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ECD PEPTIDES INHIBIT IN VITRO FERTILIZATION IN MICE

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Abstract: Cyclic and linear peptides containing the conserved ECD sequence from the disintegrin domain of the β-subunit of fertilin were synthesized and tested as inhibitors of sperm-egg fusion in in vitro mouse fertilization assays. At 500 μM concentrations, reduced ECD peptides inhibited fertilization, whereas oxidized dimers did not. Control peptides suggest that more than three amino acids of the disintegrin domain are recognized by its receptor.

In our study of the putative binding loop of the disintegrin domain in the β-subunit of fertilin, we sought to determine the minimal recognition element for the binding of fertilin to its receptor. Fertilin is an extracellular, membrane-bound, heterodimeric protein present on the surface of sperm and a member of the ADAM family (A Disintegrin and Metalloprotease) of recently discovered cellular proteins containing both metalloprotease and disintegrin domains.[1,2] The mature form of fertilin contains one disintegrin domain in the β-subunit. Prior to this discovery, disintegrin domains were only known to occur as soluble snake toxins, the snake venom metalloproteases (SVMP).[3-5] These toxins are antagonists of αIIbβ3 integrin. There are two classes of SVMP that contain disintegrin domains, P-II and P-III.[1] Both types of disintegrin domain are rich in cysteine and the cysteine distribution and content of the two classes is identical except in the binding loop. The ADAM disintegrin domains are similar to the P-III class of the SVMP disintegrins. We designed small linear and cyclic peptide analogs of fertilin-β to probe the importance of differences between the classes of disintegrins and to aid in the identification of the fertilin receptor.

The wealth of structural information for SVMP P-II disintegrins from NMR[6-10] and inhibition studies[11-14] offers insight into the relationship between structure and activity in the disintegrins. The cysteines are oxidized in intramolecular disulfide bridges and form a rigid cross-linked structure that serves as a framework for presenting a 10-amino acid binding loop. The consensus binding sequence of the SVMP P-II disintegrins, RGD, is presented at the tip of this poorly structured hairpin loop. Many small peptides and peptido-mimetics of the RGD sequence have been designed, synthesized, and shown to be antagonists of αIIbβ3 integrin, a platelet receptor that binds fibrinogen. Thus, the minimal recognition element of SVMP P-II disintegrins is RGD.

The ADAM disintegrins show high amino acid identity (>50%) to the SVMP P-II disintegrins except in the RGD binding loop. The RGD sequence is not present, and the loop contains an extra amino acid, cysteine (see Figure 1). If the minimum binding sequence in the β-fertilin disintegrin is comprised of a three amino acid sequence analogous to RGD, there are two possibilities. The first possibility is that the amino acid sequence directly aligned with RGD (e.g., QDE in mice and TDE in guinea pig) represents the recognition element. The second possibility is that the conserved sequence, ECD, is the minimal binding element. This sequence is also conserved between P-II and ADAM disintegrins. At the time this study was initiated, members of the SVMP P-III family were the only known homologs of fertilin. Sequence alignment of fertilins from various species reveals that E/DECD are the conserved amino acids in the putative binding loop of β-fertilin. Small peptides containing

the QDE sequence or both sequences (TDECD, in guinea pig) of the ADAM disintegrin loop sequence have been shown to inhibit sperm-egg fusion and/or binding in mice[15,16] and in guinea pigs.[17]

Kopf and coworkers have synthesized linear and cyclic peptides (CAQDEC) and tested them as inhibitors of fusion in mice.[16] Their cyclic peptides used the loop cysteine as the handle for cyclization. Only the linear, and *not* the cyclic peptides, inhibited in vitro fertilization at 500 μM concentrations. These results suggested that either the free thiol is important for binding or that cyclization locked the peptide in an unfavorable conformation.

SVMP P-II disintegrins		
	Kistrin[18]	CKFSRAGKICRIP**RGD**-MPDDRCTGQSADC
	Echistatin[18]	CKFLKEGTICKRA**RGD**-DMDDYCNGKTCDC
	Barbourin[19]	CRFMKKGTVCRVA**KGD**-WNDDTCTGQSADC
SVMP P-III disintegrins		
	HR1B[20]	CRFRTAGTECRAAES**ECD**IPESCTGQSADC
	Jararhagin[21]	CKFSKSGTECRASMS**ECD**PAEHCTGQSSEC
	Atrolysin E[22]	CKFTSAGNVCRPARS**ECD**IAESCTGQSADC
ADAM disintegrins		
1	Fertilin α mouse[2]	CTFKKKGSLCRPAED**VCD**LPEYCDGSTQEC
2	Fertilin β mouse[2]	CKLKRKGEVCRLAQD**ECD**VTEYCNGTSEVC
2	Fertilin β monkey[23]	CLFMSQERVCRPSFD**ECD**LPEYCNGTSASC
2	Fertilin β guinea pig[24]	CEFKTKGEVCRESTD**ECD**LPEYCNGSSGAC
2	Fertilin β human[25]	CLFMSKERMCRPSFE**ECD**LPEYCNGSSASC
3	Cyritestin mouse[26]	CTIAERGRLCRKSKD**QCD**FPEFCNGETEGC
4	ADAM 4 mouse[2]	CKFAPTGTICRDKNG**ICD**LPEYCSGASEHC
5	ADAM 5 mouse[2]	CTVKMNDVVCRKSVD**ECD**LLEYCNGKDPYC
7	EAP I monkey[27]	CQIKKAGSICRPAED**ECD**FPEMCTGHSPAC
8	MS2 mouse[28]	CKVKPAGEVCRLSKD**KCD**LEEFCDGRKPTC

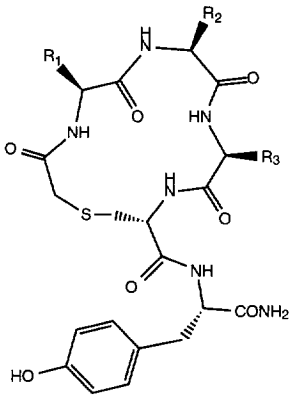
Figure 1. Sequence alignment of the residues adjoining the binding loop of representative disintegrins. The putative consensus binding sequence is bold-faced, and the loop residues are underlined.

White and coworkers demonstrated that a 14-mer peptide (CRLAQDEADVTEYC) with the loop cysteine substituted with an alanine in either the linear or cyclized form still inhibited sperm binding in mice, (IC_{50} = 250 μM).[15] This experiment demonstrated that the "loop" is involved in binding. It is still unclear, however, whether the cysteine in the loop is unimportant, or if the additional 10 amino acids compensate for loss of binding upon substitution of cysteine with alanine. In guinea pig in vitro fertilization assays,[17] peptides comprised of both recognition elements (STDECDLK) or part of both elements (CSTDEC) inhibited sperm egg fusion.

All of these experiments demonstrated that the putative binding loop is involved in sperm-egg fusion. The role of the extra cysteine, however, remained ambiguous. We sought to clarify its role in binding and inhibition of fusion. We postulated that this odd cysteine remains a free thiol. Furthermore, cyclization of the consensus binding sequence of RGD disintegrins has led to very effective inhibitors of αIIbβ3 integrin and consequently, platelet aggregation.[11] Presumably, improved inhibition occurs because the conformational entropy of the peptide analog is reduced prior to binding. We reasoned that cyclization of the ECD motif might improve binding in a

similar fashion. We have synthesized the series of peptides shown in Figure 2 and tested them as inhibitors of sperm-egg fusion.

Peptides **1a** and **2a** are the target mimics, cyclized and linear, respectively. The remainder are control peptides. We chose a thioether cyclization methodology for two reasons. First, the thioether bridge allowed easy entry into cyclized peptides containing free thiols; the thioether functionality was compatible with the free thiol in the loop. The synthesis simply required differential protection of the free cysteine and the thioether cysteine thiols. Second, thioether RGD mimics have shown good affinity for αIIbβ3 integrin and consequently, good inhibition of platelet aggregation.[29] The scrambled mimics **3a** and **4a** test the importance of sequence. The scrambled sequence control is particularly important for an in vitro assay, in which the effects of small amounts of contaminants (e.g., from solvents) are difficult to distinguish from real competitive inhibition. The serine mimic **5** tests the role of the cysteine. Dimers **1b–4b** test the importance of the free thiol and serve as controls for thiol oxidation during the in vitro assay. The tyrosine was incorporated to facilitate peptide quantitation.



	R_1	R_2	R_3
1a	CO_2H	SH	CO_2H
1b	CO_2H	S-S	CO_2H
3a	SH	CO_2H	CO_2H
3b	S-S	CO_2H	CO_2H
5	CO_2H	OH	CO_2H

2a Ac - E - C - D - A - Y - NH_2

2b Ac - E - C - D - A - Y - NH_2 (S–S) Ac - E - C - D - A - Y - NH_2

4a Ac - C - D - E - A - Y - NH_2

4b Ac - C - D - E - A - Y - NH_2 (S–S) Ac - C - D - E - A - Y - NH_2

Figure 2. Peptides synthesized and tested as inhibitors of in vitro fertilization. All amino acids were of L-configuration.

We synthesized peptides **1–5** as the N-terminal amides using standard solid phase methodology with the PAL linker and MBHA resin,[30,31] FMOC-amine protection and TBTU[32] as the activation agent. All peptide synthesis reagents were obtained from Advanced ChemTech (Louisville, KY). The two cysteines in the cyclic peptides **1** and **3** were differentially protected with trityl and acetamidomethyl groups. Linear peptides, **2** and **4** were acetylated at the N-terminus, cyclic peptides, **1**, **3**, and **5** were bromoacetylated. After bromoacetylation, cleavage from the resin yielded a peptide with a free thiol on the C-terminal cysteine. Upon basicification to pH 8, the peptide cyclized to form the desired thioether with an Acm-protected cysteine in the loop. The cyclized peptide was purified by HPLC. The Acm was removed with dimethyl(methylthio)sulfonium tetrafluoroborate.[33] Prior to use, the mixed disulfide was reduced with DTT. Excess salts and reducing agent were removed by C_{18} reversed-phase HPLC and the peptides were dissolved in water. The solution pH was adjusted to 7 before addition to in vitro fertilization assays. The peptides were purified by HPLC to greater than 99% purity. All peptides were analyzed by amino acid analysis (Commonwealth Biotechnologies, Richmond, VA) and had the correct amino acids incorporated in the expected proportions with the exception of cysteine, for which the ratio was low. The

exact masses were correct as determined by MALDI and HRFAB mass spectrometry. Dimers were formed by air oxidation and purified by C_{18} reversed-phase HPLC. Dimerization was confirmed by MALDI mass spectrometry. After 60 min at pH 7 in water, the reduced peptides were 8% oxidized. Our analysis indicated that <10% oxidation/dimerization occured in the time scale of the fertilization assay. There are no reducing agents present in the in vitro fertilization medium.

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 the absence of the peptides.[17] The results of the in vitro fertilization inhibition assays are presented in Figure 3. The fertilization index and rate for the no peptide control were 0.82 sperm/egg and 68% eggs fertilized, respectively.

In comparing peptides **1a** and **2a,** we saw little difference between cyclized and linear peptides (in the RGD case, there was a ten-fold improvement upon cyclization). Interestingly, both disulfide dimers show no inhibition of fertilization rate and low inhibition of fertilization index. The importance of a free thiol is further supported by the minimal inhibition by **5**, in which the reduced cysteine has been replaced by serine.

The inhibition results of peptides **3** and **4**, however, seem to contradict this conclusion. If we accept that cyclization does not markedly improve binding, we can concentrate on comparing scrambled monomers containing free thiols, **3a** and **4a**, and scrambled dimers containing disulfides, **3b** and **4b**. We observed that the scrambled peptides inhibit fertilization regardless of thiol oxidation state. The CDE sequence is very similar to the QDE sequence of mouse β-fertilin in the loop. In fact, the conserved sequence is E/DECD, not ECD. At the time of our inhibitor design, sequence analysis did not reveal that the E/D was conserved. We hypothesize that the presence of 2 adjacent acidic residues produces an inhibitory effect regardless of thiol oxidation state. This result will be used in the design of inhibitors in the future.

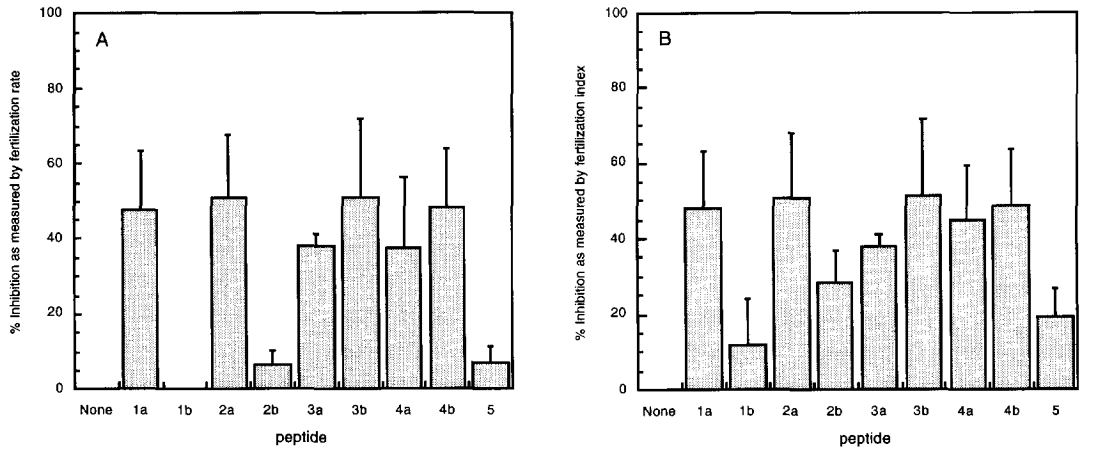


Figure 3. Effect of peptides **1–5** on sperm-egg fusion. ZP-free mouse eggs were incubated in 500 μM peptide for 30 min prior to insemination. Eggs were then inseminated with 1.0–2.0 x 10^4 sperm/mL in the presence of peptide. (a) The percentage inhibition as measured by number of eggs fertilized (fertilization rate). (b) The percentage inhibition as measured by the number of sperm fused per egg (fertilization index). The results here represent the average of 3–7 experiments per peptide ± s.e.m. and a total of 30–150 eggs per peptide.

Similar inhibition by a scrambled peptide with adjacent acidic residues was observed by Evans et al.[16] They also postulated that the adjacent residues are important for inhibition, or alternatively, that the diacid might chelate Ca^{2+}. The concentration of Ca^{2+} in the fertilization assay medium is 1.8 mM, the addition of 0.5 mM chelating peptide will at most lower the Ca^{2+} concentration to 1.3 mM. According to Evans et al.'s experiments to measure the fertilization rate dependence on Ca^{2+} concentration,[16] this change in concentration is unlikely to reduce sperm-egg fusion to the extent observed. Due to the limited number of scrambled peptide possibilities, it was not possible to construct a control that completely scrambled the position of the acidic residues.

We conclude that the binding site of fertilin is not as restricted as in the SVMP P-II RGD disintegrins. In mice, at least QDECD is required for maximum specificity (i.e., both three-amino acid sequences). The 50% inhibition observed with partial sequences supports the requirement for a minimum of 5 amino acids for maximum specificity. Furthermore, it appears that the loop cysteine exists as a free thiol. These requirements are further supported by Myles' work with peptides that inhibit in vitro fertilization in guinea pigs.[17] In that work, 100% inhibition was observed at 500 μM, with peptides comprising the extended recognition site (TDECD in guinea pig). We will further test this hypothesis of extended recognition site in future work.

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References and Notes

1. Wolfsberg, T. G.; Primakoff, P.; Myles, D. G.; White, J. M. *J. Cell Biol.* **1995**, *131*, 275.
2. Wolfsberg, T. G.; Straight, P. D.; Gerena, R. L.; Huovila, A. P. J.; Primakoff, P.; Myles, D. G.; White, J. M. *Devol. Biol.* **1995**, *169*, 378.
3. Fox, J. W.; Bjarnason, J. B. In *Zinc Metalloproteases in Health and Disease*; Hooper, N., Ed.; Ellis Horwood/Simon & Schuster: West Sussex, 1995.
4. Hite, L. A.; Jia, L.-G.; Bjarnason, J. B.; Fox, J. W. *Arch. Biochem. Biophys.* **1994**, *308*, 182.
5. Scarborough, R. M.; Rose, J. W.; Naughton, M. A.; Phillips, D. R.; Nannizzi, L.; Arfsten, A.; Campbell, A. M.; Charo, I. F. *J. Biol. Chem.* **1993**, *268*, 1058.
6. Senn, H.; Klaus, W. *J. Mol. Biol.* **1993**, *232*, 907.
7. Cooke, R. M.; Carter, B. G.; Murray-Rust, P.; Harshorn, M. J.; Herzky, P.; Hubbard, R. E. *Protein Eng.* **1992**, *5*, 473.
8. Chen, Y.; Pitzenberger, S. M.; Barsky, V. M.; Lumma, P. K.; Sanyal, G.; Baum, J. *Biochemistry* **1991**, *30*, 11625.
9. Adler, M.; Lazarus, R. A.; Dennis, M. S.; Wagner, G. *Science* **1991**, *253*, 445.
10. Atkinson, R. A.; Saudek, V.; Pelton, J. T. *Int. J. Pept. Protein Res.* **1994**, *43*, 563.
11. Ojima, I.; Chakravarty, S.; Dong, Q. *Bioorg. Med. Chem.* **1995**, *3*, 337.
12. Blackburn, B. K.; Gadek, T. R. *Ann. Reports Med. Chem.* **1993**, *28*, 79.
13. Cook, N. S.; Kottirsch, G.; Zerwes, H.-G. *Drugs Fut.* **1994**, *19*, 135.
14. Weller, T.; Alig, L.; Müller, M. H.; Kouns, W. C.; Steiner, B. *Drugs Fut.* **1994**, *19*, 461.

15. Almeida, E. A. C.; Huovila, A. P. J.; Sutherland, A. E.; Stephens, L. E.; Calarco, P. G.; Shaw, L. M.; Mercurio, A. M.; Sonnenberg, A.; Primakoff, P.; Myles, D. G.; White, J. M. *Cell* **1995**, *81*, 1095.

16. Evans, J. P.; Schultz, R. M.; Kopf, G. S. *J. Cell Sci.* **1995**, *108*, 3267.

17. Myles, D. G.; Kimmel, L. H.; Blobel, C. P.; White, J. M.; Primakoff, P. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 4195.

18. Dennis, M. S.; Henzel, W. J.; Pitti, R. M.; Lipari, M. T.; Napier, M. A.; Deisher, T. A.; Bunting, S.; Lazarus, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 2471.

19. Scarborough, R. M.; Rose, J. W.; Hsu, M. A.; Phillips, R. R.; Fried, V. A.; Campbell, A. M.; Nannizzi, L.; Charo, I. *J. Biol. Chem.* **1991**, *266*, 9359.

20. Takeya, H.; Oda, K.; Miyata, T.; Omori-Satoh, T.; Iwanaga, S. *J. Biol. Chem.* **1990**, *265*, 16068.

21. Paine, M. J. I.; Desmond, H. P.; Theakston, R. D. G.; Crampton, J. M. *J. Biol. Chem.* **1992**, *267*, 22869.

22. Hite, L. A.; Shannon, J. D.; Bjarnason, J. B.; Fox, J. W. *Biochemistry* **1992**, *31*, 6203.

23. Ramarao, C. S.; Myles, D. G.; White, J. M.; Primakoff, P. *Molecular Reproduction and Development* **1996**, *43*, 70.

24. Blobel, C. P.; Wolfsberg, T. G.; Turck, C. W.; Myles, D. G.; Primakoff, P.; White, J. M. *Nature (London)* **1992**, *356*, 248.

25. Gupta, S. K.; Alves, K.; Palladino, L. O.; Mark, G. E.; Hollis, G. F. *Biochem. Biophys. Res. Commun.* **1996**, *224*, 318.

26. Heinlein, U. A. O.; Wallat, S.; Senftleben, A.; Lemaire, L. *Dev. Growth Differ.* **1994**, *36*, 49.

27. Perry, A. C. F.; Jones, R.; Barker, P. J.; Hall, L. *Biochem. J.* **1992**, *286*, 671.

28. Yoshida, S.; Setoguchi, M.; Higuchi, Y.; Akizuki, S.; Yamamoto, S. *Int. Immunol.* **1990**, *2*, 585.

29. Barker, P. L.; Bullens, S.; Bunting, S.; Burdick, D. J.; Chan, K. S.; Deisher, T.; Eigenbrot, C.; Gadek, T. R.; Gantzos, R.; Lipari, M. T.; Muir, C. D.; Napier, M. A.; Pitti, R. M.; Padua, A.; Quan, C.; Stanley, M.; Struble, M.; Tom, J. Y. K.; Burnier, J. P. *J. Med. Chem.* **1992**, *35*, 2040.

30. Albericio, F.; Barany, G. *Int. J. Peptide Protein Res.* **1987**, *30*, 2016.

31. Albericio, F.; Kneib-Cordonier, N.; Gera, L.; Hammer, R. P.; Hudson, D.; Barany, G. In *Peptides: Chemistry and Biology*; Marshall, G. R., Ed.; Escom: Leiden, 1988; pp 159–161.

32. Knorr, R.; Trzeciak, A.; Bannwarth, W.; Gillessen, D. *Tetrahedron Lett.* **1989**, *30*, 1927.

33. Bishop, P.; Jones, C.; Chmielewski, J. *Tetrahedron Lett.* **1993**, *34*, 4469.

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