Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 4195–4198, May 1994
Cell Biology

Identification of a binding site in the disintegrin domain of fertilin required for sperm–egg fusion

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Communicated by M. J. Osborn, January 31, 1994

ABSTRACT **Fertilization and certain later stages in mammalian embryonic development require fusion between membranes of individual cells. The mechanism of eukaryotic cell–cell fusion is unknown, and no surface molecules required for this process have been unequivocally identified. The role of the sperm surface protein fertilin in sperm–egg fusion was tested by using peptide analogues of a potential integrin binding site in the fertilin β subunit. Peptide analogues that include a TDE sequence from the disintegrin region of fertilin β are able to bind to the egg plasma membrane and strongly inhibit sperm–egg fusion. These results show that the disintegrin domain of fertilin β binds to the egg plasma membrane and that this binding is required for membrane fusion.**

Membrane fusion is important in many different cellular functions. The majority of membrane fusion events involve the membranes within a single cell, and these processes are being intensively studied. Much less is known about the molecular mechanism of fusion between the plasma membranes of two cells. Our investigations have focused on the fusion between the sperm and the egg plasma membranes, a key event in development.

Our initial antibody inhibition studies identified a protein on the guinea pig sperm surface called fertilin that is involved in sperm–egg membrane fusion (1), but the exact role of fertilin remained unsolved. Fertilin (originally named PH-30 because of its localization to the posterior head domain of the sperm) is a heterodimeric protein. Analysis of the cDNA sequence of guinea pig fertilin α and β subunits revealed that the N-terminal region of the mature β subunit has high homology with a family of integrin ligands, the disintegrins, and that the α subunit contains a putative fusion peptide, analogous to the fusion peptides of viruses (2). The presence of the disintegrin sequence led to the hypothesis that fertilin is a novel type of cell surface integrin ligand in that the disintegrin domain of fertilin β might bind to an egg integrin and this binding might be required for sperm–egg fusion. To test this hypothesis we examined the ability of peptide analogues derived from the putative fertilin β binding site to bind to the egg plasma membrane and to block sperm–egg fusion. The results of this study indicate that sperm bind to the egg plasma membrane through the disintegrin domain of fertilin β and that this binding step is required for sperm–egg fusion.

MATERIALS AND METHODS

Peptides. Peptides were a generous gift of Christopher Turck (University of California, San Francisco) or were prepared by the Yale University Peptide Synthesis Facility (New Haven, CT).

Covasphere Binding. Violet MX Covaspheres, 0.8-μm (Duke Scientific, Palo Alto, CA), were conjugated with CSTDEC or CTESDC peptide at 1 mg/ml (200 μg of peptide per 50 μl of Covaspheres), following the manufacturer's instructions. Unreacted sites were blocked with 1% glycine. Covaspheres (10 μl) were added to oocytes in a 100-μl drop of modified Tyrode's solution (mT), mixed well, and incubated under mineral oil for 3 h at 37°C with 95% air/5% CO_2. Oocytes were washed free of excess beads by pipetting through five 50-μl drops of mT and were mounted on slides in 25 μl of mT, compressed slightly with a coverslip, and photographed using a 345/425-nm filter set, and micrographs were scored for the total number of fluorescent beads bound per half-oocyte. Because few Covaspheres bound over the cortical granule-free region of the oocytes, this region was bisected for counting.

***In Vitro* Fertilization Assays.** Guinea pig oocytes were collected from ovaries and matured, and the zonae were removed as previously described (1). For zona-intact oocytes, matured oocytes were briefly treated with 0.25% hyaluronidase (Sigma) to remove the cumulus. Oocytes were washed after enzymatic treatment by pipetting through four 400-μl drops of mT and then put into a 100-μl drop with or without peptide, followed by incubation at 37°C in 95% air/5% CO_2 for 30 min. Sperm were capacitated, the acrosome reaction was induced, and fusion assays were carried out as previously described (1, 3). Sperm concentrations were in the range 1–5 × 10^4 per ml for zona-free eggs and 2.5–5 × 10^5 per ml for zona-intact eggs. Fusion was scored by the presence of swollen sperm heads after acetolacmoid staining (4). We determined that this method gave results equivalent to those obtained by following a protocol in which fusion was scored by preloading the eggs (5) with the fluorescent stain 4′,6-diamidino-2-phenylindole (DAPI) dihydrochloride (Polyscience), which stains the nuclei of fused sperm.

RESULTS AND DISCUSSION

Comparison of the deduced amino acid sequence of fertilin β to the sequence of other members of the disintegrin family was used to design test peptides (Fig. 1). Snake venom small peptides, the first identified members of the disintegrin family, bind to the platelet integrin GPIIb-IIIa ($\alpha_{IIb}\beta_3$) and inhibit platelet aggregation (18, 19). The sequences of most of these small disintegrins include the tripeptide RGD as part of the binding site (kistrin, bitistatin, echistatin, barbourin; Fig. 1*a*) (18–20). The disintegrin family also includes larger proteins from snake venom [jararhagin (10), HR1B (11), and

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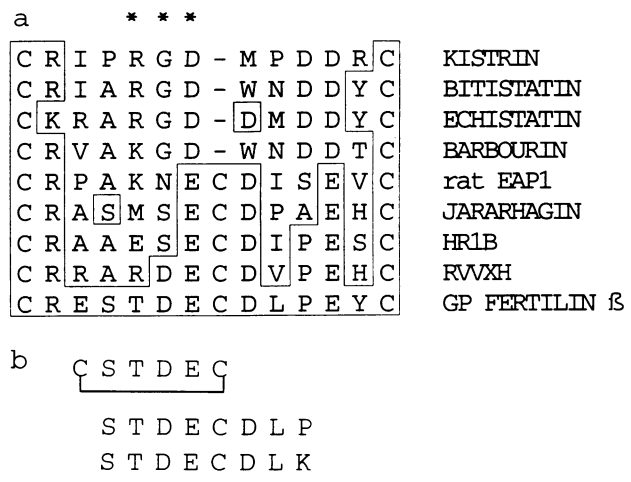

FIG. 1. Sequence comparison of known binding regions of small disintegrins with putative binding regions of disintegrin domains of guinea pig fertilin β and of large snake venom proteins (*a*) and fertilin β peptides tested in this study (*b*). Shown are relevant sequences from small disintegrins (48–83 residues) isolated from snake venoms [kistrin (6), bitistatin (7), echistatin (8), barbourin (9)] and larger snake venom proteins [jararhagin (10), HR1B (11), RVV-X heavy chain (12)] and the surface protein rat EAP1 (13) that share with fertilin β the disintegrin domain and two other domains (14). The sequences shown are the 13 amino acids that form the RGD-containing loop of kistrin (15) and echistatin (16, 17) and the corresponding 13 or 14 amino acids of the other disintegrin domains. The whole disintegrin domain of fertilin β is the N-terminal 90 amino acids of mature fertilin β (2). The percentage of identical amino acids in the whole disintegrin domain compared with fertilin β is 44% for kistrin; 54% for bitistatin; 36% for echistatin; 47% for barbourin; 54% for jararhagin; 54% for HR1B; 58% for the heavy chain of RVV-X, RVVXH; and 44% for rat EAP1. The peptides tested are shown in *b*, aligned with the sequence of the putative binding site shown in *a*.

RVV-X (12)] and a few cell surface proteins of unknown functions—for example, the mammalian surface protein EAP1 (13). Like fertilin β, these other proteins have alternative amino acids aligned with the RGD sequence. These alternative amino acids are followed by a cysteine not found in the smaller snake venom disintegrins (Fig. 1*a*). Fertilin β has a substitution of TDE in place of the RGD tripeptide (Fig. 1*a*). Two peptides were chosen to be tested on the basis of the amino acid sequence in this region: CSTDEC and STDECDLP (Fig. 1*b*). In addition, a variant of STDECDLP with a K substituted for the final P was tested (this variant peptide was originally synthesized for a separate study).

The CSTDEC peptide was cyclized prior to testing because a cyclized peptide might better mimic the native binding site.

Table 1. Peptide-coated Covasphere binding to zona-free eggs

Peptide	No. of eggs	No. of expts.	No. of Covaspheres bound per egg: Mean	No. of Covaspheres bound per egg: Mean − background
None	49	4	34 ± 3	—
CTESDC	102	8	42 ± 25	8
CSTDEC	102	8	86 ± 23	52

The number of peptide-coated Covaspheres bound was compared with the number of control beads bound (with no peptide). Results are mean ± SD. The confidence level for a significant difference between CSTDEC and no peptide is greater than 95%. The control scrambled peptide (CTESDC) showed no significant difference when compared with no peptide ($P = 0.04$).

Structural studies of the snake venom small disintegrins have demonstrated that the RGD tripeptide is located at the tip of a flexible hairpin loop created by disulfide bridges (15–17, 21) and that the binding activity of peptides to integrins is greater when the loop conformation is maintained (20). The additional cysteine (TDEC) that occurs in the potential binding site of the disintegrin domain of fertilin β and the larger snake venom proteins (Fig. 1*a*) could be free or disulfide bridged. The CSTDEC peptide was cyclized by oxidation, thereby mimicking either a loop conformation or disulfide bonding of the TDEC cysteine.

To determine if cyclized CSTDEC bound to the egg plasma membrane, we tested if CSTDEC-coated fluorescent Covaspheres would bind to the plasma membrane of zona-free eggs. Binding was compared to Covaspheres that were coated with a control cyclized peptide (CTESDC), containing the same amino acids but in a rearranged (scrambled) order. Covaspheres conjugated to the CSTDEC peptide bound to eggs at a level 6.5-fold higher than Covaspheres conjugated to the control scrambled peptide (Table 1). The finding that a peptide from the predicted binding site of fertilin β binds to the egg suggests that sperm can bind to the egg through fertilin β.

Because the PH-30 monoclonal antibody recognizes the β subunit of fertilin (22) and inhibits sperm–egg fusion (1), fertilin β-mediated binding would be expected to lead to fusion. To focus our experiments exclusively on physiologically competent sperm that bind to and then fuse with the egg, we tested the ability of TDE-containing peptides to inhibit sperm–egg fusion. Both the percentage of eggs fused with at least one sperm (fertilization rate) and the the mean number of sperm fused per egg (fertilization index) were scored. The control peptides tested were scrambled versions of both test peptides, with the same amino acids but in a

Table 2. Inhibition of sperm fusion with zona-intact eggs

Peptide	No. of eggs	No. of expts.	% of eggs fused (FR)	% inhibition of fusion as measured by FR	Mean no. of sperm fused per egg (FI)	% inhibition of fusion as measured by FI
None	195	16	83 ± 16	—	1.06 ± 0.32	—
CSTDEC	73	6	11 ± 14	87	0.11 ± 0.14	90
STDECDLP	47	4	16 ± 8	81	0.20 ± 0.10	81
STDECDLK	55	4	2 ± 4	98	0.02 ± 0.04	98
CTESDC	25	3	72 ± 15	13	0.80 ± 0.02	25
PDCTESDL	40	4	75 ± 17	10	0.90 ± 0.13	15
GRGES	35	3	68 ± 32	18	0.91 ± 0.27	14

TDE-containing peptides tested were cyclized CSTDEC and linear peptides STDECDLP and STDECDLK. Control peptides were either scrambled versions of test peptides (cyclized CTESDC and linear PDCTESDL) or an irrelevant peptide (GRGES). All peptides were tested at 500 μM. Results are mean ± SD. Confidence levels for a significant difference between all TDE-containing peptides and all controls, including no-peptide controls, are ≥95% ($P < 0.0001$, for both the fertilization rate and the fertilization index). Control peptides showed no significant difference when compared with no-peptide controls; P values for fertilization rate and fertilization index, respectively, were as follows: CTESDC, $P = 0.29$ and 0.19; PDCTESDL, $P = 0.38$ and 0.35; GRGES, $P = 0.22$ and 0.46.

Table 3. Inhibition of sperm fusion with zona-free eggs

Peptide	No. of eggs	No. of expts.	% of eggs fused (FR)	% inhibition of fusion as measured by FR	Mean no. of sperm fused per egg (FI)	% inhibition of fusion as measured by FI
None	165	13	74 ± 16	—	1.55 ± 0.49	—
CSTDEC	45	4	11 ± 10	85	0.13 ± 0.11	92
STDECDLK*	44	4	16 ± 14	78	0.23 ± 0.25	85
CTESDC	84	7	77 ± 17	0	2.10 ± 0.67	0
GRGES	35	3	78 ± 20	0	2.40 ± 2.5	0

Peptides are the same as those used in Table 2. All peptides were tested at 500 μM, except where noted. Results are mean ± SD. Confidence levels for a significant difference between all TDE-containing peptides and no peptide controls are ≥95% ($P < 0.0001$, for both fertilization rate and the fertilization index). Control peptides did not inhibit fusion.
*Two of these four experiments were carried out at a peptide concentration of 250 μM.

rearranged order, and an irrelevant peptide, GRGES. In sperm–egg fusion assays we tested both zona-intact and zona-free eggs.

The experiments with zona-intact eggs provide a test of the inhibitory activity of peptides on eggs that have not been treated with protease to remove the zona, a treatment that could alter the egg plasma membrane (23, 24). When zona-intact eggs were incubated with peptide prior to incubation with sperm, fusion of sperm with eggs was inhibited 81–98% in both the fertilization rate and the fertilization index in comparison with eggs incubated in the absence of peptide

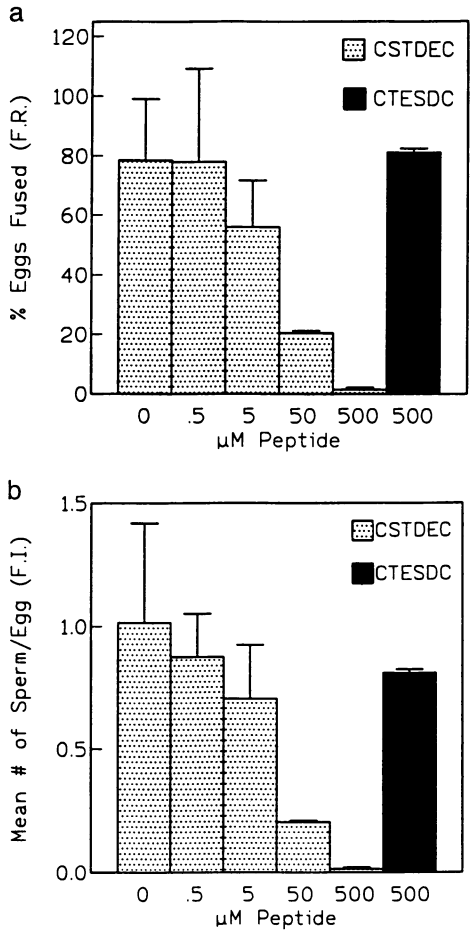

FIG. 2. Dose-dependent lowering of fertilization rate (*a*) and fertilization index (*b*) of zona-intact oocytes with the CSTDEC peptide. The results are the mean of two experiments for each peptide concentration from 0.5 to 500 μM, with the total number of eggs being 25 to 34 per peptide concentration. Inhibition at 500 μM peptide is taken as maximal and is close to 100%. Error bars represent SEM.

(Table 2). Control peptides inhibited at a much lower level (10–25%).

The experiments with zona-free eggs rule out the possibility of peptide inhibiting at the level of sperm adhesion to, or penetration through, the zona. In this case the TDE-containing peptides also strongly inhibited fusion as measured by the fertilization rate and the fertilization index (Table 3). The decrease of the fertilization rate caused by TDE peptides was 78–85% and the decrease of the fertilization index was 85–92%, when compared with eggs where no peptide was present. Control peptides caused no decrease in either the fertilization rate or the fertilization index. We do not know why we observed no effect of control peptides on either the fertilization rate or index in fusions with zona-free eggs, while there was a low inhibition in fusions with zona-intact eggs. There could be a low-level nonspecific inhibition by peptides of sperm–zona binding or penetration. This low-level inhibition is not observed in all experiments with zona-intact eggs (Fig. 2*a*).

The lowering of both the fertilization rate and the fertilization index of zona-intact eggs by CSTDEC was dose dependent. Half-maximal inhibition was between 5 and 50 μM (Fig. 2). This concentration is comparable to that required for inhibition of ligand–integrin GPIIb-IIIa binding by short RGD peptides that also inhibit in the micromolar range (18).

These experiments provide direct evidence for the role of the predicted fertilin β binding sequence (TDE) in sperm–egg binding and fusion. The results indicate that sperm fertilin binds to the egg plasma membrane by a mechanism that leads to sperm–egg fusion. In analogy to viral fusion proteins, fertilin β binding could result in a conformational change leading to the exposure of a hydrophobic fusion peptide in fertilin α (2) that could then promote membrane fusion (25).

The binding of fertilin β through its disintegrin domain is consistent with the hypothesis that the egg surface receptor for fertilin is an integrin. Since additional cell surface proteins with disintegrin domains have been reported (13, 26), fertilin may be a representative of an additional class of cell surface integrin ligands. Recent work has demonstrated the presence of several integrins on the surface of mammalian oocytes (24, 27). Inhibition of sperm fusion with hamster eggs has been observed with RGD-containing peptides (28) that may bind to one or more integrins, possibly competing with the binding of fertilin β on hamster sperm. The RGD sequence is not specific for a unique integrin, as is, for example, the KGD sequence of barbourin (9), and RGD-containing peptides can inhibit non-RGD ligand binding to integrins (29–31). Sperm binding to an egg integrin would mean that a potential pathway for sperm to signal the initiation of development (egg activation) would be through integrin-initiated signaling (32).

We are grateful to Dr. Laurinda A. Jaffe (University of Connecticut Health Center) for suggestions on the manuscript and to Ms. Lorri Goldsmith for help in its preparation. This research was funded

by National Institutes of Health Grants HD16580 and HD29125 to D.G.M. and GM48739 to J.M.W.

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