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Dimyristoylated Peptides Incorporated into Liposomes Are Polyvalent Fertilin β Mimics

Suparna Gupta and Nicole S. Sampson*

Department of Chemistry, State University of New York, Stony Brook, New York 11794-3400

Nicole.sampson@sunysb.edu

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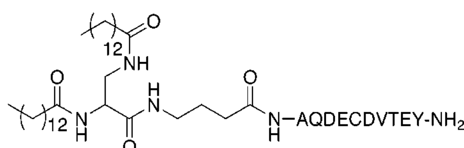
ABSTRACT



Fertilin β is an integral membrane sperm protein that is involved in sperm binding to the egg plasma membrane. We synthesized a dimyristoylated fertilin β peptide and incorporated it into POPC liposomes at 1 mol %. The concentration of fertilin β peptide required for 50% inhibition is reduced 100-fold to 5.2 ± 1.6 μM relative to a monomeric control. Moreover, in contrast to the inhibition observed with monomeric peptides, we obtain complete inhibition with the peptidic liposomes.

Fertilin β is a protein present on the equatorial membrane of fertilization competent sperm that is involved in sperm binding to the egg plasma membrane. It is present in all mammalian species sequenced to date.[1] It is clear from a combination of photoaffinity labeling experiments[2] and cell-binding assays with recombinant wild-type and mutant proteins[3] that fertilin β binds directly to the $\alpha_6\beta_1$ integrin receptor on the egg plasma membrane.[4,5] This interaction may be inhibited with small peptides that contain the binding sequence of fertilin β or recombinant protein fragments that correspond to the disintegrin domain of fertilin β.[3,6,7] However, complete inhibition of sperm binding or fusion is never observed with these mimics.

Incomplete inhibition by peptides or fragments that mimic fertilin β may be a result of the intricacy of this system or simply because the high avidity of the sperm for $\alpha_6\beta_1$ integrin receptors is not completely inhibited by moderate affinity monovalent mimics. Genetic "knock-out" experiments suggest that there may be redundant ligand−receptor pairs in sperm−egg binding.[8] However, the regulation of gene expression is quite complex in the sperm; for example, deletion of a single gene results in the disappearance of multiple sperm surface proteins.[9] To address the second possibility that high avidity is required for complete inhibition, we synthesized polyvalent fertilin β mimics.

There are many different approaches to designing and synthesizing polyvalent inhibitors. These include the use of linkers, tethering ligands to polymer backbones or dendrimers, and incorporation of ligands into liposomes or onto

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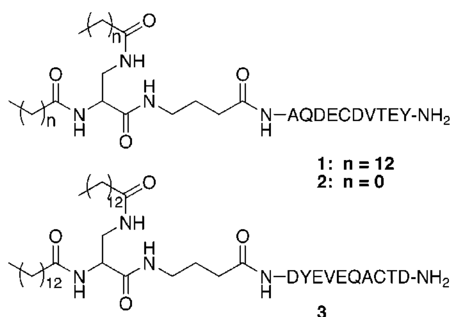
monolayer surfaces.[10] We report here our application of liposomes to study the polyvalent problem of sperm−egg adhesion. This polyvalent approach to receptor−ligand binding has been used successfully, for example, with selectins,[11] hemagluttinin,[12] Sendai virus,[13] enkephalin,[14] and antibodies.[15] For our purposes, liposomes allow easy variation of the surface concentration, i.e., mole fraction, of ligand and the combinations of ligand present. Thus, the use of liposomes will allow us to investigate the density required for maximal inhibition, and to include other sperm ligands, for example, cyritestin, that might also be involved in sperm−egg binding. Here, we present initial data demonstrating that a polyvalent fertilin β mimic is a much more potent inhibitor than its monovalent homologue.

We synthesized dimyristoylated fertilin β peptide **1** that contained the amino acid sequence from the fertilin β binding loop. In addition, we prepared a diacetylated monomeric control, **2**, as well as a dimyristoylated scrambled sequence control, **3**. These peptides were synthesized using standard



Fmoc solid-phase peptide chemistry on a Pal-Peg resin on a 0.2 mmol scale.[16] For peptides **1** and **3**, the myristic acid was coupled to the appropriate peptide chain while it was still attached to the resin, and the dimyristoylated peptide was deprotected and cleaved from the resin using trifluoroacetic acid/ethanedithiol/water (95:2.5:2.5). The peptide was dissolved in neat trifluoroacetic acid (TFA), diluted with CH_2Cl_2, and purified by flash chromatography (5 μ silica, 10 mm × 10 cm). The column was washed with $CHCl_3$/MeOH (90:10, 50 mL) and a gradient of TFA in CH_3CN (1−5%, 150 mL) to remove cleavage scavengers, and the peptide was eluted with 10% TFA in CH_3CN (50 mL). Peptide **2** was purified using reversed-phase HPLC (C18, 0.1% TFA, linear gradient of 5% CH_3CN/H_2O to 95% CH_3CN/H_2O). The purity of the peptides was assessed by reversed-phase HPLC (C4, 0.1% TFA, linear gradient of 5% CH_3CN/H_2O to 95% CH_3CN/H_2O) to be >99%. The identity of the peptides was confirmed by MALDI/TOF mass spectroscopy.

Peptides were analyzed by analytical HPLC immediately before use to confirm that the cysteine had not oxidized to form disulfide dimer. Either peptide **1** or **3** was mixed with 1-palmitoyl-2-oleoylphosphatidyl choline (POPC) in a 1:99 molar ratio in chloroform and dried. The lipid mixture was redissolved in EtOH to make a 100 mM stock solution. The lipid EtOH solution (100 μL) was added to a rapidly stirring solution of M16 media, 3% BSA (900 μL) to form small unilamellar vesicles (SUVs). A sample of the unilamellar vesicles was analyzed by dynamic light scattering. The vesicle size was homogeneous with an average diameter of 40 ± 5 μm. Aliquots were added to the IVF buffer (100 μL) containing mouse eggs to obtain the appropriate concentration. Peptide **2** was dissolved in water (10 mM), and an aliquot was added directly to the IVF assay. The IVF assay was performed as previously described using mouse eggs and sperm.[7] Briefly, the peptides or liposomes were incubated with eggs for 1 h, sperm were added, and after 1 h the eggs scored for number of sperm bound and fused, and number of eggs fertilized.

The IC_{50} for inhibition by peptide **2** was the same as previously observed for other monomeric fertilin β peptides, 524 μM (Table 1). Thus, the addition of the diaminopropionic acid and GABA linker to the N-terminus of the peptide does not affect inhibition. When the dimyristoylated peptide **1** is incorporated into SUVs at 1 mol %, the concentration of fertilin β peptide required for 50% inhibition is reduced 100-fold (Table 1). Peptide **3**, which presents the same amino acids in scrambled sequence, showed no inhibition up to 10 μM (1 mol %, 1 mM POPC). We were unable to add a higher concentration of lipid vesicles to the IVF assay. 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 appear to be accessible to the $\alpha_6\beta_1$ integrin receptor on the surface of the egg and block sperm binding, although they may be blocking a sperm surface receptor as well.

Table 1. IC_{50}'s for Inhibition of Sperm−Egg Adhesion as Measured by Number of Sperm Fused/Egg (Fertilization Index, FI)

peptide	IC_{50} (μM)	max inhibition obsd (%)
1 (1 mol % with POPC)	**5.2 ± 1.6**[a]	**98 ± 2**
2	**524 ± 3**	**71 ± 3**
3 (1 mol % with POPC)	**n.i.**[b]	
POPC vesicles	**n.i.**[c]	

[a] Reported as concentration of peptide, not liposomes. [b] No inhibition observed up to 50 μM **3**. [c] No inhibition observed up to 500 μM POPC.

In addition to improved potency of inhibition, the concentration dependence of inhibition demonstrated complete inhibition at 10 μM peptide **1** (Figure 1). This is in

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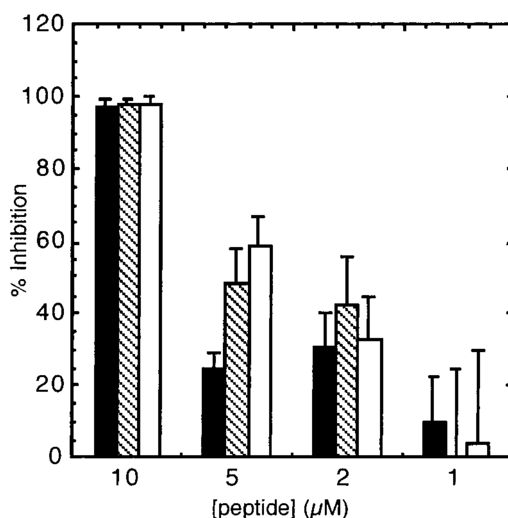

Figure 1. Concentration dependence of IVF inhibition by 1 mol % peptide **1** in POPC liposomes: solid bars, no. of eggs fertilized (FR); striped bars, no. of sperm fused/egg (FI); open bars, no. of sperm bound/egg. Percent inhibition is relative to a buffer control in which 70% of the eggs were fertilized and an average of 1.5 sperm were fused per egg. Error bars are SEM where $n = 5$, 70−100 eggs.

marked contrast to monomeric peptide **2** and other monomeric peptides[4,6,7,17] that do not inhibit more than 70−80% in IVF assays. By mimicking the polyvalent sperm surface display of fertilin β with a liposome, we obtain complete inhibition.

The exact mechanism of inhibition by the peptidic liposomes is unclear. Improved inhibition may be a result of an improved avidity of the peptidic liposomes for the egg in comparison to monomeric fertilin β mimics. Alternatively, improved inhibition may be due to steric blocking of the egg cell surface by the liposomes.[18] A third scenario is that inhibition may be improved due to clustering of the integrin receptor on the egg surface by the liposome. The receptor clustering could, in turn, be activating a signaling pathway within the egg that modulates the affinity of other egg surface receptors that are required for binding the sperm.[19] Future studies will address the importance of ligand density, lipid bilayer fluidity, liposome surface charge, and presentation of different sperm ligands for blocking sperm−egg binding as well as investigate the mechanism of polyvalent disintegrin inhibition in fertilization. The use of these polyvalent inhibitors will allow us to probe the molecular circuitry of sperm−egg adhesion.

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