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Characterization of Fertilinβ-Disintegrin Binding Specificity in Sperm–Egg Adhesion

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Abstract—An essential step leading to fertilization is the binding of a sperm to the egg plasma membrane. Fertilinβ, a membrane bound protein on the extracellular surface of sperm, partially mediates this binding via the $\alpha_6\beta_1$ integrin. Fertilinβ is a member of the still expanding family of ADAM proteins (a disintegrin and metalloprotease) that are implicated in many cellular functions ranging from neurogenesis to myoblast fusion and cytokine processing. Fertilinβ contains a highly conserved motif (D/E)ECD in the disintegrin domain. This suggests that (D/E)ECD could be the consensus sequence for recognition of disintegrins by $\alpha_6\beta_1$ integrins. Previously, it has been demonstrated that small peptides containing different moieties of this consensus sequence are inhibitors of in vitro fertilization. In the present study, we sought to determine whether a four amino acid peptide sequence with two adjacent acidic residues improved inhibition, and investigated the importance for inhibition of a cysteine versus a cystine. A series of linear and cyclic peptides were synthesized, in which either one or both adjacent acidic residues in the sequence DECD were mutated to their corresponding amides (N or Q). To explore the required oxidation state of the cysteine in the (D/E)ECD sequence, it was protected as a mixed disulfide. Our results indicate that only one acidic residue is required for inhibition of fertilization and a reduced C is required.

Introduction

An essential step leading to fertilization is the binding of a sperm to the egg plasma membrane. Fertilinβ, a membrane-bound protein on the extracellular surface of sperm, is thought to mediate this binding.[1–3] Small peptides that mimic the binding loop of fertilinβ inhibit fertilization in vitro.[4–10] In this work, we investigated the amino acid requirements for inhibition.

Fertilinβ is a member of the still expanding family of ADAM proteins (a disintegrin and metalloprotease) that are implicated in many cellular functions ranging from neurogenesis to myoblast fusion and cytokine processing.[11–13] The ADAM proteins are related to the soluble snake venom metalloproteases (SVMPs), and together they comprise the reprolysin super-family of proteins.[14] Both the ADAMs and SVMPs are modular and contain metalloprotease and disintegrin domains. In the ADAMs and some SVMPs, the disintegrin domain is followed by a cysteine-rich region and lectin-like or EGF-like region. The ADAMs have a transmembrane domain and cytosolic C-terminal tail. As new members of the ADAM family are characterized, more variations may be identified. However, the metalloprotease and disintegrin domains are present throughout the reprolysin super-family.

The disintegrin domains of the SVMPs were the first disintegrins to be characterized structurally and functionally. The P-II class of SVMP disintegrins are ligands for the integrin receptor $\alpha_{IIb}\beta_3$ on platelets.[15] Analysis of the P-II disintegrin amino acid sequences and their NMR structures revealed that 3 conserved amino acids, Arg-Gly-Asp (RGD), are present at the tip of a hairpin loop.[16–21] Moreover, small peptides and peptidomimetics containing the consensus binding sequence RGD of the disintegrin-binding loop inhibit the $\alpha_{IIb}\beta_3$-fibrinogen interaction, and consequently inhibit platelet aggregation. Thus, it is well established that the minimum recognition element for the $\alpha_{IIb}\beta_3$ integrins is the RGD sequence of the snake venom disintegrin domain. In addition, this recognition is dependent on the conformation of the peptide. Some RGD disintegrins bind to different integrin receptors, e.g. $\alpha_v\beta_3$. Conformationally constrained peptide mimetics have been designed that depending on their structure are specific for either $\alpha_{IIb}\beta_3$ or $\alpha_v\beta_3$.[22,23]

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The high (approx 50%) amino acid identity of ADAM disintegrins to the snake venom disintegrins suggested that the ADAM disintegrins might also be a family of extracellular ligands for integrin receptors. Despite the overall high similarity of amino acids between the ADAMs and the SVMPs, the binding loops of most ADAM disintegrins do not contain the RGD sequence. This difference suggested that the disintegrins might be specific for different integrin receptors depending on their binding loop sequence.

In fact, several investigations have provided evidence that the fertilinβ disintegrin domain binds to the $\alpha_6\beta_1$ integrin receptor on the egg membrane during sperm–egg binding and fusion. First, these integrin subunits, as well as others, have been identified on the surface of mammalian eggs.[5,24–27] Second, small peptides corresponding to the disintegrin binding loop of fertilinβ inhibit fertilization in vitro in guinea pigs,[4] mice,[5–8] monkeys[9] and humans.[10] Third, sperm will only bind to somatic cells that express both the α_6 and the β_1 integrin subunits.[5] Fourth, a 13-amino acid photoaffinity peptide corresponding to the disintegrin binding loop of fertilinβ inhibits in vitro fertilization and exclusively labels the $\alpha_6\beta_1$ integrin in mouse eggs.[28] Fifth, monoclonal antibodies to β_1 integrin inhibit sperm and recombinant fertilinβ binding to mouse eggs.[5,29]

These investigations all support the conclusion that the disintegrin domain of the extracellular sperm protein fertilinβ binds to $\alpha_6\beta_1$ integrin on the plasma membrane of the egg. However, the minimal peptide motif for binding to $\alpha_6\beta_1$ (analogous to RGD) has not yet been ascertained. The sequence alignment between the disintegrin domains of SVMPs and ADAM proteins (Fig. 1) reveals that the QDE (in mice) and TDE (in guinea pig) sequences of fertilinβ disintegrin directly align with the RGD sequence in the snake venom disintegrins. Therefore, it was originally postulated that these could be the putative binding sites for fertilinβ. To test this hypothesis, many small peptides containing the QDE or TDE sequence were synthesized by different researchers and tested as inhibitors of sperm-egg fusion.[4–9] Inhibition by these peptides provided evidence for the involvement of fertilinβ disintegrin domain in the mammalian sperm–egg fusion.

However, as more and more sequences of fertilinβ from different species were determined, their alignment revealed that the ECD sequence is highly conserved in the binding loop of fertilinβ. The alignment suggested that ECD could be the minimal recognition sequence for $\alpha_6\beta_1$ integrin on the egg. In a previous study conducted in our laboratory, a series of cyclic and linear peptides comprising the sequence ECDAY and their dimers were synthesized and tested for their inhibitory effects. The linear and cyclic forms of the ECDAY peptides inhibited sperm-egg fusion 50% at 500 μM relative to a non-peptide control. Interestingly, the disulfide dimers did not inhibit in vitro fertilization in mice, nor did an ESDAY mutant. These results supported the importance of the free thiol of the cysteine residue for inhibition. However, a control peptide with a scrambled sequence, CDEAY, also inhibited fertilization. In addition, the disulfide dimer of CDEAY was also an inhibitor. It appeared that two adjacent acidic residues might be sufficient to inhibit sperm binding. As more fertilinβ sequences became available, the alignments hinted that perhaps (D/E)ECD was actually the consensus sequence. At that time, we hypothesized that the CDEAY mimicked the DE of the DECD sequence and that the dimerization of CDEAY did not interfere with binding.

SVMP P-II disintegrins		
	Kistrin[15]	CKFSRAGKICRIP**RGD**-MPDDRCTGQSADC
SVMP P-III disintegrins		
	Atrolysin E[38]	CKFTSAGNVCRPAR**SECD**IAESCTGQSADC
SVMP P-IV disintegrins		
	RVVX[39]	CKIKTAGTVCRAAR**DECD**VPEHCTGQSAEC
ADAM disintegrins		
1	mouse (fertilinα)[6]	CTFKKKGSLCRPAE**D**V**CD**LPEYCDGSTQEC
2	mouse (fertilinβ)[6]	CKLKRKGEVCRLAQ**DECD**VTEYCNGTSEVC
2	monkey[40]	CLFMSQERVCRPSF**DECD**LPEYCNGTSASC
2	guinea pig[3]	CEFKTKGEVCREST**DECD**LPEYCNGSSGAC
2	human [41]	CLFMSKERMCRPSF**EECD**LPEYCNGSSASC
2	cow [42]	CAFIPKGHICRGST**DECD**LHEYCNGSSAAC
2	rat[43]	CNLKAKGELCRPANQ**ECD**VTEYCNGTSEVC
2	rabbit [44]	CTFKERGQSCRPPVG**ECD**LFEYCNGTSALC
3	mouse (cyritestin) [45]	CTIAERGRLCRKSK**D**Q**CD**FPEFCNGETEGC

Figure 1. Sequence alignment of the residues comprising the binding loop region of representative disintegrins. Conserved cysteines are outlined, the binding loop is underlined, and the consensus residues are boldfaced.

In the present study, we sought to clarify whether two adjacent acidic residues improved inhibition and to further investigate the importance of a free thiol for inhibition. A series of linear and cyclic peptides was synthesized (Fig. 2), in which either one or both adjacent acidic residues in the sequence DECD were mutated to their corresponding amides (N or Q). The cysteine free thiols were prepared, as well as the thiomethyl disulfide of DECD.

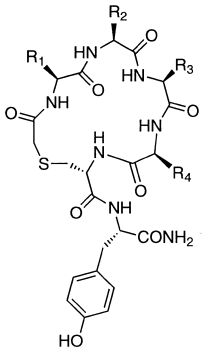
Results

Peptide synthesis

Peptides corresponding to the disintegrin domain of mouse fertilinβ were assembled using solid-phase synthesis and α-amino Fmoc protection.[30] All peptides were purified by preparative RP-HPLC, and their purity was assessed by analytical RP-HPLC. Nucleophilic displacement of bromine from an α-bromoacetyl moiety yielded the cyclic peptides **1b** and **3**. These cyclizations were effected by employing a dual protection scheme for the two Cys residues at the C-terminus and the N-terminus using trityl and acetamidomethyl resepctively. The identity of intermediate and final peptides was confirmed by mass spectrometry (Table 1).

In vitro fertilization assay

An in vitro assay using mouse eggs and sperm was employed to test the effect of the peptides on sperm binding and fusion to the egg. Zona free eggs and in vitro acrosome-reacted sperm were used in order to study the interactions occurring at the plasma membrane. The concentration of sperm used (5×10^4 sperm/mL) was chosen so that the control had on average one sperm fused per egg, i.e. a physiologically relevant level of sperm–egg fusion. Eggs were preloaded with Hoechst 33342, a dye that fluoresces when intercalated with DNA. Thus upon excitation with UV light, egg chromosomes fluoresce blue. If a sperm fuses with a preloaded egg, the dye leaks into the sperm cytoplasm and the sperm chromosomes also fluoresce. A sperm was scored fused if a sperm head was observed with phase-contrast microscopy, and if it fluoresced.



1a Ac - D - E - C - D - A - Y - NH_2

2 Ac - N - Q - C - D - A - Y - NH_2

4 Ac - D - Q - C - D - A - Y - NH_2

5 Ac - E - C - D - A - Y - NH_2

	R_1	R_2	R_3	R_4
1b	CO_2H	CO_2H	SH	CO_2H
3	CO_2H	CO_2H	S-SMe	CO_2H

Figure 2. Peptides synthesized and tested as inhibitors of fertilization.

Table 1. Chromatographic and mass spectrometric analyses of peptides **1–5**

Peptide	Formula	$[MNa^+]$[a]	t_R[b] (min)	Purity (%)[c]
1a	$C_{30}N_7O_{14}H_{40}S = 755.1$	778.1	17.1	99
1b	$C_{30}N_7O_{14}H_{39}S_2 = 785.1$	808.1	8.7	98
2	$C_{30}N_9O_{12}H_{43}S = 753.2$	776.2	13.0	98
3	$C_{31}N_7O_{14}H_{41}S_3 = 831.1$	854.1	19.8	97
4	$C_{30}N_8O_{13}H_{42}S = 754.1$	777.2	12.1	95
5	$C_{25}N_6O_{11}H_{36}S = 640.1$	663.1	12.4	98

[a]Molecular ion observed in mass spectrum obtained by MALDI–TOF.
[b]Analytical RP-HPLC; gradient 5–65% CH_3CN in H_2O (containing 0.1% TFA) over 60 min.
[c]By integration of RP-HPLC chromatograms (λ = 220 nm).

The inhibition properties of the peptides were measured in two ways. Both the average number of sperm fused per egg (fertilization index) and the percentage of eggs fertilized (fertilization rate) were measured in the presence and absence of peptides. The average fertilization rate and index for the non-peptide control were 68% and 1.2 sperm/egg respectively.

Each peptide was tested in 3–7 independent trials. The results of these assays are presented in Table 2. Peptides **1a**, **1b**, **4** and **5** all inhibited in vitro fertilization. Peptides **2** and **3** did not inhibit fertilization. Peptides **1a** and **5** were assayed in the same trial and with the same control. There was no significant difference in their potency. Inhibition by peptide **1a** was concentration dependent as shown in Figure 3.

Discussion

Many integrins recognize ligand amino acid sequences which contain an acidic residue that is essential for receptor binding. For instance, many integrins recognize the Arg-Gly-Asp (RGD) motif that is present in extracellular matrix proteins, e.g. fibronectin, vitronectin, and von Willebrand factor. The RGD motif in these extracellular ligands can bind to a variety of

Table 2. Linear and cyclized peptides evaluated in this study and their effect on in vitro fertilization of mouse eggs

Peptide	% Inhibition by FR[a]	% Inhibition by FI[b]
1a	64 ± 5	78 ± 5
1b	59 ± 6	79 ± 7
2	−0.75 ± 16	18 ± 9
3	4 ± 25	16 ± 3
4	70 ± 9	80 ± 7
5	70 ± 6	93 ± 3

[a]The ratio of fertilization in the presence of 500 μM peptide as measured by number of eggs fertilized (fertilization rate) relative to a non peptide control. The average FR for the no peptide control was 68%. Errors are ± SEM. Each peptide was tested with 50–100 eggs and 3–7 independent trials were performed with each.
[b]The ratio of fertilization in the presence of 500 μM peptide as measured by the number of sperm fused per egg (fertilization index) relative to a non peptide control. The average FI for the no peptide control was 1.2. Errors are ± SEM.

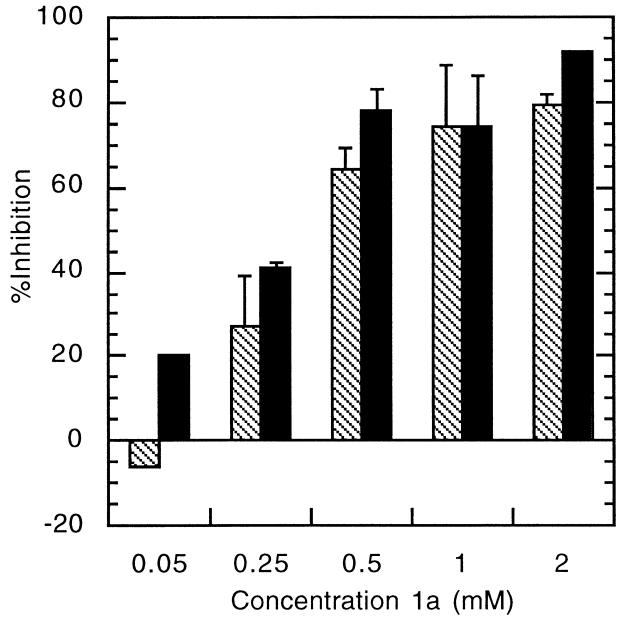


Figure 3. Concentration dependence of inhibition of in vitro fertilization by **1a**. Striped bar, inhibition of fertilization in the presence of peptide as measured by the number of eggs fertilized (fertilization rate) relative to the non peptide control. Solid bar, inhibition of fertilization in the presence of peptide as measured by the number of sperm fused per egg (fertilization index) relative to the non-peptide control. Error bars are SEM.

integrins, i.e. $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_1$, $\alpha_v\beta_5$ and $\alpha_2\beta_1$.[31] A second peptide motif Leu-Asp-Val (LDV) is present in integrin binding proteins in the immunoglobulin family and this motif binds to $\alpha_4\beta_1$, $\alpha_5\beta_1$ and $\alpha_4\beta_7$ integrins.[32] In addition to RGD and LDV, a number of other binding motifs have been identified in extracellular matrix molecules such as KQAGDV in the fibrinogenγ chain that binds to $\alpha_{IIb}\beta_3$ integrin, and LRE in laminin that binds to $\alpha_6\beta_1$ and $\alpha_7\beta_1$.[33] It is not yet clear whether all of the amino acids in the highly conserved motif (D/E)ECD of fertilinβ are required for sperm–egg adhesion. Previously, only peptides corresponding to part of this sequence or containing 2–4 flanking residues on either side of the sequence have been tested. In mice, these include QDEC,[6] ECDAY,[7] and AQDECDVT.[8] We wanted to compare side by side peptides that contained the two conserved adjacent acidic residues DE, to a peptide that only contained the E. Elucidation of the importance of individual amino acids is important for the development of better peptidomimetic inhibitors. The aim of this study was to establish the minimal number of amino acids in the putative binding loop of the disintegrin fertilinβ required for inhibition of sperm adhesion to the egg.

The peptides designed contained the four amino acid sequence DECD. At the penultimate C-terminal position, a cysteine was incorporated for cyclization to thioethers **1b** and **3**. Peptide **1a** is a linear control for cyclic peptide **1b** and includes an alanine in place of the penultimate cysteine. A tyrosine was included at the C-terminus for facile quantitation. This aromatic residue does not reduce binding affinity or alter binding specificity. The ECDAY peptide, for example, has the same IC_{50} as an 8-mer AQDECDVT that contains only fertilinβ residues.[7,8] Moreover, this peptide competitively inhibits binding of the 13 amino acid photoaffinity label used to identify $\alpha_6\beta_1$ as the fertilinβ receptor.[28] Peptide **2** was designed as a mutant in which the two adjacent acidic residues (D and E) were replaced by their corresponding amides (N and Q). In the mutant peptide **4**, only one of the two acidic residues (E) was replaced by its corresponding amide (Q). Peptide **3** was designed to examine the importance of a free thiol for inhibition in the sequence DECD, by protecting the Cys residue as a mixed disulfide that has minimal steric hindrance.

The cyclic peptides **1b** and **3** were synthesized because cyclization should reduce the peptide's molecular entropy, and if constrained to the correct conformation, increase its binding affinity for its receptor. In the case of the snake venom disintegrins, this is the case.[34] The binding affinity of RGD peptides to integrins is greater when the peptides are constrained to be cyclic using a thioether linkage.

First, peptide **1a** was compared directly to the previously studied inhibitor, **5**.[7] A minimal difference was observed in the inhibitory effects of these two peptides. It was concluded, therefore, that inclusion of an acidic residue (D) to form a 4-mer sequence (DECD) does not lead to improved inhibition over the 3-mer sequence (ECD). This is consistent with the results of Yuan et al.[8] who tested an octapeptide, AQDECDVT, that contained the DECD sequence. A high concentration (500 μM) of this peptide was also required for 50% inhibition. Moreover, inhibition by peptide **1a** was concentration dependent (Fig. 3).

There was no significant difference in the percent inhibition measured for the linear (**1a**) and cyclic (**1b**) forms of the DECD peptide. This minimal difference in inhibition between peptides **1a** and **1b** could be a result of constraining the peptide into the wrong conformation for binding. We examined the conformational heterogeneity of **1a** and **1b** in D_2O by ^1H NMR. The α-hydrogen region of the **1b** spectrum clearly shows that either a single conformer is present or different conformers are rapidly interconverting on the NMR time scale, there is no evidence for conformational populations that cannot rapidly equilibrate. The α-hydrogen region of the **1a** spectrum shows the same. The reason for no improvement of inhibition upon cyclization is most likely that we have constrained the peptide into a conformation or subset of rapidly interconverting conformations that do not correspond to the optimal receptor-bound conformation. A definitive explanation awaits full structure determination of both the cyclic and linear peptides, as well as direct binding assays to purified receptors.

The control peptide **2**, in which both the adjacent acidic residues (D and E) in the sequence (D/E)ECD were replaced by their corresponding amides, showed no inhibition of in vitro fertilization. The control peptide **4**, in which only one acidic residue (E) is substituted by its

corresponding amide (Q), was designed to form the sequence DQCD. This is the sequence found in the disintegrin binding loop of another ADAM protein, cyritestin (ADAM 3), that is also found on the surface of fertilization competent sperm. It had been reported previously that, in addition to fertilinβ, cyritestin is also involved in sperm–egg plasma membrane adhesion and fusion.[8,35] An octapeptide, SKDQCDFP, corresponding to the cyritestin binding loop is a reasonable inhibitor of sperm binding to egg membrane.[8] In our studies, inhibition of fertilization by peptide **4** demonstrates that the flanking amino acid residues in the octapeptide reported before are not required for inhibition, although they may confer receptor specificity. This result, in combination with earlier results, clearly indicates that at least one of the adjacent acidic residues (D or E) in the sequence (D/E)ECD is required for inhibition.

Inhibition of fertilization by peptides derived from cyristestin, i.e. **4** and SKDQCDFP, strongly suggests that more than one disintegrin on the sperm surface is involved in egg adhesion. Future work will address whether cyritestin binds to the $\alpha_6\beta_1$ integrin receptor like fertilinβ, or if it interacts with a different integrin receptor. Until the receptor for cyritestin is identified, we can not say whether only one of the two adjacent acidic residues (D or E) in the binding sequence (D/E)ECD is required for binding to the integrin $\alpha_6\beta_1$.

Peptide **3** was made to test the importance for inhibition of the free thiol of the conserved cysteine residue. This peptide showed no inhibition of in vitro fertilization. This result clearly shows that the conserved cysteine is required as a free thiol for inhibition of in vitro fertilization. Lack of inhibition by peptide **2**, that also contains a free thiol, argues that peptides **1a**, **1b**, **4** and **5** are inhibitors because of their full sequence, not because a thiol(ate) inactivates sperm-egg adhesion.

Conclusion

In conclusion, we observed that good inhibition of sperm-egg binding requires at least one of the conserved adjacent acidic residues (D or E), along with the conserved cysteine in a reduced state in the binding sequence (D/E)ECD of fertilinβ. It is known that integrin ligand binding and receptor activation are dependent on divalent cations, and the ligand binding sites within integrins coincide with the putative cation binding sites.[31] It is possible that a divalent cation could be coordinated simultaneously with the ECD ligand motif and the active site within the integrin, with the acidic residue providing a cation coordinating group. This requirement explains the observation that an acidic residue is critical for integrin binding during fertilization. Moreover, the LDV binding site in $\alpha_4\beta_1$ has been mapped and it corresponds to the RGD binding site in $\alpha_{IIb}\beta_3$.[36] Thus, it appears that there is a common ligand-binding pocket in these integrins. The requirement for inclusion of acidic residues from the (D/E)ECD sequence of fertilinβ, suggests that the fertilinβ disintegrin binding site will map to the homologous region in $\alpha_6\beta_1$. Our work corroborates the work of others[8] that suggested more than one ADAM protein is involved in sperm–egg adhesion during fertilization. In order to completely understand the specificities of small peptide inhibitors, binding studies with isolated receptors will be necessary.

Experimental Procedures

Materials

Amino acids were purchased from Advanced Chemtech (Louisville, KY). Peptide synthesis resin and *O*-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) were purchased from Perseptive Biosystems (Framingham, MA). Solvents were from Fisher Scientific, Inc. (Springfield, NJ). Other reagents were purchased from Sigma-Aldrich Co. (Milwaukee, WI).

General

Peptides were purified by preparative RP-HPLC using a Vydac C_{18} Column (218TP1022, 1.0×25 cm). Gradient elution was performed at 10 mL/min, with an increasing concentration of CH_3CN in H_2O. The eluent contained a constant concentration of TFA (0.1%). Column effluent was monitored at 220 nm and 274 nm. Fractions containing the desired peptide were collected and lyophilized. They were analyzed by analytical RP-HPLC using a Vydac C_{18} column (218TP54, 0.46 × 25 cm) at 1 mL/min and gradient elution as described above. MALDI–TOF mass spectrometry was carried out using a Bruker Protein TOF instrument at the CASM facility at SUNY Stony Brook. The matrix used was α-cyano-4-hydroxycinnamic acid. Mass calibrations were performed using internal standards with appropriate molecular weights.

Peptide synthesis

Peptides were synthesized on a Rainin PS3 synthesizer using N^{α}-9-fluorenylmethyloxycarbonyl-[5-(4-aminomethyl)-1-(3,5-dimethoxyphenoxy)-valerate]-polyethylene glycol-polystyrene (Fmoc-PAL-PEG) or Fmoc-PAL resins. A typical synthesis was performed on a 0.2 mmol scale. All amino acids were of L-configuration and were Fmoc protected at the N-terminus. Fmoc deprotection was effected with 20% piperidine in DMF followed by washing with DMF. The amino acids were coupled with HATU activation in a 4-fold molar excess with 8% diisopropylethylamine (DIPEA) in DMF as solvent. The first amino acid was coupled twice. The coupling times were typically 20 min. Before deprotection of the Fmoc, capping was effected using a solution of 5% acetic anhydride and 8% DIPEA in DMF. The amino acid side-chains of Asp, Glu, and Tyr were *t*-butyl protected. Cys was protected with trityl (Trt) or acetamidomethyl (Acm) depending on its position in the sequence. The final protected peptide on the resin was washed with CH_2Cl_2 and Et_2O and dried in vacuo.

Cleavage and deprotection of the resin was effected by treatment of the resin with a cleavage cocktail containing anisole, thioanisole, H_2O and TFA (10:3:3:84) for 2–3 h. The resin was removed by filtration and washed with small quantities of TFA. The combined filtrate and washings were concentrated under a stream of N_2. The crude peptides were obtained by trituration with Et_2O and collection of the precipitate, followed by further washing with Et_2O. After purification by preparative RP-HPLC, the molecular masses of the peptides were confirmed by mass spectrometry and their purity assessed by analytical RP-HPLC.

Cyclized peptides (1b,3)

The peptidyl resin was assembled as described above. The C-terminal Cys and the N-terminal Cys were introduced as Fmoc-Cys(Trt)-OH and Fmoc-Cys (Acm)-OH, respectively. The capping of the N-terminus of the peptides was effected with bromoacetic acid. After the synthesis of each peptide, the resin was cleaved and deprotected to afford peptides with a reduced-Cys at the C-terminus. The cyclization of the crude peptides was achieved under dilute alkaline conditions (pH 8), forming a thioether linkage through the deprotected Cys. After purification of the cyclized peptides, the Acm group of the N-terminus Cys was removed with dimethyl(methylthio)sulfonium tetrafluoroborate (DMMT)[37] and treated with dithiothreitol (DTT) to afford cyclized material with a reduced Cys. The reaction mixture was concentrated by lyophilization and chromatographed (5–65% CH_3CN over 1 h) to provide compound **1b**. Peptide **3** was formed from the precursor to peptide **1b** by deprotecting the Acm group with DMMT to form the mixed disulfide. The reaction mixture was lyophilized and chromatographed by preparative RP-HPLC to provide compound **3**.

In vitro fertilization assay

Eggs and sperm were isolated from ICR (Taconic farms) or CD-1 (Charles River Laboratories) mice as described in Yuan et al.[8] Zona pellucida were removed by treatment with acid Tyrode's solution for 30 s and recovered at 37 °C, 5% CO_2 for 1 h. Zona-free eggs were loaded with Hoechst 33342 (1 μg/mL) for 30 min and washed through six drops of M16 medium (0.5% BSA).

Eggs (20–30/40 μL) were incubated with peptide in M16 medium (3% BSA) for 45 min and then sperm were added (5×10^4/mL). After 45 min the eggs were washed in M16 medium (3% BSA) and mounted onto microscope slides. Fusion was scored by fluorescent labeling of sperm nuclei by Hoechst 33342 present in preloaded eggs. 3–7 experiments per peptide were performed and 25–100 eggs per peptide were used. A DAPI (465 nm) cutoff filter was used for fluorescence microscopy.

NMR spectroscopy

All spectra were acquired in D_2O and were externally referenced to the solvent. The spectra were acquired on a Varian INOVA 500 MHz spectrophotometer. ^1H NMR data are reported in the following manner: chemical shift in ppm (multiplicity, integrated intensity, coupling constant in hertz). **1a**: δ 6.968 (d, 2, $J=8.4$), 6.524 (d, 2, $J=8.4$), 4.766 (m, 2), 4.633 (dd, 1, $J=8.9$, 5.4), 4.485 (dd, 1, $J=7.9$, 7.2), 4.430 (dd, 1, $J=10.2$, 4.8), 4.300 (dd, 1, $J=12.9$, 7.3), 2.990 (dd, 1, $J=14.1$, 6.9), 2.893 (dd, 1, $J=14.0$, 8.5), 2.827 (m, 2), 2.765 (dd, 1, $J=7.7$, 6.9), 2.669 (m, 3), 2.510 (dd, 1, $J=15.5$, 9.4), 2.228 (dd, 2, $J=15.6$, 8.0), 2.009 (s, 3), 1.897 (m, 1), 1.283 (d, 3, $J=6.5$); **1b**: δ 7.118 (d, 2, $J=7.5$), 6.808 (d, 2, $J=7.5$), 4.735 (m, 1), 4.595 (m, 2), 4.465 (dd, 1, $J=8.5$, 5.5), 4.357 (dd, 1, $J=7.0$, 6.5), 4.323 (dd, 1, $J=7.5$, 7.0), 3.433 (d, 1, $J=15.0$), 3.264 (d, 1, $J=15.0$), 3.166 (dd, 1, $J=14.5$, 5.5), 2.840–2.996 (m, 9), 2.467 (dd, 2, $J=7.4$, 7.2), 2.281 (m, 1), 2.054 (m, 1).

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