

# A Role for the Disintegrin Domain of Cyritlestin, a Sperm Surface Protein Belonging to the ADAM Family, in Mouse Sperm–Egg Plasma Membrane Adhesion and Fusion

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**Abstract.** Sperm–egg plasma membrane fusion is preceded by sperm adhesion to the egg plasma membrane. Cell–cell adhesion frequently involves multiple adhesion molecules on the adhering cells. One sperm surface protein with a role in sperm–egg plasma membrane adhesion is fertilin, a transmembrane heterodimer ( $\alpha$  and  $\beta$  subunits). Fertilin  $\alpha$  and  $\beta$  are the first identified members of a new family of membrane proteins that each has the following domains: pro-, metalloprotease, disintegrin, cysteine-rich, EGF-like, transmembrane, and cytoplasmic domain. This protein family has been named ADAM because all members contain a disintegrin and metalloprotease domain. Previous studies indicate that the disintegrin domain of fertilin  $\beta$  functions in sperm–egg adhesion leading to fusion. Full length cDNA clones have been isolated for five ADAMs expressed in mouse testis: fertilin  $\alpha$ , fertilin  $\beta$ , cyritestin, ADAM 4, and ADAM 5. The presence of the disintegrin domain, a known integrin ligand, suggests that like fertilin  $\beta$ , other testis ADAMs could be involved in sperm adhesion to the egg membrane. We

tested peptide mimetics from the predicted binding sites in the disintegrin domains of the five testis-expressed ADAMs in a sperm–egg plasma membrane adhesion and fusion assay. The active site peptide from cyritestin strongly inhibited (80–90%) sperm adhesion and fusion and was a more potent inhibitor than the fertilin  $\beta$  active site peptide. Antibodies generated against the active site region of either cyritestin or fertilin  $\beta$  also strongly inhibited (80–90%) both sperm–egg adhesion and fusion. Characterization of these two ADAM family members showed that they are both processed during sperm maturation and present on mature sperm. Indirect immunofluorescence on live, acrosome-reacted sperm using antibodies against either cyritestin or fertilin  $\beta$  showed staining of the equatorial region, a region of the sperm membrane that participates in the early steps of membrane fusion. Collectively, these data indicate that a second ADAM family member, cyritestin, functions with fertilin  $\beta$  in sperm–egg plasma membrane adhesion leading to fusion.

**T**HE interaction of sperm with the egg culminating in sperm–egg membrane fusion, is a multi-step process in mammals. After penetrating the cumulus cell layer and the zona pellucida, a sperm adheres to the egg plasma membrane and fuses. Only acrosome-reacted sperm can fuse with the egg. During the acrosome reaction, the outer acrosomal membrane fuses at multiple points with the anterior head sperm plasma membrane, and the fused membranes are released along with the soluble acrosomal contents. The inner acrosomal membrane that is incorporated into the sperm plasma membrane during the acrosome reaction remains a distinct membrane domain. The region of the sperm membrane that makes the initial contact with the egg plasma membrane is the inner acrosomal

membrane (IAM),<sup>1</sup> followed by the equatorial/posterior head region of the membrane (Shalgi and Phillips, 1980; Talbot and Chacon, 1980; Koehler et al., 1982; Yanagimachi, 1994). The fusion begins with the sperm head plasma membrane, but only the equatorial/posterior head region actually fuses with the egg plasma membrane. The IAM region is incorporated into the egg cytoplasm by a phagocytosis-like process. The sperm tail also eventually fuses with the egg plasma membrane and contributes to the zygote membrane in most mammals (Yanagimachi, 1994).

The molecular mechanisms of these interactions between the sperm and egg plasma membranes are not well understood. Our initial studies of sperm–egg fusion in guinea pigs indicated that fertilin, a heterodimeric ( $\alpha$  and

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1. *Abbreviations used in this paper:* FI, fertilization index; FR, fertilization rate; IAM, inner acrosomal membrane; MAP, multiple antigenic peptide.

$\beta$  subunits) sperm membrane protein, is involved in the fusion process. This protein is located on the posterior head of acrosome-reacted guinea pig sperm. Both a monoclonal antibody to fertilin  $\beta$  (Primakoff et al., 1987) and peptide mimetics of a predicted binding site in the disintegrin domain of fertilin  $\beta$  (Myles et al., 1994) inhibited sperm-egg fusion. Similar studies using fertilin  $\beta$  active site peptides to inhibit sperm-egg fusion in mouse have been reported (Almeida et al., 1995; Evans et al., 1995). Thus a role for fertilin  $\beta$  in sperm adhesion to the egg plasma membrane is well supported.

The evidence indicating participation of fertilin  $\beta$  in the process of sperm-egg fusion does not provide information as to whether other sperm surface molecules may also function in this process. In the adhesion process between other cell types, multiple receptors and counter receptors are active. For example, in neutrophil adhesion to endothelial cells, there are five pairs of adhesion partners that participate. From the selectin family, L-selectin (on the neutrophil) and E- and P-selectin (on the endothelial cell) bind to carbohydrate ligands on the adhering cell. After these interactions, the integrins  $\alpha$ L $\beta$ 2 and  $\alpha$ M $\beta$ 2 on the neutrophil bind to adhesion partners, ICAM-1 and/or ICAM-2 on the endothelial cell (for review see Springer, 1994).

Fertilin  $\alpha$  and  $\beta$  are the first identified members of a new family of membrane proteins, the ADAM family (a disintegrin and metalloprotease). These proteins share the same multidomain structure, including pro-, metalloprotease, disintegrin, cysteine-rich, EGF-like, transmembrane and cytoplasmic domains (Wolfsberg et al., 1995). At least 17 full length ADAM cDNAs have been cloned and sequenced from mammals (Yagami-Hiromasa et al., 1995; Wolfsberg et al., 1995; Gupta et al., 1996; Weskamp et al., 1996), and ADAM sequences have also been found in other species including *Xenopus laevis* (Blobel, C., personal communication), *Drosophila melanogaster* (Rooke et al., 1996), and *Caenorhabditis elegans* (Podbilewicz, 1996). This family of proteins has sequence homology with soluble snake peptides and proteins that contain one or more of the above first four domains (Blobel et al., 1992). Disintegrin peptides from snake venom are known to bind to platelet integrin  $\alpha$ Ib $\beta$ 3 (GP IIb/IIIa) and prevent binding of fibrinogen and subsequent clotting (Adler et al., 1991; Beer et al., 1992). By analogy, it is likely that fertilin  $\beta$  binds through its disintegrin domain to an egg integrin. Several different integrins have been identified on mammalian eggs, and at least one of them ( $\alpha_6\beta_1$ ) appears to participate in sperm adhesion to the egg membrane (Almeida et al., 1995).

Five members of the ADAM protein family, all of which are expressed in testis, have been sequenced in mouse (Heinlein et al., 1994; Wolfsberg et al., 1995) and mapped to distinct chromosomes (Lemaire et al., 1994; Cho et al., 1996): fertilin  $\alpha$ , fertilin  $\beta$ , cyritestin, ADAM 4, and ADAM 5. Fertilin  $\alpha$ , ADAM 4, and ADAM 5 are expressed in testis and other tissues, whereas fertilin  $\beta$  and cyritestin (cyritestin sequence data are available from Genbank/EMBL/DBJ under accession number X64227) are testis specific (Heinlein et al., 1994; Wolfsberg et al., 1995). Because each of these proteins has a potential integrin ligand site in the disintegrin domain, we characterized and studied their

functions in sperm-egg adhesion and fusion. Our data suggest that in addition to fertilin  $\beta$ , a second ADAM family member, cyritestin, is also involved in sperm-egg plasma membrane adhesion and fusion.

## Materials and Methods

### Synthesis of Predicted Active Site Peptides from the Disintegrin Domain of ADAM Proteins

Peptides with sequences from the predicted active sites of the disintegrin domains of ADAM proteins were synthesized by the W.M. Keck Biotechnology Resource Center (Yale University, New Haven, CT). The sequences of the peptides were chosen by sequence alignment with the known active sites of snake disintegrins (RGD) and guinea pig fertilin  $\beta$  (TDE; Fig. 1). The peptides synthesized were each linear, eight residue peptides from mouse fertilin  $\alpha$ , fertilin  $\beta$ , cyritestin, ADAM 4, and ADAM 5. Their sequences are underlined in Fig. 1. All of the peptides were purified by HPLC, and their sequences were confirmed by mass spectroscopy.

### Preparations of Antibodies against Fertilin $\beta$ and Cyritestin

Antibodies to the cytoplasmic tail domains of fertilin  $\beta$  and cyritestin were generated by synthesizing peptides containing the COOH-terminal 15 amino acids of fertilin  $\beta$  and cyritestin. The peptides, conjugated to *Diphtheria* toxin, were used as antigens for the immunization of rabbits by Chiron Mimotopes Peptide Systems (Victoria, Australia). Sequences of the peptides are: fertilin  $\beta$ , FSSEEQFESESESKD, and cyritestin, PYRSIP-EDGNSQQ. The corresponding antisera are termed m $\beta$ -CT1 (mouse fertilin  $\beta$  COOH terminus 1) and mCyri-CT1 (mouse cyritestin COOH terminus 1; Table I). m $\beta$ -CT1 and mCyri-CT1 antibodies were affinity purified using the corresponding peptide and a ProtOn™ Kit 1 (Chiron Mimotopes Peptide Systems, Raleigh, NC) following the producer's protocols and application guide. A second antibody to the cytoplasmic tail domain of cyritestin was generated using a peptide containing cyritestin residues 787–800: GNTDQNFMTVPGSF. A multiple antigenic peptide (MAP) with this sequence was prepared by Research Genetics (Huntsville, AL) and used to immunize rabbits. The resultant antiserum is termed mCyri-CT2. mCyri-CT2 antibodies were affinity purified as described.

To produce antibodies to the active site region of fertilin  $\beta$  and cyritestin, chimeric peptides containing a T cell epitope and a B cell epitope were used. The chimeric peptide sequences were: fertilin  $\beta$ , PSDKHIEQY-LKKAKGEVCRLAQDEADVTEYCNGTSE, and cyritestin, PSDKHIEQYLLKARGRLCRKSKDQADFPEFCNGETE. In these peptides, the NH<sub>2</sub>-terminal sequence PSDKHIEQYLLKK is a T cell epitope from the malaria circumsporozoite protein which elicits a strong T cell response and thus obviates the need for conjugating the peptide to a carrier protein (Good et al., 1987). The T cell epitope is followed by a single A as a spacer and then a COOH-terminal sequence (23 residues) from the disintegrin domain active site region, for fertilin  $\beta$ , KGEVCRLAQDEADVTEYC-NGTSE, and for cyritestin, RGRLCRKSKDQADFPEFCNGETE (Heinlein et al., 1994; Wolfsberg et al., 1995). The chimeric peptides were synthesized and cyclized using the two cysteines in the disintegrin domain by the W.M. Keck Biotechnology Resource Center. The residue after the QDE in fertilin  $\beta$  and the KDQ in cyritestin is also a cysteine in the wild-type sequence but was substituted with alanine (A) in these peptides to

Snake:	
kistrin	CRIPRGD-MPDDRC
bitistatin	CRIAARGD-WNDYDC
Guinea pig:	
fertilin $\beta$	CRSTDECDLPEYC
Mouse:	
fertilin $\alpha$	CRPAEDVCDLPEYC
fertilin $\beta$	CRIAQDECDVTEYC
cyritestin	CRKSKDQCDPEFC
ADAM 4	CRDKNGICDLPEYC
ADAM 5	CRKSYDECDLLEYC

**Figure 1.** Sequence alignment of disintegrin domain active sites of snake disintegrins and guinea pig fertilin  $\beta$  with mouse ADAM family members. The sequences shown are the 13 amino acids that form the RGD-containing

loop of two snake venom disintegrins, kistrin and bitistatin, and the corresponding 14 amino acids of guinea pig fertilin  $\beta$  and mouse ADAM family proteins. Italicized residues are those which align with RGD, and the underlined sequences are the peptide sequences tested in the adhesion and fusion assay.

**Table 1. Antibodies to Fertilin  $\beta$  and Cyritestin**

Antibody name	Antigen	Species
m $\beta$ -CT1	Fertilin $\beta$ cytoplasmic tail (amino acids 702–716)	Rabbit
m $\beta$ -AS1	Fertilin $\beta$ active site (amino acids 428–450)	Mouse
mCyri-CT1	Cyritestin cytoplasmic tail 1 (amino acids 809–823)	Rabbit
mCyri-CT2	Cyritestin cytoplasmic tail 2 (amino acids 787–800)	Rabbit
mCyri-AS1	Cyritestin active site (amino acids 453–475)	Mouse

prevent the formation of a disulfide bond with one of the other cysteines. The cyclized peptides were purified by HPLC, and their sequences were confirmed by mass spectroscopy. B10.A(4R) mice (The Jackson Immunoresearch Laboratory, Inc., Bar Harbor, ME) were immunized on day 0 intraperitoneally with 100  $\mu$ g of chimeric peptide emulsified in Complete Freund's Adjuvant (Sigma Chemical Co., St. Louis, MO) followed by two additional intraperitoneal injections (56 d and 89 d) with 100  $\mu$ g chimeric peptide emulsified in Incomplete Freund's Adjuvant (Sigma Chemical Co.). Serum was collected by tail bleeds from the mice 2 wk after the third injection. These antisera to the active site regions of fertilin  $\beta$  and cyritestin are termed m $\beta$ -AS1 and mCyri-AS1.

### Sperm and Egg Isolation for the In Vitro Adhesion and Fusion Assay

Sperm for the in vitro adhesion and fusion assay were isolated from the cauda epididymis and vas deferens of 10–12-wk-old male ICR mice (Harlan Sprague Dawley, Inc., Indianapolis, IN). Dissected cauda and vas were diced with scissors, and the sperm were released into Whittingham's medium (Whittingham, 1971) containing 3% BSA. Released sperm were incubated at 37°C, 5% CO<sub>2</sub>, for 3–4 h in the same medium for capacitation and acrosome reaction. This procedure resulted in a population of 60–70% acrosome-reacted sperm (acrosomal status was determined by Coomassie blue staining; Moller et al., 1990).

Eggs were collected from the oviducts of 6–8-week-old superovulated female ICR mice (Harlan Sprague Dawley, Inc.). Mice were superovulated by the injection of 10 IU of pregnant mare's serum gonadotropin (PMSG; Sigma Chemical Co.) followed 48 h later by an injection of 5 IU of human chorionic gonadotropin (hCG; Sigma Chemical Co.). About 12 h after hCG injection, mice were killed and their oviducts removed and put in prewarmed Whittingham's medium with 0.3% BSA. Cumulus-egg complexes were collected and transferred to 500- $\mu$ l drops of Whittingham's medium containing 300  $\mu$ g/ml Type I-S hyaluronidase (Sigma Chemical Co.) under mineral oil. After a 3–5 min incubation at 37°C, 5% CO<sub>2</sub>, cumulus-free metaphase II eggs (eggs with one polar body) were collected, transferred to a 100- $\mu$ l drop of medium, and then washed through two more 100- $\mu$ l drops. Zona pellucidae of metaphase II eggs were removed by gently passing through a narrow bore pipette after treating with 10  $\mu$ g/ml chymotrypsin (Sigma Chemical Co.) for 2–4 min. Zona-free eggs were washed three times with medium, and then preloaded with 4',6-diamidino-2-phenylindole (DAPI) dihydrochloride (Polysciences, Inc., Warrington, PA) at 10  $\mu$ g/ml for 15 min at 37°C, 5% CO<sub>2</sub>.

### In Vitro Adhesion and Fusion Assay

To test the peptides' effects on sperm-egg adhesion and fusion, peptides were added to eggs at varying concentrations 30 min before addition of sperm and were present throughout the sperm-egg incubation. Capacitated and acrosome-reacted sperm were added to eggs in 500  $\mu$ l Whittingham's medium with 3% BSA under mineral oil to produce a final concentration of 2–4  $\times$  10<sup>4</sup> sperm/ml. After 30 min incubations at 37°C, 5% CO<sub>2</sub>, eggs were gently washed through three 100- $\mu$ l drops of Whittingham's medium with 3% BSA, and then the eggs were mounted onto microscope slides (Corning Glass Works, Corning, NY). Sperm binding was scored under the light microscope with 20 $\times$  magnification. Fusion was scored by the fluorescent labeling of sperm nuclei by DAPI present in the preloaded eggs (Kline and Kline, 1992). Two measures of fusion were used: fertilization index (FI, mean number of fused sperm per egg) and fertilization rate (FR, percentage of eggs fused with at least one sperm).

To test the effects of anti-peptide antibodies on sperm-egg adhesion and fusion, antisera (1:50 final dilution) were added to capacitated and acrosome-reacted sperm and incubated for 30 min at 37°C, 5% CO<sub>2</sub>. Zona-

free eggs were added to the preincubated sperm. Subsequent steps were the same as above.

### Immunoblot Analysis

To study the molecular forms of fertilin  $\beta$  and cyritestin on spermatogenic cells or sperm at different developmental stages, three populations of cells were analyzed. The first was a pool of all testicular spermatogenic cells, referred to as "testicular cells" (Blobel et al., 1990). The second population consisted of isolated testicular sperm, the most fully developed cells in the testis (Blobel et al., 1990). These two populations were isolated through 52% Percoll (Sigma Chemical Co.) gradients (Phelps et al., 1990). The third population was "epididymal sperm," which are sperm gently expressed from the cauda epididymis and vas (Phelps et al., 1990). These three populations of cells were collected from 10–12-wk-old male ICR mice, washed twice with PBS, resuspended in 1 $\times$  SDS sample buffer, heated at 100°C for 4 min, and pelleted in a microcentrifuge at 14,000 rpm for 10 min. The supernatants were used for electrophoresis.

SDS-PAGE was conducted on 10% resolving gels with 5% stacking gels. About 10<sup>6</sup> cells were loaded in each lane. After electrophoresis, proteins were transferred to nitrocellulose membranes (0.2  $\mu$ m; Bio-Rad Laboratories, Richmond, CA), which were then blocked with 5% nonfat dry milk in TBS. Primary anti-peptide antibodies, followed by alkaline phosphatase-conjugated secondary antibodies (Promega Biotech, Madison, WI) were added in TBS containing 3% BSA and 10% FBS. Alkaline phosphatase activity was detected by color developed with 5-bromo, 5-chloro indolyl phosphate, and nitroblue tetrazolium (BCIP/NBT; Sigma Chemical Co.).

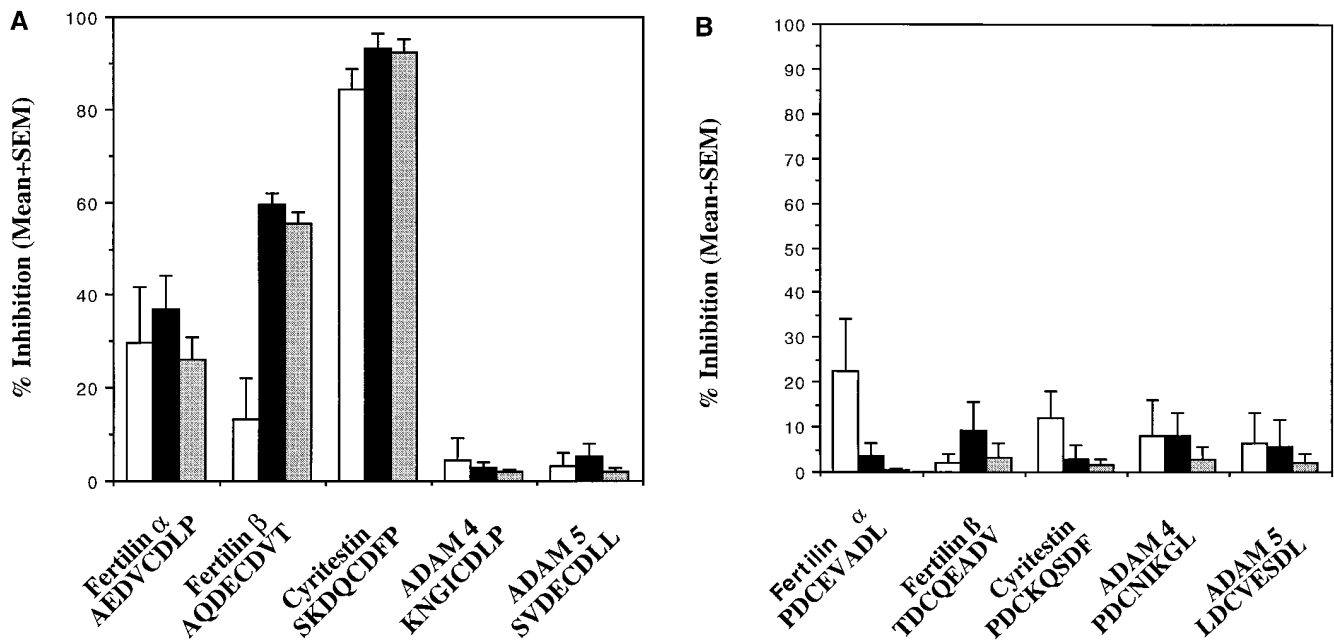
### Sperm Immunofluorescence

Epididymal sperm were collected as described above in Whittingham's medium containing 3% BSA. After 15-min incubations at 37°C, 5% CO<sub>2</sub>, sperm were washed with PBS three times. Both acrosome-intact and acrosome-reacted sperm were used for immunofluorescence. About 5–10% of sperm in the acrosome-intact populations had acrosome reacted. The acrosome reaction was induced as described or by treatment with 10  $\mu$ M A23187 (Sigma Chemical Co.) for 15 min. Preimmune serum, m $\beta$ -AS1 or mCyri-AS1, the antiserum to the active site region of fertilin  $\beta$  or cyritestin, at 1:50 final dilution, was mixed with a 50- $\mu$ l sperm sample (10<sup>7</sup>/ml) in PBS. After 30 min incubation at room temperature, sperm samples were layered onto 0.5 ml of PBS with 3% BSA and pelleted gently. A Fab fragment of goat anti-mouse IgG conjugated with rhodamine (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) was added to the sperm at 1:75 dilution in PBS and incubated for 30 min at room temperature in the dark. Sperm were washed through PBS with 3% BSA as described. Before observation, the sperm were fixed with 1.5% paraformaldehyde for 10 min and then washed once with PBS. Fluorescence with prefixed sperm was done as described by Linder et al. (1995). Images were acquired with a scanning confocal microscope (LSM410; Carl Zeiss, Inc., Thornwood, NY) with 100 $\times$  magnification. Paired phase-contrast images were acquired simultaneously with a transmitted light detector. Images were printed without processing. Sperm fluorescence was also checked with an Axiophot microscope (Carl Zeiss, Inc.) with 60 $\times$  magnification. 50 sperm were checked to determine the percentage of stained sperm for each experiment.

## Results

### Inhibition of Sperm-Egg Plasma Membrane Adhesion and Fusion by Active Site Peptides

We asked if the disintegrin domain of any testis-expressed ADAM family member other than fertilin  $\beta$  has a role in sperm-egg membrane adhesion. We tested the ability of peptides from the putative active site in the disintegrin domain of each protein to inhibit an in vitro adhesion and fusion assay. Three parameters were measured: (a) the number of sperm bound per egg; (b) the FI, and (c) the FR. Structural studies of snake disintegrins have shown that the RGD tripeptide is the integrin-binding site and is located at the tip of a flexible loop created by disulfide bonds (Adler et al., 1991; Chen et al., 1991; Calvete et al.,



**Figure 2.** Effects of ADAM active site peptides on sperm-egg binding and fusion. Both experimental (*A*) and control (*B*) peptides were tested at 500  $\mu$ M. *FI*, fertilization index; *FR*, fertilization rate. The number of eggs tested for each peptide was between 43 and 82. For the controls without peptides, 292 eggs were tested; the average number of sperm bound per egg was 11.6; the *FR* was 88.4%, and the *FI* was 1.17. The sequences of each peptide are under their names. Error bars represent SEM.  $\square$ , Percentage inhibition of binding;  $\blacksquare$ , percentage inhibition of *FI*;  $\text{▨}$ , percentage inhibition of *FR*.

1992; Saudek et al., 1992). The active-site regions of the five mouse testis proteins were predicted by sequence alignment with the snake disintegrin binding loop and guinea pig fertilin  $\beta$  (Fig. 1). Linear, eight residue peptides covering this region (Fig. 1, *underlined*) were tested.

The mouse fertilin  $\beta$  peptide inhibited sperm-egg fusion (Fig. 2 *A*), as expected from previous work with guinea pig fertilin  $\beta$  peptides (Myles et al., 1994) and mouse fertilin  $\beta$  peptides (Almeida et al., 1995; Evans et al., 1995). The fertilin  $\beta$  peptide gave a 59% inhibition in *FI* and a 55% inhibition of *FR*, but only a slight inhibition of sperm-egg binding (13%; Fig. 2 *A*). The cyritestin peptide inhibited sperm-egg fusion more strongly than the fertilin  $\beta$  peptide and additionally inhibited sperm binding to the egg plasma membrane (Fig. 2 *A*). The peptide from cyritestin resulted in 84% inhibition of sperm-egg binding, 93% inhibition of the *FI*, and 92% inhibition of the *FR*. The fertilin  $\alpha$  peptide showed limited inhibition ( $\sim$ 30%) of sperm-egg binding and fusion. Whether or not this is biologically significant is not yet clear. In comparison, the other two family members, ADAM 4 and 5 peptides, showed essentially no inhibition (Fig. 2 *A*). The control peptides for all of the five proteins, which contained the same eight amino acids but in a rearranged (scrambled) order, showed only slight or no inhibition (Fig. 2 *B*).

Inhibition by the fertilin  $\beta$  and cyritestin peptides was dose dependent. Relative to the fertilin  $\beta$  peptide, the cyritestin peptide inhibited to a greater extent at high concentration (500  $\mu$ M) and was also a more potent inhibitor at lower concentration (Fig. 3, *A-C*). For example, using the peptides, 50% inhibition of *FI* was obtained with  $\sim$ 400  $\mu$ M fertilin  $\beta$  and  $\sim$ 70  $\mu$ M cyritestin, and 50% inhibition

of *FR* was obtained with  $\sim$ 470  $\mu$ M fertilin  $\beta$  and  $\sim$ 80  $\mu$ M cyritestin (Fig. 3, *B* and *C*).

Additional control experiments were done to check if inhibiting peptides had an adverse effect on the eggs. Eggs were incubated with the cyritestin peptide as in the previous experiments and were then washed into peptide-free medium before addition of sperm. Under these conditions, sperm-egg binding and fusion were not inhibited.

### Characterization and Processing of Fertilin $\beta$ and Cyritestin Proteins

Because these peptide studies indicated a role for both mouse fertilin  $\beta$  and cyritestin in sperm-egg fusion, we used antibodies made against unique peptide sequences from these two proteins (Table I) to determine their relative molecular weights and if the proteins were proteolytically processed after synthesis. We have previously found that guinea pig fertilin  $\alpha$  and  $\beta$  are first expressed as large precursors in the testis and are subsequently processed (Phelps et al., 1990; Blobel et al., 1990; Wolfsberg et al., 1993). Such processing appears to be required for protein function in some ADAMs (Yagami-Hiromasa et al., 1995). To determine whether mouse fertilin  $\beta$  and cyritestin also undergo processing during sperm development, we immunoblotted testicular cells and sperm cells from different developmental stages with antibodies against fertilin  $\beta$  or cyritestin.

Detergent extracts were prepared from three cell populations: testicular cells (TC), testicular sperm (TS), and epididymal sperm (ES). All extracts were electrophoresed by SDS-PAGE, and then subjected to immunoblot analysis with

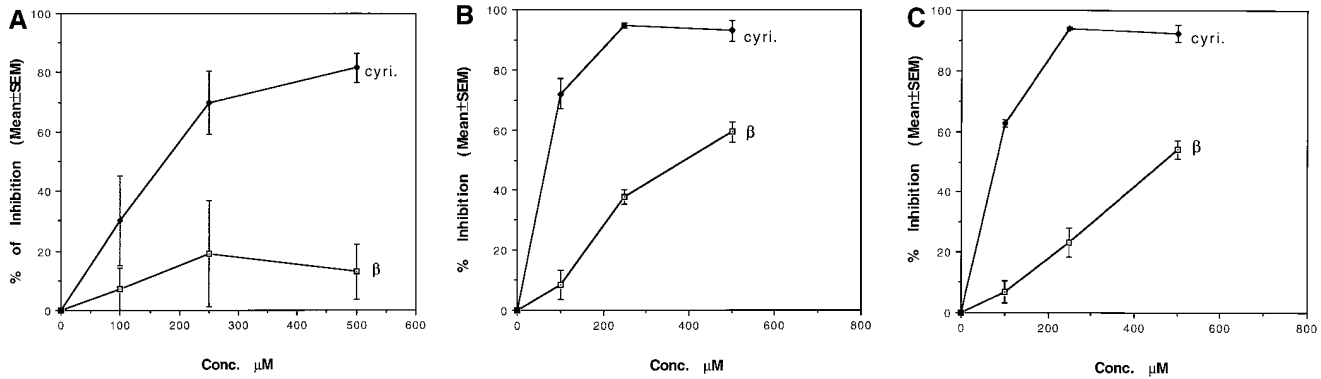


Figure 3. Dose-dependent inhibition of sperm–egg binding (A), FI (B), and FR (C) with fertilin  $\beta$  and cyritestin peptides. Peptide concentrations tested ranged from 100 to 500  $\mu\text{M}$ . Error bars are SEM.

the anti-peptide antibodies. We used antibodies directed against the COOH-terminal cytoplasmic tail (Table I), because the processing of guinea pig fertilin  $\beta$  and meltrin  $\alpha$  occurs in the  $\text{NH}_2$ -terminal extracellular domain at the junction of the metalloprotease and disintegrin domains (Blobel et al., 1992; Yagami-Hiromasa et al., 1995). If a similar pattern of processing occurs for mouse fertilin  $\beta$  and cyritestin, then the COOH-terminal antibodies should be able to recognize both the precursors and processed forms. Using the m $\beta$ -CT1 antibody, we found two bands in the testicular cell extract with molecular mass of 101 and 88 kD (Fig. 4 A). At the testicular sperm stage, there were two bands observed: one at 101 kD and the other at 55 kD. At the epididymal sperm stage, there was only one band that ran at 55 kD (Fig. 4 A). To test the specificity of these bands, 50  $\mu\text{g}/\text{ml}$  of the same peptide used for generating the antibody was added during the first antibody incubation in the immunoblot. The peptide inhibited binding of m $\beta$ -CT1 to all the bands in all three cell populations (Fig. 4 A').

Like fertilin  $\beta$ , cyritestin was expressed as a larger precursor that was processed on epididymal sperm, but the processing was not as complete as with fertilin  $\beta$ . Using the mCyri-CT1 antibody (Table I) as primary antibody in the immunoblot gave a major band at 110 kD in all three cell populations (Fig. 4 B). There was an additional band at 55 kD observed in epididymal sperm (Fig. 4 B). Previously it had been reported that cyritestin was completely processed to the 55-kD form on epididymal sperm (Linder et al., 1995). To understand the difference between our results and this previous report, we also used a second anti-cytoplasmic tail antibody (mCyri-CT2; Table I) made against the identical peptide sequence used previously (Linder et al., 1995). Consistent with their result, this antibody (mCyri-CT2) predominantly recognized the band at 55 kD (Fig. 4 B, ES'). These results indicate that the mCyri-CT1 antibody can recognize both processed and unprocessed cyritestin on epididymal sperm, whereas the mCyri-CT2 antibody recognizes mainly the processed form. There are two additional strong bands with relative molecular weight of 30–35 kD in testicular cells and testicular sperm (Fig. 4 B). All the bands recognized by the cyritestin antibodies disappeared after the addition of 50  $\mu\text{g}/\text{ml}$  of the immunizing peptide during first antibody incubation (Fig. 4 B').

By analogy with fertilin  $\alpha$  and meltrin  $\alpha$ , the cyritestin band of 55 kD is the expected size of a cyritestin processed

form, beginning at the  $\text{NH}_2$  terminus of the disintegrin domain and ending with the cytoplasmic tail COOH-terminal residues recognized by mCyri-CT1. Like meltrin  $\alpha$  on myoblasts, cyritestin on epididymal sperm is only partially

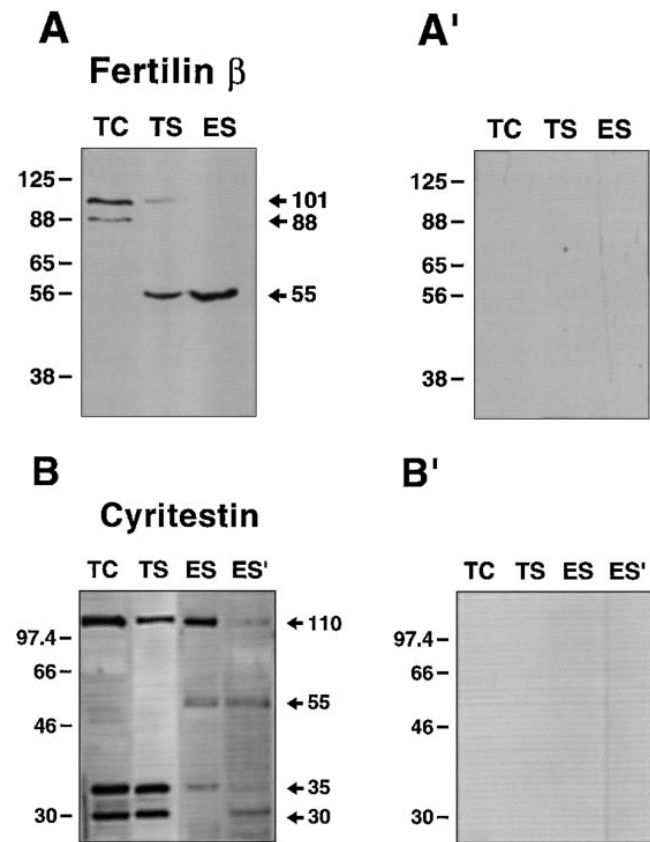
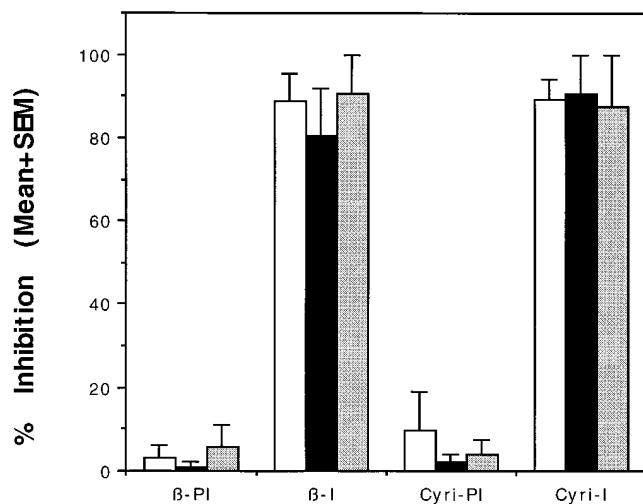


Figure 4. Expression of fertilin  $\beta$  and cyritestin during sperm maturation. Cells at different developmental stages were run on reducing SDS-PAGE and blotted with affinity-purified anti-peptide antibodies (10  $\mu\text{g}/\text{ml}$ ). About  $10^6$  cells were loaded per lane. A, fertilin  $\beta$ ; B, cyritestin. Antibodies for the blots were fertilin  $\beta$ , m $\beta$ -CT1; cyritestin, mCyri-CT1 (TC, TS, ES), and mCyri-CT2 (ES'). The panels on the left (A and B) show the test immunoblots. The panels on the right (A' and B') show control blots done in the same way as the tests, except 50  $\mu\text{g}/\text{ml}$  of peptide was added during the incubation with anti-peptide antibody. TC, testicular cell; TS, testicular sperm; ES, epididymal sperm.



**Figure 5.** Inhibitory effects on sperm–egg binding and fusion of antibodies to the active sites of fertilin  $\beta$  or cyritestin. Both pre-immune and immune sera were tested at 1:50 dilution. *FI*, fertilization index; *FR*, fertilization rate. Approximately 30 eggs were tested with each antiserum. For the controls without serum, 41 eggs were tested; the average number of sperm bound per egg was 5.95; the FI was 1.12, and the FR was 85.1%. Error bars represent SEM. □, Percentage inhibition of binding; ■, percentage inhibition of FI; ▨, percentage inhibition of FR.

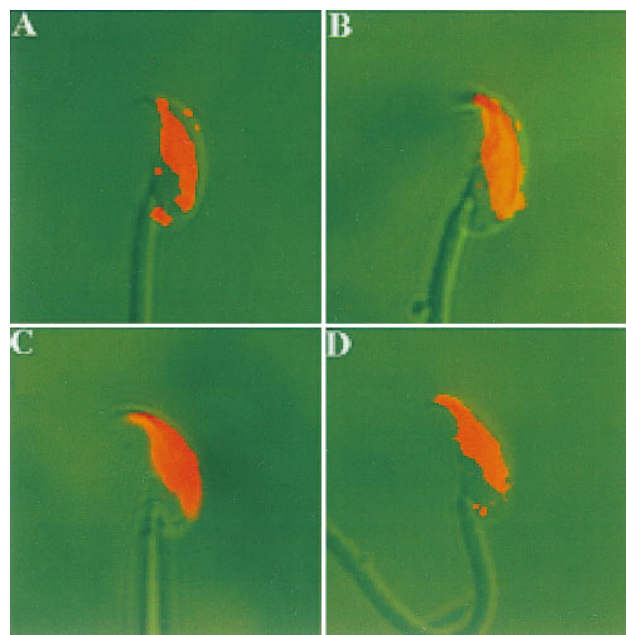
processed to this 55-kD form, and some precursor remains. The two bands of 30–35 kD appear to be proteolytic products whose size indicates loss of the disintegrin domain, and these fragments are of unknown significance.

### Effects of Active Site Antibodies on In Vitro Sperm–Egg Adhesion and Fusion

An alternative approach to using peptide mimetics as inhibitors of the fusion assay was tested. We generated antibodies to the active site region of fertilin  $\beta$  or cyritestin by immunizing mice with chimeric peptides comprised of a malaria T cell epitope and a B cell epitope(s) containing 23 amino acids from the disintegrin active site. These antisera, m $\beta$ -AS1 and mCyri-AS1, immunoblotted fertilin  $\beta$  and cyritestin, respectively, and bound specifically to sperm extracts in an ELISA, i.e., binding was inhibited in the presence of the immunizing chimeric peptide (data not shown). The ability of these anti-active site antisera to inhibit sperm–egg adhesion and fusion was tested. Sperm were induced to acrosome react by incubating for 3 h in capacitating medium. Acrosome-reacted sperm were incubated for 30 min with m $\beta$ -AS1 or mCyri-AS1, and then eggs were added to the sperm. Both m $\beta$ -AS1 and mCyri-AS1 inhibited sperm–egg binding and fusion dramatically (80–90%). The preimmune sera did not have any observable effect on binding or fusion (Fig. 5).

### Localization of Fertilin $\beta$ and Cyritestin on the Sperm Surface

Because interaction of the sperm and egg membranes is a regional process, it is important to know the localization of fertilin  $\beta$  and cyritestin. From staining of permeabilized



**Figure 6.** Indirect immunofluorescence of fertilin  $\beta$  and cyritestin on sperm surface. Live sperm were stained with antisera m $\beta$ -AS1 or mCyri-AS1 at dilution 1:50 followed by the Fab fragment of rhodamine-conjugated goat anti-mouse IgG. The images are representative of the major pattern observed in each sperm population. Micrographs are a combined transmission image (green) and rhodamine-stained image (red). Fertilin  $\beta$ , *A*, acrosome intact sperm; *C*, acrosome reacted sperm. Cyritestin, *B*, acrosome intact sperm; *D*, acrosome reacted sperm.

sperm, it was previously reported that cyritestin was located exclusively on the IAM (Linder et al., 1995). This localization was observed using an anti-cytoplasmic tail antibody. When we stained permeabilized sperm with antibody mCyri-CT2 raised against the same peptide, no difference was found between preimmune and immune sera. The reason for this discrepancy is unclear. Using antibodies to the active site regions of fertilin  $\beta$  and cyritestin, we obtained localized staining patterns on the plasma membrane. Live, swimming, acrosome-intact sperm were stained with the active site antibodies m $\beta$ -AS1 or mCyri-AS1. With either m $\beta$ -AS1 or mCyri-AS1, we observed bright, fluorescent staining restricted to the equatorial region on the majority of sperm (Fig. 6, *A* and *B*). The pre-immune serum controls for both fertilin  $\beta$  and cyritestin did not show any fluorescence.

Because only acrosome-reacted sperm are able to fuse with the egg plasma membrane, we also stained live sperm after they had been induced to acrosome react by incubation in capacitating medium for 3 h or treatment with 10  $\mu$ M A23187. After induction of the acrosome reaction, the main staining pattern (80% of the sperm) showed unchanged localization of cyritestin, i.e., restricted to the equatorial region (Fig. 6 *D*). A small population of the sperm (10–20%) had both equatorial segment and IAM staining after acrosome reaction. The antibody against fertilin  $\beta$ , on the other hand, was localized in both the equatorial and IAM regions of  $\sim$ 70% of the sperm (Fig. 6 *C*). The pre-immune serum controls for both fertilin  $\beta$  and cyritestin also

did not show any fluorescence on acrosome-reacted sperm. The staining of sperm with mCyri-AS1 was abolished in the presence of the immunizing cyritestin chimeric peptide, but was unchanged in the presence of the fertilin  $\beta$  chimeric peptide, indicating that mCyri-AS1 binds specifically to the active site of cyritestin on the sperm surface.

Because antibodies could potentially induce changes in the localization patterns by causing antigen clustering, we also stained sperm after they had been fixed. The same patterns were observed for both fertilin  $\beta$  and cyritestin.

## Discussion

Common domain structures of the ADAM family of membrane proteins indicate that family members may have similar functions. Two of the proteins in this family that have been previously studied, functionally appear to participate in cell–cell fusion: fertilin in sperm–egg fusion (Primakoff et al., 1987; Blobel et al., 1992; Myles et al., 1994; Almeida et al., 1995; Evans et al., 1995), and meltrin  $\alpha$  in myoblast fusion (Yagami-Hiromasa et al., 1995). Because other ADAM proteins were found to be expressed in mouse testis, we asked if any of these additional family members were also involved in sperm–egg adhesion and fusion. We focused on the hypothesis that the predicted active site in the disintegrin domain of these proteins may participate in sperm–egg adhesion and fusion. To test this, we carried out peptide and antibody inhibition studies using an *in vitro* adhesion and fusion assay.

Our studies indicated that cyritestin, one of the ADAM family proteins whose expression is limited to testis, participates in sperm–egg binding and fusion along with fertilin  $\beta$ . Both the predicted active site peptide and an antibody to a peptide from the active site region could inhibit sperm–egg binding and fusion strongly (80–90%). The cyritestin active site peptide was a more potent inhibitor of both binding and fusion than the corresponding fertilin  $\beta$  peptide. 50% inhibition of sperm–egg fusion was obtained with a fivefold lower concentration of cyritestin peptide than fertilin  $\beta$  peptide. Most of the eggs displayed no sperm bound when incubated with the cyritestin active site peptide at saturating concentrations or when sperm were preincubated with anti–active site antibody. If the cyritestin peptide–treated eggs were washed into peptide-free medium, these eggs could be subsequently fertilized.

Sperm–egg fusion was also inhibited by the active site peptide from fertilin  $\beta$ , a result consistent with our previous results in guinea pig (Myles et al., 1994) and with results using shorter (Evans et al., 1995) and longer fertilin  $\beta$  peptides in mouse (Almeida et al., 1995). Additional evidence supporting the role of fertilin  $\beta$  in mouse gamete fusion comes from the experiment with the anti–active site antibody for fertilin  $\beta$ . This antibody strongly inhibits (80–90%) both sperm–egg binding and fusion.

The inhibitory potency of a peptide mimetic of the disintegrin active site of an ADAM protein will depend upon the sequence of the peptide and its affinity for the corresponding receptor on the egg. The cyritestin peptide, SKDQCDFP, and fertilin  $\beta$  peptide, AQDECDVT, are identical at underlined residues 3 = D, 5 = C, and 6 = D. Although these residues may be necessary for binding activity, they are not sufficient for the inhibitory effect.

These same residues are also present in the ADAM 5 peptide, SVDECDLL, which did not inhibit either sperm–egg binding or fusion.

Because specific sperm surface domains are associated with specific functions, the localizations of sperm surface proteins must be consonant with their proposed activities. Localization of cyritestin in the equatorial region is consistent with its participation in sperm–egg fusion. Although both the equatorial regions and posterior head of the sperm membrane fuse with the egg membrane, the initiation of fusion has been reported in some species to occur in the equatorial region (for reviews see Yanagimachi, 1981, 1988, 1994). Fertilin  $\beta$  is also located in the equatorial region of mouse sperm, although it is restricted to the posterior head of guinea pig sperm (Primakoff et al., 1987). This may reflect the fact that, unlike mouse sperm, in guinea pig sperm the equatorial region is very narrow and fusion occurs in the central region of the sperm head, which could include the anterior part of the posterior head as well as equatorial region (Noda and Yanagimachi, 1976).

We found that both mouse fertilin  $\beta$  and cyritestin were made as large precursors (101 and 110 kD, respectively) and processed during sperm maturation to 55-kD forms. The processing of fertilin  $\beta$  was complete, whereas only a portion of the cyritestin precursor was processed. This processing could be a prerequisite for function of these proteins. For the ADAM family member meltrin  $\alpha$ , only the processed form was functional in myoblast fusion (Yagami-Hiromasa et al., 1995).

Our studies indicate at least two testis-specific ADAM family members, cyritestin and fertilin  $\beta$ , have roles in sperm–egg adhesion leading to sperm–egg fusion. Participation of multiple members of a protein family in a single cell–cell adhesion event has been observed in other cases. One system that has been intensely studied is the adhesion of leukocytes to endothelial cells that leads to leukocyte extravasation (for review see Springer, 1994; Frenette et al., 1996). As with sperm adhesion to the egg plasma membrane, this system involves a moving leukocyte attaching to a stationary endothelial cell. When, for example, a neutrophil binds to the endothelial cell, L-selectin on the neutrophil and P- and E-selectin on the endothelial cell initially bind to carbohydrate ligands on the adhering cell. Subsequently, members of the integrin family on the neutrophil,  $\alpha$ L $\beta$ 2 and  $\alpha$ M $\beta$ 2, bind to one or two endothelial ligands, ICAM-1 and ICAM-2, members of the Ig super family.

It is likely that cyritestin and fertilin  $\beta$  bind via the disintegrin domain to egg integrins (Almeida et al., 1995). We do not yet know if either cyritestin or fertilin  $\beta$  bind to one or more (integrin) receptors on the egg. In addition, the two proteins could bind to the same or different sets of receptors. In neutrophil–endothelial cell adhesion, the ligand ICAM-1 binds to both  $\alpha$ L $\beta$ 2 and  $\alpha$ M $\beta$ 2 integrins, whereas the ligand ICAM-2 binds only to  $\alpha$ L $\beta$ 2 (for review see Springer, 1994).

Cyritestin and fertilin  $\beta$  may be acting in sperm adhesion sequentially or concurrently. Sequential action could involve the first binding step activating the egg receptor or sperm ADAM for the second binding step. Concurrent action could involve cooperativity in promoting cell adhesion. In either case, disruption of either adhesion results in a strong block to sperm–egg fusion, indicating both pro-

teins are required. This finding of unexpected complexity in the sperm adhesion process may provide a key to understanding how adhesion occurs and how it leads to fusion of the two gametes.

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