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Fertilinβ peptidic liposomes inhibit fertilization by steric blockage

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Abstract—Liposomes presenting fertilinβ peptides are inhibitors of mouse in vitro fertilization. We undertook a study of the relationship between IC_{50} and mole fraction of fertilinβ in the liposome, and the size dependence of inhibition of fertilization to understand the mechanism of their inhibition. Our results indicate that a small number of multivalent contacts are required for efficient attachment of inhibitor to receptor on the target membrane, and that, as designed, the liposomes target the egg membrane but not the sperm membrane. The size dependence of the liposome inhibition demonstrates that the liposomes physically block access of the sperm to the egg membrane thereby preventing sperm binding to all egg receptors not just the fertilinβ receptor.

1. Introduction

A complex series of events results in sperm–egg fusion. These events include sperm binding to the zona pellucida, acrosome reaction of the sperm, and sperm binding to the egg plasma membrane. Many proteins are involved in the latter step that immediately precedes fusion. One of the best characterized is fertilinβ that is an integral membrane protein on the surface of the acrosome-reacted sperm.

Fertilinβ is a member of the ADAM (A Disintegrin and Metalloprotease) family of proteins[1,2] and is present in all mammalian species sequenced to date.[2] On the mature sperm, the pre-, pro-, and metalloprotease domains have been proteolytically cleaved, with the disintegrin, cysteine-rich, and EGF-like domains remaining on the extracellular moiety. Both recombinant fertilinβ and peptides corresponding to the putative fertilinβ disintegrin binding loop inhibit fertilization in vitro. Male fertilinβ knockout mice are infertile and the levels of fertilin β-/-sperm binding to wild-type eggs are reduced 8-fold compared to wild-type sperm in vitro.[3] These experiments all support a functional role for fertilinβ in sperm-egg binding.

Previous work in our laboratory, as well as others, identified the three amino acid sequence, glutamate–cysteine–aspartate, ECD, of the fertilinβ disintegrin domain as the minimum sequence required for inhibition of sperm-egg binding.[4–7] The aspartate is the most important residue for binding as judged by mutagenesis studies using recombinant fertilinβ disintegrin domain.[6,7] Many different linear peptides containing the ECD sequence have been synthesized, and the IC_{50} for inhibition of sperm–egg binding is typically 500 μM. These peptides have been tested in a variety of species, for example, guinea pig, mouse, monkey and human, and inhibit fertilization in all of them. Thus, ECD is a promising pharmacophore for development of inhibitors of fertilization.

Work from several laboratories has identified integrin $\alpha_6\beta_1$ as the egg plasma membrane integrin receptor for fertilinβ.[6,8–11] A combination of photoaffinity labeling experiments[11] and cell-binding assays with recombinant wild-type and mutant proteins[6,8] suggest that fertilinβ binds directly to integrin $\alpha_6\beta_1$. This interaction may be inhibited with small peptides that contain the binding sequence of fertilinβ or recombinant protein fragments that correspond to the disintegrin domain of fertilinβ.[4,5,11–15] On the basis of these experiments, it has been proposed that fertilinβ-$\alpha_6\beta_1$ binding is a precursor to sperm–egg fusion. In addition, it has recently been demonstrated that fertilinβ, along with several other ADAM proteins, binds to $\alpha_9\beta_1$ integrin,[16,17] yet integrin $\alpha_9\beta_1$ has not been detected on the mouse egg surface or in the ovary.[17,18] However, female mice with a conditional knockout of integrin β_1 in their oocytes are fertile in vivo and in vitro.[19] One reconciliation of the discrepancy between the biochemical and genetic experiments is that there may be redundancy of ligand-receptor pairs in wild-type fertilization, that is, more than one egg receptor

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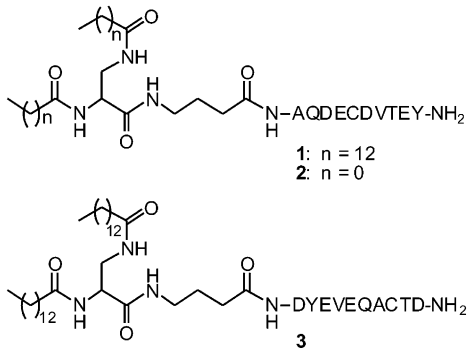
type may be operating. This proposed redundancy is not surprising considering that survival of an organism depends on successful fertilization.

Both linear peptides[4,5,14,15] and existing peptide mimics[20] of fertilinβ based on the ECD motif are modest inhibitors of sperm–egg binding with IC_{50}'s around 500 μM. We hypothesized that the modest inhibition observed with linear peptides might be due to a monovalent binding interaction that is of low affinity and have been pursuing the syntheses and analyses of polyvalent liposome inhibitors. Previously, we demonstrated that when the dimyristoylated peptide **1** is mixed with 1-palmitoyl, 2-oleoylphosphatidyl choline (POPC) at 1 mol% to form small unilamellar liposomes, the concentration of fertilinβ peptide required for 50% inhibition is reduced 200-fold compared to its monomeric counterpart **2** (Table 1).[21] Scrambled sequence **3** showed no inhibition up to 50 μM (1 mol%, 5 mM POPC). In addition, POPC vesicles that had no myristoylated peptide incorporated did not inhibit sperm–egg binding or fusion. Thus, the dimyristoylated peptides on the surface of the SUVs are accessible to their receptor on the surface of the egg and block sperm binding.[21] In addition, to improved potency of inhibition, the concentration dependence of inhibition demonstrated complete inhibition at 10 μM peptide **1** in 1 mol% liposomes. This is in marked contrast to monomeric peptide **2**, other monomeric peptides,[4,5,14,15] and polymeric peptides[22] that do not inhibit more than 70–80% in IVF assays. The incomplete inhibition by fertilinβ monomers and the more potent polymers was in agreement with the evidence that there might be two sperm receptor types on the egg surface, and suggested that the fertilinβ peptides could only competitively block one, presumptively $\alpha_6\beta_1$ integrin. We postulated that the size of our fertilinβ liposomes was sufficiently large to sterically block access of the sperm to additional sperm receptors on the egg without competitively blocking the receptors' binding sites, thereby leading to complete inhibition of sperm–egg binding. Here we describe our investigation



of this hypothesis by varying the density of fertilinβ ligands in the liposome and the size of the liposomes.

We first undertook a study of the relationship between IC_{50} and mole fraction of fertilinβ in the liposome. We found that as the average number of peptides in the outer monolayer decreased, the IC_{50} in terms of peptide concentration also decreased (Table 1). At first, this may seem counter-intuitive, but if one considers the IC_{50} in terms of vesicle concentration instead, the IC_{50} remains nearly constant within experimental error.[23] Thus, it appears that inhibition is dependent on the number of vesicles added and that a small number of fertilinβ peptides is sufficient to efficiently attach the liposome to the surface. However, multivalent presentation of fertilinβ peptide is required for liposome attachment. Liposomes presenting on average one peptide in the outer leaflet (0.013 mol%) did not inhibit fertilization at liposome concentrations (1 mM POPC) twice the highest IC_{50} observed for higher density liposomes.

These data are consistent with a mechanism of inhibition in which the liposomes block sperm binding by physically coating the surface and would explain the complete blocking of sperm binding obtained. If steric stabilization is the primary mechanism, then one would predict that blocking would require fewer larger liposomes. We next assayed 400 nm liposomes with 0.5

Table 1. IC_{50}'s for inhibition of fertilization by fertilinβ liposomes[a]

Liposome	No. peptides in outer leaflet[b]	IC_{50} (μM peptide) by FR	IC_{50} (μM peptide) by FI	IC_{50} (liposomes/mL)$\times 10^{12}$ by FR	Maximal % inhibition observed
5 mol% **1**, 40 nm[c]	385	5.0 (45±2.6%)	5.0 (51±4.2%)	4.2±0.4	n.d.[d]
1 mol% **1**, 40 nm	77	2.5±0.3	2.4±1.9	11 ±1.3	97±2
0.5 mol% **1**, 40 nm	39	0.34±0.12	0.50±0.5	2.8±1.0	93±7
0.1 mol% **1**, 40 nm[c]	8	0.50 (39±5%)	0.50 (52±10%)	21±2.7	n.d.
0.013 mol% **1**, 40 nm	1	n.i.[e]	n.i.[e]	n.i.	n.d.
0.5 mol% **1**, 400 nm	3900	0.75±0.01	0.92±0.46	0.059±0.001	n.d.
2	—	500±26	524±3	—	71±3
1 mol% **3**, 40 nm	80	n.i.[f]	n.i.[f]	n.i.	n.i.
POPC, 40 nm or 400 nm	0	n.i.[g]	n.i.[g]	n.i.	n.i.

[a] The ratio of fertilization in the presence of peptide as measured by number of zona-free eggs fertilized (FR, fertilization rate) or average number of sperm fused per egg (FI, fertilization index) relative to a non peptide control. The average FR for the no peptide control was 76% eggs fertilized and the average FI was 1.6 sperm fused per egg. Error bars are s.e.m. for a fit at 5–10 different concentrations, with 30–75 eggs per concentration.
[b] We calculated the number of peptides present based on an average surface area of 65 $Å^2$/PC molecule, an outer diameter of 40/400 nm, and an inner diameter of 37/397 nm to determine the number of lipid molecules per liposome, and from the mol% of peptide used.
[c] % Inhibition was determined at the single concentration given with 30–50 eggs per concentration, and the % inhibition±s.e.m. observed is given in parentheses.
[d] Not determined.
[e] No inhibition observed at 125 nM peptide (1 mM POPC).
[f] No inhibition observed up to 50 μM peptide.
[g] No inhibition observed up to 500 μM POPC.

mol% fertilinβ peptide prepared size selectively by extrusion, and found that the IC_{50} in peptide is 0.75 μM, however the IC_{50} in number of vesicles *decreases* to 0.059×10^{12} vesicles/mL. (With the larger liposomes, there is more peptide/liposome and thus the peptide IC_{50} increases.) If one approximates the egg surface area as a sphere and assumes that the vesicles and receptors are uniformly distributed, a very rough approximation because the egg surface is replete with microvilli and has an amicrovillar region over the meiotic spindle, a surface area calculation predicts that for a 10-fold increase in liposome diameter, one would expect a 100-fold reduction in number of vesicles required to coat the surface. We observe that 47-fold fewer 400 nm vesicles are required than 40 nm vesicles. These numbers are gratifyingly close considering the uncertainties and assumptions in the calculations, and support our hypothesis that the liposomes sterically block the egg surface. That is, the efficiency of inhibition increases as the size of the liposome increases.

We investigated the localization of liposome binding using fluorescence microscopy. We prepared control liposomes containing 0.1 mol% POPE-Oregon Green, 99.9 mol% POPC. Our control liposomes do not bind to the surface of the egg (Fig. 1A and 1D), but do show non-specific background staining. We then prepared liposomes containing 1 mol% **1**, 0.1 mol% POPE-Oregon Green, 98.9 mol% POPC. The liposomes bind to the surface of the egg (Fig. 1B,C,E and F). However, these

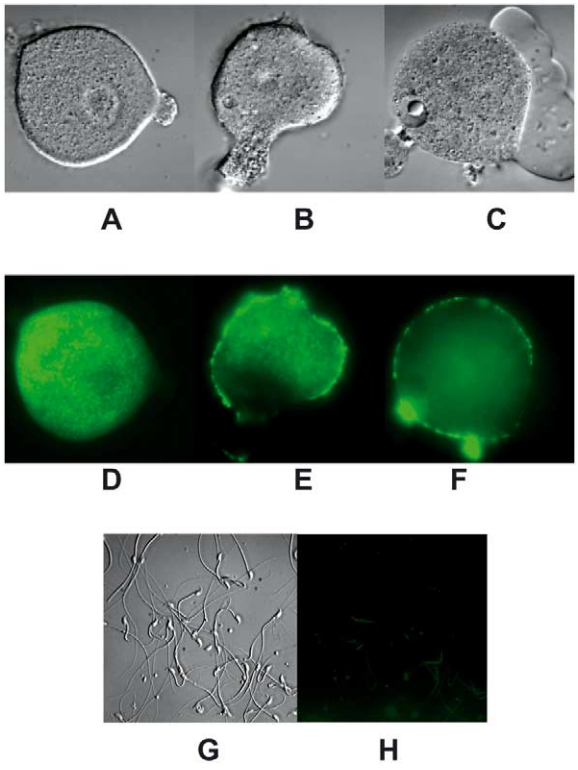


Figure 1. Visualization of liposome binding to zona-free eggs (A–F) and sperm (G–H) (60X, 1.40 NA objective). DIC images: A–C, and G. Epi-fluorescence images of liposomes with oregon green phosphatidylethanolamine (0.1 mol%): D–F, and H. A and D stained with POPC liposomes (0.5 mM in lipid) (no peptide **1**). B–C and E–H stained with 1 mol% **1** POPC liposomes (0.5 mM in lipid).

complexes are not stable for long incubation times (data not shown). Fertilinβ peptide containing liposomes do not bind to the sperm surface (Fig. 1G and H). Liposome inhibition is clearly due to blocking of the sperm from binding to the egg surface by the attachment of the liposome to the egg. Our mole fraction dependence data indicate that a very small number of fertilinβ peptides is required for efficient attachment of the liposome to the egg surface, but that once bound, the liposomes can present a steric barrier that prevents sperm binding to all egg receptors.

Our data suggest a general strategy to inhibiting cell–cell interactions without knowledge of all physiologically relevant receptors. It is only necessary to identify a receptor-ligand pair for which the ligand may be presented on the surface of a liposome. After attachment of the liposome via the known receptor to one cell surface, a physical barrier will be created that prevents approach and adhesion of a second cell. In combination with kinetically stabilized liposome structures, this inhibition methodology may lead to improved therapeutic technologies.

2. Experimental procedures

2.1. Peptide synthesis and liposome preparation

Peptides were synthesized, purified and incorporated into 40 nm liposomes as described.[21] After 5 freeze (−176 °C)/thaw (37 °C) cycles, 400 nm liposomes were prepared by extrusion[24] through two stacked 400 nm filters (Costar, Cambridge, MA) ten times using a nitrogen gas pressure of 350–400 psi to provide a homogeneous batch of vesicles.

2.2. In vitro fertilization assay

Inhibitors were assayed as described.[5]

2.3. Fluorescence microscopy

Zona-free oocytes were isolated from female mice as described.[5] Oocytes were incubated for 1 h with liposomes (0.5 mM lipid) containing 98.9 mol% POPC, 1 mol% peptide **1**, and 0.1 mol% oregon green-PE. Eggs were transferred to a 0.3 mL drop of M16 medium (0.5% BSA) and shaken for 10–15 min at 30 rpm. Then eggs were fixed in a 100 μL drop of formaldehyde (3.7%, 0.1% polyvinylpyrrolidinone, 1X PBS) for 10–15 min and mounted in Vecta Shield mounting medium (5 μL). The samples were viewed by epifluorescence microscopy. Incubations with control liposomes were performed in an identical manner.

Sperm were isolated from the cauda epididymis of 2 male mice and incubated in M16 medium (3% BSA) at 37 °C for 3 h. They were incubated for 1 h at 37 °C with fertilinβ liposomes (0.5 mM lipid) prepared as described above. Sperm were washed twice in M16 (3% BSA). The sperm were then fixed in 3.7% formaldehyde (PBS) for 10–15 min, mounted in Vecta Shield mounting medium and the sample was viewed by epifluorescence microscopy.

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