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β_1 Integrin is an Adhesion Protein for Sperm Binding to Eggs

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Abstract

We investigated the role of β_1 integrin in mammalian fertilization and the mode of inhibition of fertilin β -derived polymers. We determined that polymers displaying the Glu-Cys-Asp peptide from the fertilin β disintegrin domain mediate inhibition of mammalian fertilization through a β_1 integrin receptor on the egg surface. Inhibition of fertilization is a consequence of competition with sperm binding to the cell surface, not activation of an egg-signaling pathway. The presence of the β_1 integrin on the egg surface increases the rate of sperm attachment, but does not alter the total number of sperm that can attach or fuse to the egg. We conclude that the presence of β_1 integrin enhances the initial adhesion of sperm to the egg plasma membrane and that subsequent attachment and fusion are mediated by additional egg and sperm proteins present in the β_1 integrin complex. Therefore, the mechanisms by which sperm fertilize wild-type and β_1 knockout eggs are different.

Introduction

In mammals, for fertilization to be successful, a single sperm out of thousands must traverse the zona pellucida to reach the perivitelline space to first bind to, and then fuse with, the egg plasma membrane. This interaction between gamete membranes induces egg activation. One of the earliest events associated with egg activation is an increase in intracellular calcium. Later events such as cortical granule exocytosis, blockages to polyspermy that occur at both the zona pellucida and the egg plasma membrane, resumption of meiosis, pronuclei formation, and development of the zygote are all dependent upon the calcium response (3-7). Before sperm binding to the egg plasma membrane can occur, sperm are activated by binding to the zona pellucida, an interaction that is species selective (8).

Sperm protein fertilin β (ADAM2) plays a role in egg plasma membrane binding that leads to fertilization. Fertilin β is a type 1 integral membrane protein located in the equatorial region of the sperm head, and is a member of the ADAM (A Disintegrin and A Metalloprotease domain) family of proteins (9,10). The fertilin β disintegrin domain is highly conserved across species. Its domain structure is partially shared by the snake venom metalloproteases (SVMP's) (11,12). The disintegrin domains of SVMPs bind integrin receptors with high affinity and inhibit integrin-mediated platelet aggregation and cell-matrix attachment. A short peptide sequence present in the disintegrin domain of fertilin β , Glu-Cys-Asp (ECD), is important for the protein's function in egg adhesion (13-17). Antibodies raised against fertilin β block sperm-egg binding and fusion (18). Knockout of the fertilin β gene resulted in

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SUPPORTING INFORMATION Supporting Information Available Supplementary data for inhibition assays and immunofluorescence microscopy. This material is available free of charge via the Internet.

reduced binding of sperm to the egg plasma membrane *in vitro* (19,20). However, later knockout experiments suggested that the simultaneous loss of multiple ADAM proteins is responsible for this phenotype (21-23).

Molecular probes, which mimic the disintegrin domain and incorporate the ECD motif of sperm protein fertilin β , have been designed and tested. Peptides containing the ECD sequence inhibit sperm adhesion to zona pellucida free eggs with IC₅₀'s in the 500 μ M range (13-15,24-27). Multivalent polymers were developed in our laboratory to further probe the protein-protein interactions that occur upon sperm-egg binding (1,28). The most potent inhibitors to date are polymers that contain multiple copies of ECD displayed on a polynorbornene scaffold with IC₅₀'s of 3-5 μ M in peptide (1).

$\alpha_6\beta_1$ Integrin on the mouse egg plasma membrane was identified as the ECD binding partner on the egg (14,29,30). Adhesion and inhibition studies suggested that fertilin β mediates sperm adhesion via $\alpha_6\beta_1$ integrin on the egg (14,17,29,31). A linear peptide containing 12 amino acids of the fertilin β binding sequence, including Glu-Cys-Asp, and *p*-benzoylphenylalanine was used to photoaffinity label the $\alpha_6\beta_1$ integrin, providing evidence for a direct interaction between the ECD ligand and the integrin receptor (30).

The role of egg $\alpha_6\beta_1$ integrin as the egg receptor for sperm fertilin β was further tested by genetic mutation. *In vitro*, eggs in which either the α_6 integrin gene (32) or the α_3 integrin gene (33) is disrupted are fertilizable. Moreover, mice with a conditional knockout of the β_1 integrin in their eggs are fertile *in vivo* (33). Antibodies selected to block α_v or β_3 integrin-matrix binding failed to inhibit fertilization of the β_1 integrin knockout eggs. In light of these results, it was concluded that none of the integrins present on mouse eggs are essential for fertilization. The observed inhibition by ECD-containing constructs was suggested to be a consequence of either low specificity binding to a non-integrin receptor or due to alteration of the egg membrane rendering sperm fusion inhibited (33). More recently, it has been suggested that the presence of $\alpha_6\beta_1$ integrin on the sperm can compensate for the loss of egg integrins by membrane exchange (34). However, this is unlikely because this mechanism requires that the egg and sperm be in contact before exchange can occur. Moreover, there is no fertilin β present on the egg that could function in trans (35,36).

In order to resolve these conflicting observations, we investigated the mechanism of inhibition by polymers containing the ECD peptide (Figure 1). Inhibition by both multivalent polymers and monomeric ECD peptide probes requires the β_1 integrin on the egg. We hypothesized two possible mechanisms of inhibition by ECD polymers. In the first mechanism, ECD polymers may directly compete with or block sperm binding sites on the egg plasma membrane. A second possible mechanism is that the multivalent polymers trigger an intracellular signal, which activates the egg membrane block to polyspermy. ECD polymers compete directly with sperm binding to the egg plasma membrane and egg activation is not responsible for inhibition. Investigation of sperm binding kinetics revealed that β_1 integrin on the egg increases the binding rate of sperm, but is not required for sperm-egg fusion. We conclude that egg β_1 integrin is an adhesion partner for sperm ADAM proteins containing the ECD binding motif and that the mechanism of sperm-egg binding is different in wild-type and β_1 integrin knockout eggs.

Results and Discussion

Polymers Used in These Studies

Three norbornenyl-derived polymers were used in this work: **1**₁₀, **2**₁₀, and **1****2**₁₃ (Figure 1). Polymers like **1**₁₀ that can span multiple receptor binding sites are more potent inhibitors of fertilization than polymers containing 2-3 copies of the ECD peptide in close proximity (3 Å

along the backbone) or inhibitors that incorporate only a single copy of ECD (1,2). Multivalent polymer **1**₁₀ contains on average 10 copies of the ECD peptide whereas control polymer **2**₁₀ contains 10 copies of a mutated sequence Glu-Ser-Ala (ESA) that does not inhibit fertilization. We mutated the cysteine and aspartate residues because they are critical for binding (16,17,24). In this work, we used a mutated sequence rather than a scrambled sequence because tripeptide polymers are more synthetically accessible and the position of the two charges in the ECD tripeptide cannot be truly scrambled. Previously, we demonstrated inhibition is sequence dependent because a scrambled pentapeptide (Cys-Thr-Glu-Val-Asp) incorporated into a polymer does not inhibit fertilization, whereas the native sequence (Glu-Cys-Asp-Val-Thr) does (28). Polymer **1**₂**2**₁₃, containing on average two ECD peptides at one terminus of the polymer, was used as a low valency control as well as an aggregation control. If supramolecular structures form in solution, the valency of a polymer would be higher than designed and inhibition might be due to the supramolecular structure. We found that polymer **1**₂**2**₁₃ was no more effective an inhibitor than a monomeric ECD peptide (Figure S1). Thus, supramolecular aggregates are not responsible for the inhibition observed in the experiments described below.

Inhibition of Fertilization by **1**₁₀

We tested whether the β_1 integrin is required for inhibition of fertilization by ECD polymers. Eggs homozygous for the β_1 integrin knockout allele ($\text{Cre}^+\beta_1^f/f$, KO), eggs heterozygous for the β_1 integrin knockout allele ($\text{Cre}^+\beta_1^{f/+}$, HET) and wild-type ($\text{Cre}^-\beta_1^{+/+}$, WT) eggs were obtained as previously described (33). Immunofluorescence microscopy with anti- β_1 and anti- α_6 integrin antibodies confirmed that the β_1 integrin knockout eggs had no β_1 integrin or α_6 integrin on the plasma membrane (Figure S2).

We chose to examine inhibition of fertilization using zona pellucida (ZP)-free eggs in order to isolate our observations to the egg plasma membrane. Previous research has shown that ECD peptides are inhibitors of both ZP-intact and ZP-free *in vitro* fertilization (13,37). Thus, removal of the ZP does not introduce an artifact into inhibition of sperm-egg binding by these mimics. Moreover, we ensured ample recovery time after removing the ZP in order that egg fertilizability was not impaired (38).

ZP-free KO, HET, and WT eggs were assayed with varying concentrations of **1**₁₀. In these assays, the cumulus cells and the zona pellucida layers surrounding the egg were removed in order to test interactions at the egg plasma membrane. The number of eggs fertilized (Figure 2) and the average number of sperm fused per egg (Figure S3) were determined. As previously observed, polymer **1**₁₀ inhibited fertilization of wild-type eggs, and inhibition was concentration dependent. The approximate IC_{50} was 2.5 μM polymer. This IC_{50} is different than previously reported (1) because the polymer length and stereochemistry were different due to the use of a newer ROMP precatalyst (2,39,40). Polymer **1**₁₀ inhibited fertilization of KO eggs $19 \pm 6\%$ at the highest concentration used (50 μM in polymer, 500 μM in peptide), as compared with $73 \pm 4\%$ inhibition of WT egg fertilization at the same concentration. In experiments described below (Figure 5b), when 1% DMSO was included in the assay buffer with polymer **1**₁₀ no inhibition of KO fertilization was observed, whereas, the inhibition of WT fertilization was unchanged. This result suggests that the small amount of inhibition detected in KO fertilization is due to non-specific hydrophobic binding. Negative control polymer **2**₁₀ containing ESA peptides was assayed and did not inhibit fertilization even at 500 μM polymer (5 mM peptide, Figure 2c). These data indicate that inhibition of fertilization by **1**₁₀ is mediated by the β_1 integrin on the egg membrane. Moreover, transfer of sperm β_1 integrin to the egg membrane (34) cannot fully compensate for β_1 integrin deletion.

Inhibition of Fertilization by **1₂2₁₃**

We considered the possibility that the β_1 integrin is required for binding polymer inhibitor to the egg surface, but that β_1 integrin is not required for sperm binding. If the higher abundance β_1 integrin were to act as an anchor for **1₁₀** and tether it to the egg surface, the avidity of terminal ECD ligands binding to a second, lower abundance sperm receptor would increase. In this scenario, inhibition by a non-avid, monovalent or low valency inhibitor that blocked the second unknown receptor used by sperm would be equipotent in WT and KO fertilization. Therefore we tested **1₂2₁₃**, a low valency polymer, as an inhibitor of fertilization in WT and KO eggs (Figure 2b). Polymer **1₂2₁₃** inhibited fertilization of WT eggs 100-fold less potently than polymer **1₁₀** as expected based on previous work. Polymer **1₂2₁₃** inhibits WT fertilization 51% at 500 μ M, whereas only 20% inhibition is observed in KO fertilization. This difference is statistically significant ($p < 0.05$) and indicates that ECD peptide binding to β_1 integrin on the WT egg blocks sperm binding. We observed that inhibition of HET fertilization by polymer **1₁₀** is equipotent to inhibition of WT fertilization (Figures S4 and S5). Therefore, the loss of inhibition is not due to differences in the genetic backgrounds of the mice. Thus, β_1 integrin-mediated avidity for a second sperm receptor is not responsible for inhibition of WT fertilization.

Activation of Eggs by **1₁₀**

We next sought to address whether inhibition occurs through egg activation. During fertilization, egg activation triggers a complex sequence of events, one of which is the establishment of the egg membrane's block to polyspermic fertilization (6,41). A downstream consequence of egg activation and intracellular calcium release from the ER, is resumption of meiotic cell division and formation of the pronuclei (42).

We tested if **1₁₀** could induce pronuclei formation in ZP-intact WT and KO eggs. Sperm were allowed to capacitate, but not acrosome react, prior to insemination so that they could bind and penetrate the ZP. After insemination or polymer treatment, ZP-intact eggs were scored for pronuclei formation (Figure 3). WT and KO eggs were both activated by **1₁₀**, but less efficiently than sperm activate eggs. Polymer **1₁₀** appeared to activate more WT eggs than KO eggs, but the difference was not statistically reliable. No significant activation was observed with control polymer **2₁₀**.

Next, we tested whether polymer **1₁₀** could induce calcium oscillations. WT and KO eggs that were harvested no later than 12 hours after superovulation with hCG were treated with polymer **1₁₀**. Polymer **1₁₀** induced calcium oscillations in both WT and KO eggs and the peak frequencies, durations, and intensities were similar (Figure S7). The control polymer **2₁₀** did not induce oscillations in either egg type.

Activation versus Competition Model for Inhibition

If inhibition is due to activation of the egg membrane block to polyspermy, the block is not expected to be reversible (6,41,43). Therefore, we tested whether inhibition by polymer **1₁₀** was reversible. A multivalent ECD fluorescently-tagged polymer is not internalized into eggs (Figure 6), and after three washes the polymer is not detected on the surface of the egg (data not shown). Moreover, washing eggs six times does not affect egg penetrability (Figure 4).

Eggs were treated with **1₁₀** and inseminated immediately after washing or three hours after washing to allow the egg membrane block to reach a maximum (6,43). Washing completely eliminated inhibition regardless of insemination time. Reversible inhibition is consistent with a competitive binding mechanism and not an activation mechanism.

To further test whether egg activation was responsible for inhibition by polymer, we blocked the egg activation pathway. Calcium signaling and cytoskeletal rearrangement are required for establishing the membrane block to polyspermy (6,43). ZP-free eggs were treated with BAPTA-AM, a calcium chelator, or cytochalasin D, which perturbs actin polymerization, prior to polymer addition and insemination. As expected, WT eggs treated with BAPTA-AM or cytochalasin D fused with nearly twice as many sperm as untreated eggs (Figure 5a) (6). Treatment of KO eggs with BAPTA-AM resulted in the same increase of sperm fusion (Figure 5b). Thus, β_1 integrin is not required for sperm initiation of the membrane block. Importantly, blocking the egg activation pathway in WT eggs did not reduce the inhibition potency of **1**₁₀ (Figure 5a). No inhibition by **1**₁₀ of sperm fusion to KO eggs was observed under these conditions. These data indicate that inhibition of fertilization is not caused by polymer initiating the egg's membrane block to polyspermy. We conclude that **1**₁₀ directly blocks sperm from binding to the β_1 integrin.

The egg signaling events and the inhibition of fertilization observed when eggs are treated with **1**₁₀ are not related. Egg activation by **1**₁₀ does not appear to be sufficient to induce an egg-membrane block to polyspermy. We used fluorescent versions of **1**₁₀ and **2**₁₀, polymers **5** and **6**, respectively, to image the polymer binding to the egg (Figure 6 and supporting information). The ECD polymer clearly binds to the WT egg and is not internalized as previously mentioned (Figure 6a and 6b). However, little binding to the KO egg is observed (Figure 6c and 6d) and the signal seen is not significantly greater than for the ESA analog, polymer **6** (Figure 6e and 6f). This result suggests to us that activation is due to an indirect effect, for example, calcium chelation. The polymer has multiple carboxyl groups that may chelate calcium and induce activation. Many parthenogenetic agents are calcium ionophores or compete with calcium and these types of parthenogenetic reagents are not able to actuate the membrane block to polyspermy (41,44,45) just as we observe with polymer **1**₁₀.

Proposed Role for $\alpha_6\beta_1$ Integrin in Mammalian Fertilization

We hypothesized that if β_1 integrin was important for sperm adhesion, the kinetics of sperm binding to KO eggs would be altered. The average number of sperm bound and fused to WT and KO ZP-free eggs in a single focal plane was monitored for 20 minutes after insemination (Figure 7). Sperm binding to KO eggs was delayed 1-2 minutes ($p < 0.05$) compared with sperm binding to WT eggs and a concomitant 1-2 minute delay in sperm fusion was observed. After 5.5 minutes, no significant difference in the number of sperm bound and fused was detected. These data suggest that β_1 integrin aids sperm adhesion to the egg and imply that this adhesion step may be bypassed by attachment to other proteins in a binding-fusion complex. ECD inhibitors block sperm binding to the integrin complex, but the blocking is incomplete as evidenced by inhibition saturating at 70%.

Conclusion

ECD polymers inhibit fertilization by competition with sperm binding to the egg surface $\alpha_6\beta_1$ integrin, most likely present as the $\alpha_6\beta_1$ complex. It has been suggested that inhibition using disintegrin peptides/constructs is non-physiological and a consequence of binding to a non-integrin receptor or of activating signaling that inhibits gamete fusion (33). Our data demonstrate that the β_1 integrin is required for inhibition. Therefore, a non-integrin receptor is not responsible for the observed inhibition. Moreover, although the ECD polymers activate eggs artifactually, this activation is not responsible for inhibition of gamete fusion. Our data suggest that the mechanism by which sperm fertilize WT eggs is different than fertilization of KO eggs. That is, a second sperm-egg binding interaction can compensate for loss of β_1 integrin.

These results are in agreement with the work of Evans and coworkers that was published while the present work was under review (46).

Integrin $\alpha_6\beta_1$ is the predominant egg surface protein (47), and is known to cluster at the site of sperm contact (48). β_1 integrin is associated with tetraspanin CD9 in mammalian eggs (48-50), and eggs from CD9 null mice fuse poorly with sperm (49,51). Eggs from CD9 / CD81 double null mice do not fuse at all with sperm (52). Inclusion of the integrin within the tetraspanin cluster in wild-type eggs may improve sperm avidity for the egg surface. The incomplete blockage of sperm binding and fusion observed with ECD peptides and polymers is consistent with the role of β_1 integrin as a non-essential adhesion receptor (28). Therefore, β_1 integrin KO eggs can bypass the sperm-integrin adhesion step, but as a consequence, sperm attach and fuse to the egg plasma membrane more slowly. The reduced rate of binding does not impair fertility under laboratory mating conditions, but may confer an evolutionary advantage in the wild that results in conservation of the integrin-fertilinb binding interaction.

Methods

General Methods and Materials

All experiments performed with mice were in accordance with the National Institutes of Health and United States Department of Agriculture guidelines, and the specific procedures performed were approved by the Stony Brook University IACUC (protocol #0616). Mice containing the floxed β_1 integrin gene (53,54) were provided by Ruth Globus (NASA Ames Research Center) with permission from Reinhardt Fässler (MPI, Martinsried). Transgenic mice expressing the Cre recombinase under the control of the ZP3 promoter were obtained from Paul Primakoff (UC Davis) with permission from Jamie Marth (UC San Diego). Some mouse genotyping was performed by Transnetyx. All manipulations and incubations of eggs were performed at 37 °C, 5% CO₂ unless otherwise noted. Stock solutions of 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid acetoxymethyl ester (BAPTA-AM, Sigma) and cytochalasin D (Sigma) were prepared in dimethylsulfoxide (DMSO). Polymers were prepared with the [(H₂IMes)(3-BrPyr)₂Cl₂Ru=CHPh] precatalyst (2,55) instead of [(H₂IMes)(PCy₃)Cl₂Ru=CHPh] as previously described (1).

Generation of Oocyte-Specific β_1 Integrin Conditional Knockout Mice

Mice with the floxed β_1 integrin gene and mice with the Cre recombinase behind the ZP3 promoter were used to generate oocyte-specific β_1 integrin conditional knockout mice as previously described by He et al. (33). PCR was used to genotype progeny to identify the ZP3-Cre transgene and the presence of the floxed β_1 integrin gene. For ZP3 detection the following primers were used:

Cre12: GGA CAT GTT CAG GGA TCG CCA GGC G

Cre13: GCA TAA CCA GTG AAA CAG CAT TGC TG

To detect the presence of the floxed β_1 integrin gene the following primers were used:

T56: AGG TGC CCT TCC CTC TAG A

L1: GTG AAG TAG GTG AAA GGT AAC

L26: TAA AAA GAC AGA ATA AAA CGCAC

In vitro Fertilization Assays

Sperm were isolated from the cauda epididymis and vas deferens of 8-month-old ICR retired male breeders (Taconic). Sperm were released from dissected cauda and vas deferens into

3% BSA M16-modified Krebs-Ringer medium. Released sperm were incubated at 37 °C, 5% CO₂ for 3 h in the same medium to allow them to capacitate and acrosome react. Eggs were collected from the oviducts of 8 to 10 week old superovulated female ICR mice (Taconic) or C57 mutant progeny that were wild type (Cre⁻ β_1 +/+), heterozygous (Cre⁺ β_1 +/f), or knockouts (Cre⁺ β_1 f/f) for the β_1 allele. Mice were superovulated by injecting 5 IU PMSG (obtained through NHPP, NIDDK and Dr. A. F. Parlow), followed 48-52 hr later by an injection of 10 IU hCG. 14-16 h after hCG injection, oviducts were removed from euthanized mice and were incubated in prewarmed M16 medium with 0.5% BSA. Cumulus-egg complexes were collected and transferred to 500- μ L drops of medium containing 30 μ g/mL hyaluronidase surrounded by mineral oil. After 5 min incubation, cumulus-free metaphase II eggs were collected, transferred first to an 80- μ L drop of medium, and then washed through six 40- μ L drops of medium. Eggs were recovered for 1 h before treating with Tyrodes acid. Zona pellucidae (ZP) of metaphase II eggs were removed by incubating eggs in a 100 μ L Tyrodes acid drop for 1 min at rt followed by mechanical removal of the ZP through a pipette. ZP-free eggs were washed six times with 0.5% BSA medium, were recovered for 2 h in 0.5% BSA/M16, and then were loaded with Hoechst 33342 at 10 μ g/mL for 30 min. Eggs were washed and placed in 100- μ L drops of 3% BSA/M16. At the same time, polymers were fully reduced with 10 mM TCEP for 1 to 2 h, precipitated with 1N HCl and washed with water, and then redissolved in water adjusted to pH 7 with NH₄OH. Polymer solution was added to the egg drop (no more than 5 μ L of stock solution) and the eggs incubated for 45 min prior to sperm addition. Eggs were inseminated with 1 $\times$ 10⁵ sperm/mL for 45 min, were washed in 3% BSA/M16, and were mounted onto glass microscope slides. Sperm binding and fusion were scored by epi-fluorescence microscopy and DIC microscopy (NIKON Eclipse 400, 40X, 0.75 NA objective). Fusion was scored as the fluorescent labeling of sperm nuclei with Hoechst 33342 present in the loaded eggs. The mean number of sperm fused per egg (fertilization index, FI) and percentage of eggs fertilized (fertilization rate, FR) were measured.

Immunofluorescence Microscopy

Cumulus and ZP layers were removed as described above, and eggs were allowed to recover in 0.5% BSA/M16 for 2 h. Eggs were then treated with 30 μ g/mL rat anti- α_6 integrin mAb GoH3 (isotype IgG2a, Molecular Probes) or with 5 μ g/mL rat anti- β_1 mAb CD29 (isotype IgG2a, BD Pharmingen) for 45 min, washed in M16 for 10 min, and fixed with 4% paraformaldehyde. Then the eggs were stained with FITC (30 μ g/mL) or Alexa488-conjugated (5 μ g/mL) IgG2a goat anti-rat secondary antibody (Molecular Probes) for 45 min, washed, and mounted. Eggs were imaged on a Zeiss Axiovert with a GFP/FITC filter and 0.55 NA, 20X objective. For polymer labeled eggs, the recovered ZP-free oocytes were washed with four 60- μ L drops of 1% PVP/M16, placed in a 100- μ L drop of Alexa488-conjugated polymer solution in 1% PVP/M16, and incubated at 37 °C, 5% CO₂ for 45 min. The polymer solutions were prepared by diluting the stock polymer solutions with the buffer, and no more than 6 μ L of stock solution was diluted. The concentration of polymers in the 100- μ L drop was 20 μ M in polymer concentration. Oocytes were irradiated with UV light (λ_{max} = 350 nm, under 15 cm) at 4 °C for 15 min. The photoaffinity labeled oocytes were gently washed twice through 300- μ L drops of 1% PVP/M16 by shaking at 50 rpm for 10 min. After fixing the oocytes with a 100- μ L drop of 4% paraformaldehyde in PBS at rt for 10 min, oocytes were washed through four 60 μ L drops of 0.5% BSA/M16, mounted and imaged as described above.

Induction of Calcium Oscillations

Eggs were harvested no later than 12 h after hCG injection. After ZP removal with Tyrodes acid and recovery for 1.5-2 h, the ZP-free eggs were incubated for 30-40 min in 10 μ M Fura-2AM, 0.025% Pluronic F-127/0.05% BSA/M16. Eggs were washed and transferred to

glass bottom dishes (MatTek Corp.) which were pretreated with Cell-Tak (Sigma) and eggs were allowed to adhere for 10 min. Samples were placed on a microscope stage thermostatted at 37 °C. Polymer (30 μ M) was added to egg samples directly on the microscope stage. The ratio of fluorescence emission at 510 nm with excitation at 340 nm and 380 nm was recorded using Carl Zeiss Axiovision CD28 Software.

Measurement of Pronuclei Formation

Eggs were harvested and their cumulus cells were removed with hyaluronidase and were allowed to recover for 1 h in 1.5% BSA/M16. Eggs were placed in 100- μ L drops of the same buffer (covered with mineral oil), which contained either of the following: buffer only, capacitated sperm (1×10^5 sperm/mL), or polymers (**1**₁₀, 50 μ M; **2**₁₀, 500 μ M). After 2 h, all eggs were washed in parallel and incubated for another 6 h at which time they were scored for pronuclei formation by inspection under DIC optics. Prior to insemination and after isolation from the cauda epididymis, sperm were incubated in 1.5% BSA/M16-modified Krebs-Ringer medium 37 °C, 5% CO₂ for 1.5 h to allow capacitation without acrosome reaction.

Reversibility of Inhibition by Polymers

IVF inhibition assays were performed as described above with the exception that the ZP-free, polymer-treated (10 μ M) eggs were either inseminated without washing away polymer or were washed 6 times in 50- μ L drops of 3% BSA/ M16 prior to insemination. Capacitated and acrosome-reacted sperm were added to eggs at 2 time points: immediately after washing the eggs, or 3 h after the wash. The final concentration of sperm was 1.5×10^5 sperm/mL. Eggs were inseminated for 45 min, then they were washed with 3% BSA/M16. Eggs were mounted onto glass microscope slides, and FR and FI were scored as described above.

Inhibition of Egg Block to Polyspermy

ZP-free eggs were loaded with Hoechst 33342 in 0.5% BSA/M16 as described above. Eggs were then treated with 10 μ M BAPTA-AM or 40 μ M cytochalasin D for 60 min in 0.5% BSA/M16, 0.025% pluronic F-127. Control eggs were incubated in 1% DMSO. After 60 min of incubation with drug, BAPTA-AM treated eggs were washed 6 times in 0.5% BSA/ M16, cytochalasin D treated eggs were not washed because actin perturbation induced by cytochalasin D is reversible, and the drug can be washed out (56). Drug-loaded eggs were treated with polymer (10 μ M) as described above for 45 min. Eggs were inseminated with 1×10^5 sperm/mL for 45 min, washed with 3% BSA/M16, and the fertilization index (FI) was measured.

Sperm Binding Kinetics in β_1 Knockout Versus Wild-Type ZP-free Eggs

After ZP removal with Tyrodes acid and 1.5 h recovery in 0.5% BSA/M16, eggs were transferred to glass bottom dishes which were pretreated with Cell-Tak and the eggs were allowed to adhere for 10 min. Samples were placed on a microscope stage thermostatted at 37 °C and inseminated. The plane of focus was centered on the equator of the egg. DIC and Hoechst 33342 images were recorded every 2 sec for 20 min using Carl Zeiss Axiovision CD28 Software. Images were scored for sperm bound and sperm fused.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Structures of polymers used in this study. Multiple copies of glutamate-cysteine-aspartate (ECD) and/or glutamate-serine-alanine (ESA) peptides are displayed along a norbornene scaffold prepared using ring opening metathesis polymerization (ROMP) (1,2).

Figure 2.

Multivalent and control ECD polymers inhibit ZP-free fertilization via an egg β_1 integrin. Gray bars: WT ($\text{Cre}^-\beta_1^{+/+}$) eggs. Black bars: KO ($\text{Cre}^+\beta_1^{f/f}$) eggs. Experiments were performed with an average of 50 eggs (range 25-90) at each condition in 3-6 independent experiments. The percentage of eggs fertilized in the presence of inhibitor are shown relative to the untreated control for each condition. In the untreated controls, $96 \pm 4\%$ of WT eggs and $92 \pm 6\%$ of KO eggs were fertilized. The average number of sperm fused per egg was 1.7 ± 0.1 and 1.6 ± 0.2 , respectively. The concentrations of polymer are shown, not the concentrations of peptides. Errors are the s.e.m. * $p < 0.05$.

Figure 3.

Polymer **1₁₀** is a parthenogenetic agent. The percentage of pronuclei formed at each condition is shown relative to the sperm-treated control. After 2 hours of polymer treatment (50 μ M **1₁₀** or 500 μ M **2₁₀**) or insemination, ZP-intact eggs were washed and 6 hours later were scored for pronuclei formation. Gray bars: WT, Black bars: KO. In the sperm-treated controls, $51 \pm 7\%$ of WT eggs and $63 \pm 4\%$ of KO eggs were activated. 50-100 eggs were assayed at each condition in 3-5 independent experiments. The concentrations of polymer, not peptide, are given. Errors are the s.e.m. * $p < 0.05$. Representative microscope images are shown in Figure S6.

Figure 4.

Inhibition by polymer **1**₁₀ is reversible. ZP-free eggs were treated with polymer **1**₁₀ (10 μ M) for 45 minutes and then were inseminated after 0 or 3 hours with 1×10^5 /mL sperm or they were washed 6 times in 3% BSA/ M16 prior to insemination. Approximately 40-50 eggs per condition were tested in three independent experiments. In the unwashed control, $94\% \pm 4\%$ WT eggs were fertilized. The average number of sperm fused per egg was 1.8 ± 0.3 . Error is sem. * $p < 0.05$ versus other four conditions.

Figure 5.

Inhibition by polymer **1**₁₀ does not require egg activation. ZP-free eggs were treated with BAPTA-AM (10 μ M, gray bars) or cytochalasin D (40 μ M, white bars), followed by polymer **1**₁₀ (10 μ M) for 45 min, then inseminated with 1×10^5 /mL sperm. Error is sem. * $p < 0.05$. Approximately 60 eggs were tested at each condition in 5 independent experiments. The average number of sperm fused per egg is normalized to the untreated control. In the untreated controls, the average number of fused sperm was 1.3 ± 0.2 (BAPTA-AM) and 1.8 ± 0.3 (cytochalasin D). a) WT eggs. b) KO eggs. The difference between Bapta⁺/**1**₁₀⁻ and BAPTA⁺/**1**₁₀⁺ is not statistically significant in KO eggs.

Figure 6. Fluorescent staining of WT and KO ZP-free eggs with Alexa₄₈₈-conjugated analogs of **1₁₀** (polymer **5**) (a-d) and **2₁₀** (polymer **6**). (e-f). a, b) WT eggs; c-f) KO eggs. a, c, e) DIC images and b, d, f) epi-fluorescence images. The second polar body stains non-specifically with polymer. All images were taken with a 20X objective and a digital zoom.

Figure 7.

The presence of β_1 integrin on the egg increases the rate of sperm binding and fusion. Approximately 100 eggs were tested for each genotype in 6 independent experiments. Errors are s.e.m. a) Kinetic comparison of sperm binding to ZP-free WT and KO eggs. Data are shown for 1-minute intervals. b) Magnification of first 3 minutes from panel (a). Data are shown for 0.25-minute intervals. $p < 0.05$ for 0 - 2 minutes between WT and KO eggs. c) Kinetic comparison of sperm fusion kinetics to WT and KO eggs. Data are shown for 1-minute intervals. d) Magnification of first 7 minutes from panel (c). Data are shown for 0.25-minute intervals. $p < 0.05$ for 4 - 5 minutes between WT and KO eggs.