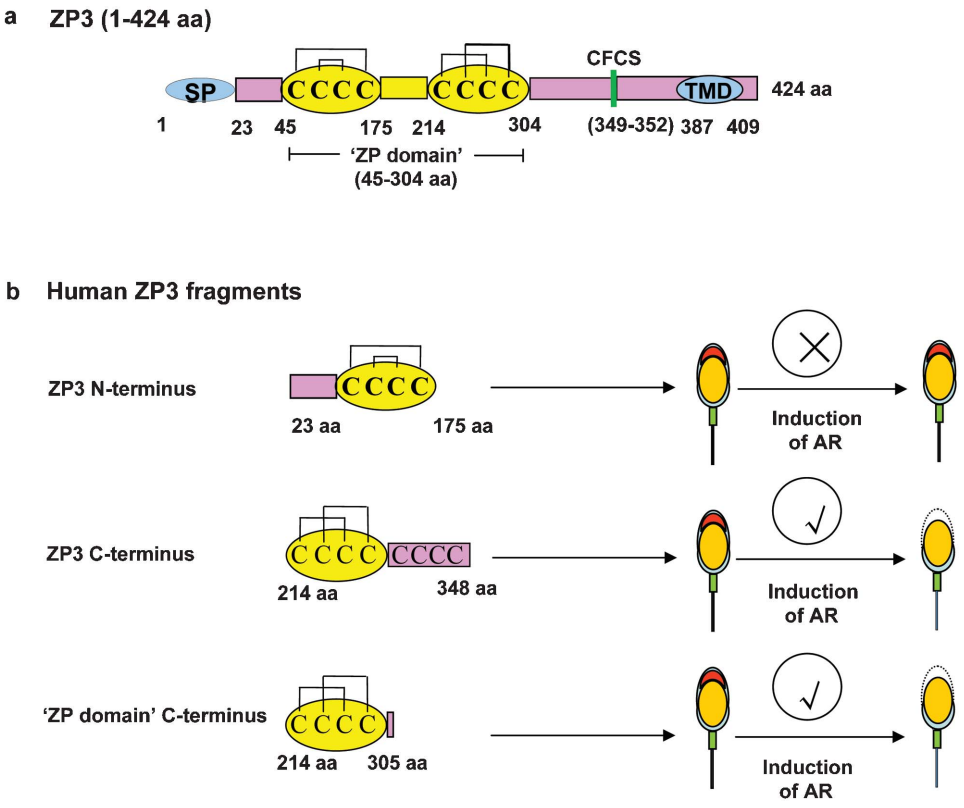

Figure 3 Schematic diagram showing the different domains of human ZP3 and their ability to induce the AR. (**a**) Human ZP3 has a 424-aa polypeptide backbone comprising an SP (1–22 aa), ZP domain (45–304 aa, yellow), CFCS (349–352 aa, green vertical bar) and TMD (387–409 aa). The ZP domain of ZP3 is composed of N- (45–175 aa) and C-terminal (214–304 aa) subdomains. Mapped disulfide linkages between different Cys residues are shown in black lines. (**b**) An N-terminal ZP3 fragment without the SP (23–175 aa), a C-terminal fragment excluding the CFCS (214–348 aa) and the ZP domain C-terminal subdomain (214–305 aa) were expressed in insect cells and purified recombinant proteins evaluated for AR induction in capacitated human spermatozoa.[83] The N-terminal fragment of ZP3 failed to induce the AR, whereas both C-terminal fragments induced the AR. AR, acrosome reaction; CFCS, consensus furin cleavage site; C, cysteine; SP, signal peptide; TMD, transmembrane-like domain; ZP, zona pellucida.

ROLE OF OLIGOSACCHARIDE MOIETIES IN SPERM–ZP INTERACTIONS AND INDUCTION OF THE AR

An understanding of the molecular basis of sperm–egg interactions is still elusive. Various models suggest that it depends on: (i) carbohydrate moieties present on the opposing gamete surfaces; (ii) protein–protein interactions; and (iii) protein–carbohydrate interactions. The protein–carbohydrate interactions are responsible for 75–80% of sperm binding to the ZP, and remaining sperm bind by protein–protein interactions.[88,89]

Table 2 Downstream signalling pathways associated with human ZP glycoprotein mediated induction of the acrosome reaction

Pathway	*Inhibitor*	*Inhibition of induction of AR mediated by*		
		ZP3	*ZP1*	*ZP4*
Extracellular Ca^{2+}	Ethylene glycol tetraacetic acid	Yes	Yes	Yes
G_i protein-coupled receptor	Pertussis toxin	Yes	No	No
T-type VOCCs	Pimozide, amiloride	Yes	Yes	Yes
L-type VOCCs	Verapamil, nifedipine	No	Yes	Yes
PKA	H-89	No	Yes	Yes
PKC	Chelerythrine	Yes	Yes	Yes

Abbreviations: AR, acrosome reaction; PKA, protein kinase A; PKC, protein kinase C; VOCC, voltage-operated calcium channel; ZP, zona pellucida.

Murine models

Chemically deglycosylated forms of mouse ZP3 fail to induce the AR, suggesting that glycosylation of ZP3 is critical for its functional activity. However, selective removal of *N*-linked oligosaccharides from mouse ZP3 by endo-β-*N*-acetyl-D-glucosamine treatment has no effect on the induction of the AR, whereas removal of *O*-linked oligosaccharides by alkaline hydrolysis abrogates its ability to induce the AR.[90] Initial studies implicated galactose in α- or β-linkages at the non-reducing terminus of *O*-linked oligosaccharides and *N*-acetylglucosamine (GlcNAc) in β-linkages as the sugar determinants on mouse ZP3 that are responsible for the binding of sperm to the ZP.[91] However, mice deficient in glycosyl transferase, which amends terminal galactose in an α-linkage, are fully fertile[92,93] implicating galactose in β-linkages or GlcNAc or both as critical residues.[57] Mannose has also been suggested to have an important role in murine sperm receptor activity.[94] Subsequently, site-directed mutagenesis revealed that glycosylation of serine residues at positions 332 and 334 is critical for the sperm receptor activity of ZP3.[73]

Human

Binding studies with various lectins suggest that the human ZP matrix has a high concentration of D-mannose.[95,96] The presence of mannose-binding sites has been reported on human sperm.[97,98] Several oligosaccharide moieties, such as mannose, GlcNAc, fucose and galac-

tose, along with complex glycoconjugates bearing selectin-like ligands, are involved in human sperm–egg binding.[99,100] On the contrary, Chapman and colleagues showed that *E. coli*-expressed recombinant human ZP3, presumably lacking glycosylation, induced the AR, suggesting that glycosylation of ZP3 may not be an absolute requirement for AR induction.[65] Our group has expressed recombinant human ZP1, ZP2, ZP3 and ZP4 using *E. coli* and baculovirus expression systems.[31,52,53] Both *E. coli*- and baculovirus-expressed recombinant human ZP1, ZP3 and ZP4 conjugated with fluorescein isothiocyanate bind to the anterior head of capacitated acrosome-intact human spermatozoa.[31,53] The binding patterns of ZP1 and ZP4 revealed that a higher percentage of sperm show binding of these proteins to the acrosomal cap as opposed to that seen with ZP3, where equatorial binding predominates in the acrosome-intact spermatozoa.[31,53] The binding profiles of *E. coli*- and baculovirus-expressed recombinant human ZP1, ZP3 and ZP4 are comparable, suggesting that glycosylation is not critical for binding *per se*. These results are corroborated by similar findings that the *E. coli*-expressed bonnet monkey ZP3 and ZP4 bind to monkey sperm.[87,101]

E. coli-expressed recombinant human ZP1, ZP3 and ZP4 fail to induce any significant increase in the AR, whereas baculovirus-expressed recombinant ZP1, ZP3 and ZP4 induce dose-dependent increases in the AR.[31,32,52,53,61] These studies suggest that glycosylation of human zona proteins is critical for induction of the AR. Expression of recombinant human ZP3 and ZP4 using a baculovirus expression system in the presence of tunicamycin made available these proteins with reduced *N*-linked oligosaccharides.[53] Incubation of capacitated human sperm with the above recombinant proteins significantly reduces the proteins ability to induce the AR, suggesting that *N*-linked glycosylation of human zona proteins are critical for AR induction.[53] The importance of *N*-linked glycosylations has been further confirmed using immunoaffinity-purified human ZP3 and ZP4 from solubilized human ZP. Removal of *N*-linked glycosides from human ZP3 and ZP4 by treatment with *N*-glycosidase F significantly decreases their respective abilities to induce the AR.[60]. Removal of *O*-linked glycans by alkali hydrolysis (β-elimination) from either baculovirus-expressed recombinant human ZP3 and ZP4 or native human ZP3 and ZP4 purified from human eggs has no significant effect on their AR induction ability.[53,60] Hence, in contrast to mouse, where *O*-linked glycosylation of ZP3 is critical for AR induction, in humans, *N*-linked glycosylation of ZP1, ZP3 and ZP4 are critical for mediating the AR.

CONCLUSION

The mouse model for the roles of individual ZP glycoproteins in binding capacitated acrosome-intact spermatozoa and subsequent induction of the AR is not tenable in other species. In mice, ZP3 is primarily responsible for AR induction. In humans (in addition to ZP3), ZP1 and ZP4 may also be involved in AR induction. In mouse, *O*-linked glycans of ZP3 are involved in the AR, whereas in humans, *N*-linked glycans of ZP1, ZP3 and ZP4 are critical for AR induction. Hence, it is imperative that each species be investigated in detail to determine the roles of zona proteins during fertilisation.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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