Fertilin α (also known as ADAM1) is a member of the ADAM (A disintegrin and A metalloprotease domain) family of proteins. In this study, we examine the mechanism of mouse fertilin α’s in adhesion of sperm to the egg plasma membrane during fertilization. We find that recombinant forms of fertilin α corresponding to either the disintegrin-like domain or the cysteine-rich domain and the EGF-like repeat can perturb sperm-egg binding, suggesting that both of these domains can participate in fertilin α-mediated adhesion events. In further examination of the fertilin α disintegrin-like domain, we find that a subdomain of disintegrin-like domain with the sequence DLEECDCG outside the putative disintegrin loop but with homology to the fertilin β disintegrin loop can inhibit the binding of both sperm and recombinant fertilin α to eggs, suggesting that this is an adhesion-mediating motif of the fertilin α disintegrin-like domain. This sequence also inhibits the binding of recombinant fertilin β to eggs and thus is the first peptide sequence found to block two different sperm ligands. Finally, a monoclonal antibody to the tetraspanin protein CD9, KMC.8, inhibited the binding of recombinant fertilin α to eggs in one type of binding assay, suggesting that, under certain conditions, fertilin α may interact with a KMC.8-sensitive binding site on the egg plasma membrane.

ADAM1 (for A disintegrin and A metalloprotease) proteins comprise a recently identified and rapidly growing molecular family of membrane proteins. To date, ~30 ADAMs have been identified in a variety of animal species, including several mammals, Xenopus laevis, Drosophila melanogaster, and Caenorhabditis elegans (1, 2). The conserved domain structure of ADAMs (see Fig. 1A) and functional analyses of these proteins indicate that members of this family have functions as proteases and/or as cell adhesion molecules. In the ADAMs that have been described to function as cell adhesion molecules, the adhesive activity generally appears to be attributable to the disintegrin-like domain, a domain with homology to integrin ligand-like snake venom polypeptides. These venom polypeptides, known as disintegrins, contain an RGD tripeptide, presented at the end of an extended loop structure called the “disintegrin loop” (3). Although ADAMs share significant sequence homology in their disintegrin-like domains to snake venom disintegrins, they do not have RGD sequences in their putative disintegrin loops (with the exception of human ADAM15). Various studies have shown that the adhesion-mediating sequence of several ADAMs appears to be a short sequence within the putative disintegrin loop, such as the ECD-containing sequences in fertilin β (4, 5), ADAM9 (6), and ADAM23 (7), or the QCD-containing sequence in cyritestin (8).

Among the cell adhesion events that are mediated by ADAMs are the interactions between mammalian gamete plasma membranes during fertilization. On mouse sperm, there are at least three ADAMs that appear to participate in sperm-egg binding: fertilin α (ADAM1), fertilin β (ADAM2), and cyritestin (ADAM5) (reviewed in Ref. 9). As noted above, fertilin β and cyritestin, as well as several other ADAMs, appear to mediate cell adhesion through short peptide sequences within the putative disintegrin loop of the disintegrin-like domain (4, 5, 8). With regard to fertilin α, studies of the putative disintegrin loop have been inconclusive, with a synthetic peptide corresponding to the fertilin α disintegrin loop (AEDVCDLP) having a modest inhibitory effect on sperm-egg interactions but with the scrambled control peptide (PDCE-VADL) also having some effect (8). Nevertheless, there are data that imply that the fertilin α disintegrin-like domain participates in cell adhesion but also that the cysteine-rich domain can mediate adhesion as well. A recombinant form of fertilin α corresponding to the complete extracellular domain (i.e. the disintegrin-like domain, the cysteine-rich domain, and the EGF-like repeat) inhibits sperm-egg binding more effectively than does a shorter form with a truncated disintegrin-like domain (10), suggesting that the presence of disintegrin-like domain in recombinant fertilin α enhances the ability of the recombinant fertilin α protein to inhibit sperm-egg binding. However, this truncated recombinant fertilin α, containing only the last C-terminal 20 amino acids of the disintegrin-like
domain, along with the cysteine-rich domain, and the EGF-like repeat (designed with respect to the original N-terminal sequence data (11)), still binds to mouse eggs and inhibits sperm-egg binding (12). In agreement with this, an antibody against a similar construct of recombinant rabbit fertilin α, with a truncated disintegrin-like domain, inhibits rabbit in vitro fertilization (IVF) (13). These data suggest that domains in fertilin α other than the disintegrin-like domain, namely the cysteine-rich domain and/or the EGF-like repeat, participate in sperm-egg adhesion. Interestingly, this is similar to another member of the ADAM family, ADAM12, which appears to be able to utilize multiple domains to mediate cell adhesion, based on the observation that recombinant forms of the ADAM12 cysteine-rich domain (14) and the ADAM12 disintegrin-like domain (15) can support cell adhesion in vitro.

The primary purpose of this study was to test the hypothesis that the fertilin α disintegrin-like domain contains amino acids that interact with egg-binding sites. Our results with recombinant fertilin α disintegrin-like domain (hereafter referred to as recombinant fertilin αD) suggest that the fertilin α disintegrin-like domain participates in fertilin α-mediated sperm-egg binding. An examination of candidate subdomains of the fertilin α disintegrin-like domain to characterize what region(s) of the disintegrin-like domain had adhesive activity identified a short adhesion-mediating sequence that has homology to the fertilin β disintegrin loop but is in a location distinct from the putative disintegrin loop region of fertilin α.

In light of this finding and other studies demonstrating that function-blocking antibodies against the α9 integrin subunit or against the tetraspan protein CD9 inhibit sperm and fertilin β binding to eggs (16–18), we also investigated the possibility that function-blocking antibodies against either of these proteins on eggs (GoH3 against α9; KMC.8 against CD9) would inhibit the binding of the recombinant fertilin αD to eggs. We find that GoH3 has no effect but that KMC.8 reduces recombinant fertilin αD binding detected by one type of assay. This observation suggests that the fertilin α disintegrin-like domain could interact with a KMC.8-sensitive site on the egg membrane but also indicates that the mode of presentation of recombinant fertilin α affects the sensitivity of fertilin α to inhibition by KMC.8.

**MATERIALS AND METHODS**

**Production of Recombinant Fertilin Proteins**—The complete extracellular portion of fertilin α (recombinant fertilin αDCE) included amino acids 406–708 (counting the start Met as amino acid 1) and was encoded by nucleotides 1216–2124 (counting the start ATG codon of full-length mouse fertilin α, assembled from the two partial cDNA sequences (accession numbers AF167406 and U22056), as nucleotides 1–3). This cDNA region was prepared by polymerase chain reaction (PCR) amplification using a 5′ primer corresponding to nucleotides 1216–1232 engineered with a XbaI restriction site (GTTCTAGAGCTGCCAATTGTGGGAA) and a 3′ primer corresponding to nucleotides 2109–2124 engineered with a stop codon and a SalI restriction site (TCTGCTGACTTTACGGTACCTCT). The region encoding the extracellular portion of fertilin α lacking the disintegrin domain (recombinant fertilin αCE, amino acids 493–708, encoded by nucleotides 1477–2124) was prepared by PCR amplification using a 5′ primer corresponding to nucleotides 1477–1493 engineered with a XbaI restriction site (GTTCTAGACGTTGATAGATTAT) and a 3′ primer corresponding to nucleotides 2109–2124 engineered with a stop codon and a SalI restriction site (TCTGCTGACTTTACGGTACCTCT). The region encoding the fertilin α disintegrin-like domain (recombinant fertilin αD, amino acids 406–493, encoded by nucleotides 1216–1478) was prepared by PCR amplification using 5′ primer corresponding to nucleotides 1216–1232 engineered with a XbaI restriction site (GTTCTAGAGCTGCCAATTGTGGG) and a 3′ primer corresponding to nucleotides 1463–1479 engineered with a stop codon and a SalI restriction site (TCTGCTGACTTTACGGTACCTCT). PCR amplifications using mouse fertilin α cDNA (pVACA-α) as template were performed in a PEC 2400 thermocycler (PerkinElmer Life Sciences) with PfuI polymerase (Promega, Madison, WI). The PCR products were then digested with XbaI and SalI and cloned into pMAL-p.2 (New England Biolabs, Beverly, MA) as described previously (10, 12, 19). The resulting plasmids were sequenced to verify that the DNA sequence encoded the correct amino acid sequence. All recombinant fertilin-maltose-binding protein (MBP) fusion proteins were expressed in DH5α Escherichia coli cells with induction by isopropyl-1-thio-β-D-galactopyranoside, then purified on an amylose affinity column, and renatured by reduction and oxidation as previously described (10, 12, 19).

**Synthetic Peptides**—Peptides corresponding to different parts of the disintegrin domain of fertilin α were synthesized by Alpha Diagnostic International (San Antonio, TX) and purified by high pressure liquid chromatography to >95% purity. The N termini were acetylated, and the C termini were amidated. The sequences were chosen based on sequence alignment to the RGD-containing disintegrin loops of snake venom disintegrins (peptide α1; AEDVCLDL, amino acids 465–472 of full-length mouse fertilin α), because of conservation between fertilin α and cytoxin (peptide α2; DLPFYCDG, amino acids 470–477) or because of homology to the disintegrin loop of fertilin β (peptide α3; DLEECDCG, amino acids 416–423). The scrambled peptide controls contained the same eight amino acids in rearranged order (α1s, EPCVDALD; α2s, EGDCPLD; α3s; CLEDGDC). Respectively, peptides were resuspended in degassed, sterile water to a stock concentration of 5 mg/μl and then lyophilized in aliquots such that a 25-μl resuspension in Whitten’s culture medium (20) would yield a final peptide concentration of 1 μM.

**Bacterial Alkaline Phosphatase-presented Peptides**—Bacterial alkaline phosphatase (BAP)-presented peptides were generated as described previously (4). Oligonucleotide sequences that were used are shown in Table I. In brief, complementary oligonucleotides were an-

| BAP-presented peptide | Oligonucleotide used to generate BAP-presented peptides |
|------------------------|-------------------------------------------------------|
| BAP-a-1                | CTAGT GCT GAG CAT GTG GTT GAC CCT CCC G               |
|                        | A CTA CTC TCA CAT ACA CAT GAG GAG GCT CCG            |
|                        | A D E V C D L P                                       |
| BAP-a-1s               | CTAGT GAG CCC TGG GTG GTT GCT GCT CTT GAC            |
|                        | A TCA GCA ACA CTA GCA GAA GCT CCG CCG CTTAG          |
|                        | E P C V D A L D                                       |
| BAP-a-1m               | CTAGT GCT GAG GTA GAT GCT GCT GCT CTT CCC G          |
|                        | A CTA CTC TCA CTC ACA CGA GAA GAG GCT CCG CTTAG      |
|                        | A E E V C D P                                        |
| BAP-a-3                | CTAGT GAC TCT GAG GTA GAT GAG GTG GAT GCC GGC G       |
|                        | A ACT AAC CTC CTC ACA ACT ACG ACC CCG CTTAG          |
|                        | D L E E C D C G                                       |
| BAP-a-3s               | CTAGT GTG GAG TGT GAC GGC GAC TCG TGC GAC GAG        |
|                        | A ACA CTC AAC CTG CCS CTG ACG CTC CTTAG              |
|                        | D C E G D C E                                        |
| BAP-a-3m               | CTAGT GAC TTT GAG TGT GAC TGC GAC TGC GAG           |
|                        | A CTG AAC CTC CTT GGT CAT ACG CCG CTTAG              |
|                        | D L E A E V C G                                       |
nealed to each other and ligated into the XbaI and BglII restriction sites in the multiple cloning site of the plasmid pHNAP, which includes a 6-histidine tag. Fusion proteins were expressed and purified on a nickel column as described previously (4).

Egg Collection, Zona Pellucida Removal, and IVF—Metaphase II-arrested eggs were released from superovulated 6–9-week-old CF-1 mice (Harlan, Indianapolis, IN); egg collection and cumulus cell removal were performed as described previously (4). All gamete cultures were performed in Whitten’s medium (20) containing 22 mM NaHCO₃ and 15 mM glucose bovine serum albumin (Albunax; J. Life Technologies, Inc.) at 37 °C in 5% CO₂ in air. For most experiments, the zona pellucida (ZPs) were removed from eggs via solubilization with a very brief incubation (∼15 s) in acidic medium-compatible buffer (4). For experiments using the monoclonal antibody GoH3 (anti-α, integrin subunit), the ZPs were removed by incubating the eggs in medium containing 10 μg/ml chymotrypsin (Sigma) for 5 min, allowing the ZP to swell, and then shearing off the ZP with a thin bore pipette. This treatment is used because GoH3 does not bind to eggs from which the ZP has been removed by acid solubilization (19). Following ZP removal, the eggs were allowed to recover for 60 min in Whitten’s medium. Sperm were collected from the caudae epididymides of C57BL/6J × SJL/J male mice (8–10 weeks old; Jackson Laboratories, Bar Harbor, ME) and capacitated in vitro for 2.5–3 h. To examine the effects of recombinant fertilin α proteins on sperm-egg interactions, ZP-free eggs (20–25 eggs/5–6.5 μl drop) were incubated for 60 min prior to insemination in Whitten’s medium containing 1.0 μM of synthetic peptide α₁, α₁s, α₂, a-2, a-3, or a-3s for 60 min. After incubation of eggs in either the peptide or the recombinant fertilin α protein, 5–6.5 μl of a suspension of capacitated sperm were added so that the final sperm concentration was 500,000 sperm/ml (adapted from Ref. 21). The final concentration of the peptides during insemination was 0.5 μM, and the final concentration of the recombinant proteins during insemination was 0.25 mg/ml. After a 15-min insemination, the eggs were washed three times to remove loosely attached sperm (adapted from Ref. 21). The eggs were then fixed in 3.7–4.0% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 for 5 min, and mounted in VectaShield mounting medium (Vector Labs, Burlingame, CA) containing 1.5 μg/ml 4',6-diamidino-2-phenylindole (Sigma) to stain the sperm DNA. The average number of sperm bound per egg was determined. The values from each experiment were normalized with respect to the appropriate control (i.e. the amount of peptide or recombinant fertilin α protein, 5–6.5 μl of a suspension of capacitated sperm were added so that the final sperm concentration was 500,000 sperm/ml) (adapted from Ref. 21). The final concentration of the peptides during insemination was 0.5 μM, and the final concentration of the recombinant proteins during insemination was 0.25 mg/ml.

RESULTS

Laminogenic Immunoassay to Quantify the Binding of Recombinant Fertilin Proteins to Eggs—To determine the effects of synthetic peptides or recombinant α proteins on the binding of recombinant fertilin α or fertilin β, ZP-free eggs were first incubated in the indicated synthetic peptide (at 0.5 μM) or BAP-presented peptide at the indicated concentration (20, 40, or 80 μM) in Whitten’s medium containing 15 mg/ml bovine serum albumin for 60 min and then in Whitten’s medium containing both 0.5 μg/ml of the indicated form of recombinant fertilin α or fertilin β and the indicated BAP-presented peptide for an additional 60 min. During these incubations, the culture medium was supplemented so that the final concentrations of CaCl₂ and MgSO₄ were 2.4 and 1.2 mM, respectively. The binding of MBP recombinant fertilin fusion proteins to ZP-free eggs was quantified as described previously (4), using an anti-MBP polyclonal rabbit antiserum (diluted 1:750; New England Biolabs), followed by an alkaline phosphatase-conjugated secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit IgG at 0.12 μg/ml; Jackson Immunoresearch, West Grove, PA). Egg-associated alkaline phosphatase activity was detected using the photon-emitting AP substrate disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2'-5'-chboro-tricyclo[3.3.1.1⁵³]decan-4-y]l)phosphate (Tropex, Bedford, MA); Photon emission was measured in raw light units/10 s in a Monolight 3100 luminometer (Analytical Luminescence Laboratory, Sparkill, NY). The digital images were normalized with respect to the negative control (indicated in the figure legends). Statistical analyses by analysis of variance with Fisher’s protected least significant difference post-hoc testing were performed using StatView version 5.0 (SAS Institute).

Assessment of the Binding of Bead-immobilized Recombinant Fertilin Proteins to Eggs—Fluorescent beads (0.2 μM yellow-green sulfate-de-derivatized latex FluoSpheres; Molecular Probes, Eugene, Oregon) were coated with recombinant fertilin αD (see Fig. 1C) or recombinant fertilin βD (4) as follows. The beads (5 μl of a 2% bead suspension) were incubated with 5 μl of 2 mg/ml anti-MBP IgG (purified by chromatography on protein G beads (Life Technologies, Inc.) from monomolecular clone MBP-17 ascites fluid (Sigma) for 2 h. The beads were then washed three times with phosphate-buffered saline followed by centrifugation at 4500 × g for 20 min. Recombinant fertilin αD or βD was then added to the beads (at a final concentration of 1 mg/ml in a total volume of 15 μl), and the beads were incubated overnight at 4 °C. The beads were then incubated with rabbit IgG (final concentration, 1 mg/ml) for 1 h at room temperature. The beads were washed three times with PBS and resuspended to a concentration of 0.2% bead suspension in Whitten’s medium compatible buffer (109.5 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 7 mM NaHCO₃, 15 mM HEPES, WHITCO). The 0.2% bead suspension was sonicated three times for 30 s immediately prior to use and then diluted to 0.02% in Whitten’s medium containing 15 mg/ml bovine serum albumin and 100 μg/ml of the indicated BAP-presented peptide. This 0.02% bead suspension was used to make 20-μl culture drops, to which ZP-free eggs were added, following a 60-min incubation in medium containing the indicated BAP-presented peptide at 100 μM. The eggs were co-cultured with beads for 60 min at 37 °C in 5% CO₂. The eggs were washed four times through 100-μl drops of Whitten’s medium to remove unbound beads and were then mounted on a microscope slide in Whitten’s medium and examined using fluorescent microscopy. Digital images were captured with a Princeton 5-mHz cooled interline CCD camera (Princeton Instruments Inc., Trenton, NJ) using IP Lab (Scananalitics, Fairfax, VA) and Photoshop (Adobe Systems Incorporated, San Jose, CA) software. The bead binding levels were assessed qualitatively, estimated as compared with binding levels in control groups, by examining eggs from the entire experimental series (three experiments/series, with 10–20 eggs/group/experiment examined). The level of recombinant fertilin βD-coated bead binding to control eggs (see Fig. 7) appeared to be higher than the level of binding of recombinant fertilin αD-coated beads (see Fig. 6); this is in agreement with results using the quantitative luminometric assay.

Effects of Different Recombinant Forms of Fertilin α on Sperm-Egg Binding—We have previously demonstrated that a form of recombinant fertilin α comprising amino acids 409–708 of mouse fertilin α, including the disintegrin-like, cysteine-rich, and EGF-like repeat domains, inhibited sperm-egg interactions (10). In that study, we also tested a form of mouse fertilin α that corresponded to the originally reported N-terminal sequence of guinea pig fertilin α (11) comprising a truncated disintegrin-like domain (the C-terminal most 20 amino acids), the cysteine-rich domain, and the EGF-like repeat (amino acids 474–708). This recombinant protein also perturbed sperm-egg binding, although not as effectively as the longer form encompassing amino acids 493–708 (10, 12), which contained a truncated disintegrin-like domain and the EGF-like repeat domains, inhibited sperm-egg interactions (10). In that study, we also tested a form of mouse fertilin α that corresponded to the originally reported N-terminal sequence of guinea pig fertilin α (11) comprising a truncated disintegrin-like domain (the C-terminal most 20 amino acids), the cysteine-rich domain, and the EGF-like repeat (amino acids 474–708). This recombinant protein also perturbed sperm-egg binding, although not as effectively as the longer form encompassing amino acids 493–708 (10). To follow up on this observation, we used a recently developed, improved assay for sperm-egg binding (21) and generated three forms of recombinant fertilin α to examine more closely the role of the disintegrin-like domain in fertilin α-mediated sperm-egg adhesion. Our updated form of recombinant fertilin αDCE corresponded to amino acids 406–708, based on the corrected N-terminal sequence of guinea pig and the N-terminal sequence of bovine fertilin α (22, 23) (please note that this differs slightly from our first version of recombinant fertilin αDCE corresponding to amino acids 409–708, based on the corrected N-terminal sequence of guinea pig and the N-terminal sequence of bovine fertilin α; please note that the form of αDCE used in these experiments does not include any portion of the disintegrin-like domain and thus differs from the form we used in previous experiments (10, 12), which contained a truncated disintegrin-like domain based on the original N-terminal sequence data for guinea pig fertilin α (11). Recombinant fertilin αD corresponded to the 88 amino acids (amino acids 406–493) of disintegrin-like domain of mouse fertilin α (Fig. 1C). In IVF

X. Zhu and J. P. Evans, unpublished data.
experiments using these proteins, we observed that sperm-egg binding was reduced in the presence of the three recombinant fertilin α forms (αDCE, αD, and αCE) as compared with sperm-egg binding in the presence of the control protein, MBP (Fig. 2). (We have previously demonstrated that sperm-egg binding and fusion are not affected by the presence of MBP in the IVF culture medium (19).) In these studies, the levels of sperm-egg binding in the presence of recombinant fertilin αDCE, αD, and αCE were not statistically significantly different from each other.

Effects of Synthetic Peptides Corresponding to Different Portions of the Fertilin α Disintegrin Domain on Sperm-Egg Binding—Because recombinant fertilin αD had an inhibitory effect on sperm-egg binding (Fig. 2), we became interested in the possibility that a specific subdomain(s) of the fertilin α disintegrin-like domain could mediate the interaction of sperm with the egg plasma membrane. To investigate this possibility, three amino acid sequences (Fig. 3) were tested as synthetic peptides for their abilities to perturb sperm-egg binding during IVF. Peptide a-1 (AEDVCDLP, amino acids 465–472) was identical to the fertilin α peptide tested by Yuan et al. (8) and corresponded to the putative disintegrin loop, lining up with the RGD-containing region of snake venom disintegrins. Peptide a-2 (DLPEYCDG, amino acids 470–477) corresponded to a stretch of eight amino acids just C-terminal from the putative disintegrin loop; this amino acid sequence was chosen because it was well conserved in all fertilin α homologs, in all cyritestin homologs, and in fertilin β homologs as well (see Table I in Ref. 9). In addition, the last four of the eight amino acids of this highly conserved DLPEYCDG sequence were present in the mature fertilin α domain (accession numbers U22056 and AF167406). The proteolytic cleavage site and the domain structure of fertilin α were predicted based on N-terminal amino acid sequence data on guinea pig and bovine fertilin α (22, 23). C, domain structures of the MBP fusion proteins corresponding to different regions of mature fertilin α.

Because recombinant fertilin αDCE binding, with protein binding levels being comparable with the levels of binding in the absence of any peptide. Peptide a-3 also reduced the binding of recombinant fertilin αD but did not have a significant effect on the binding of recombinant fertilin αDCE to −50% of control levels (control being defined as the level of binding in the absence of peptide) (Fig. 5). None of the other peptides affected recombinant fertilin αDCE binding, with protein binding levels being comparable with the levels of binding in the absence of any peptide. Peptide a-3 also reduced the binding of recombinant fertilin αD but did not have a significant effect on the binding of recombinant fertilin αDCE to −50% of control levels (control being defined as the level of binding in the absence of peptide) (Fig. 5). None of the other peptides affected recombinant fertilin αDCE binding, with protein binding levels being comparable with the levels of binding in the absence of any peptide.
Adhesion Mediated by the Fertilin α Disintegrin Domain

Disintegrin-like domains of fertilin α homologs and design of peptide sequences. A shows the alignments of the amino acid sequences of the disintegrin-like domains of mouse (M), guinea pig (GP), macaque (Mac), rabbit (Rab), rat, bovine (Bov), orangutan (Oran), and baboon (Bab) fertilin α. Shown below these is the consensus (Con) sequence. The VCD tripeptide sequence (in bold type) corresponds to the putative disintegrin loop. The EEDC sequence (in bold type and underlined) has homology to the DECD in the fertilin β disintegrin loop. The LPEYC adjacent to the VCD in the putative disintegrin loop (underlined with a dotted line) is highly conserved in all species orthologs of fertilin α (also shown in the consensus sequence). B shows the amino acid sequences of peptides a-1, a-2, and a-3 relative to where they align with respect to the mouse fertilin α amino acid sequence. Peptide a-1 corresponds to the putative disintegrin loop within disintegrin-like domains (overlapping with the RGD-containing region of true disintegrins). The numbers above the sequences refer to the numbering of the mouse fertilin α amino acid sequence (assembled from the two partial cDNA sequences; accession numbers AF167406 and U22056), counting the methionine encoded by the start codon as amino acid 1.

which we found that a BAP-presented peptide with the sequence corresponding to the fertilin β disintegrin loop sequence (AQDECDVT) inhibited the binding of recombinant fertilin β to eggs much more effectively (i.e. lower IC50) than did synthetic peptides (4). Furthermore, in contrast to our experience with some batches of synthetic peptides, we have had no problems with toxicity of BAP-presented peptides on eggs (4). We therefore used BAP-presented peptides to confirm our results with synthetic peptides a-1 and a-3 (Fig. 4). We chose to examine the a-1 sequence more closely because we were surprised that the a-1 synthetic peptide had no effect (Figs. 4 and 5). Disintegrin loop peptide sequences inhibit disintegrin-mediated adhesion in studies of other ADAMs (fertilin β (24–27), cyritestin (8, 28), ADAM9 (6, 29), ADAM15 (15, 30), and ADAM23 (7)), and we therefore wanted to verify that the a-1 sequence had no inhibitory effect on recombinant fertilin α binding by using a BAP-presented peptide form of the a-1 sequence. We surmised that the BAP-presented peptide form could be more effective than the synthetic peptide form, based on our studies of fertilin β (4). The experiments were designed as follows. BAP-presented peptide versions of a-1 and a-3 sequences (BAP-a-1 and BAP-a-3, respectively) were tested at 40 μM because this was the concentration with which we observed the maximal inhibitory effect with a BAP-presented peptide corresponding to the fertilin β disintegrin loop sequence (4). Two control BAP-presented peptides for a-1 and a-3 were used: one with scrambled amino acid sequences (BAP-a-1s and BAP-a-3s, respectively) and one with mutated sequences in which two or three amino acids were changed (BAP-a-1 m or BAP-a-3 m, respectively). Mutated residues in BAP-a-1 and BAP-a-3 were chosen based on studies of the fertilin β disintegrin loop, in which the ECD tripeptide, particularly the Asp residue, were determined to be important for adhesive activity (4, 5). Therefore, the VCD in the a-1 sequence AEDVCDLP was changed to AEDVCDLP in BAP-a-1 m, and the ECD in the a-3 sequence DLEEDCIG was changed to DLEAAVCG. BAP, expressed from the parental vector pHNAP without any modifications, was also used as a control.

The effects of the BAP-presented peptides on recombinant fertilin α binding to eggs (Table II) were similar to the effects of the synthetic peptides (Fig. 5). Neither BAP-a-1 nor its controls (BAP-a-1s and BAP-a-1 m) had a significant inhibitory effect on...
recombinant fertilin αD binding (Table II), in agreement with results with synthetic peptide a-1 on sperm-egg binding or recombinant fertilin α binding (Figs. 4 and 5). (Please note that recombinant fertilin α binding was not increased in either synthetic or BAP-presented a-1 peptide, and we do not have evidence that the sperm binding in the presence of synthetic peptide a-1 (Fig. 4) was due to any stimulatory effect on sperm adhesion.) BAP-a-3 reduced the binding of recombinant fertilin αD to eggs, and this effect appeared to be concentration-dependent (Table II). At 20 μM, BAP-a-3 reduced the binding of recombinant fertilin αD to −60% of control levels, and at 40 and 80 μM BAP-a-3 reduced the binding of recombinant fertilin αD to −50% of control levels (Table II); all of these differences were statistically significant as compared with control BAP.

Some inhibitory effects were observed with the scrambled control, BAP-a-3s, with reduced binding of recombinant fertilin αD being observed with eggs treated with 40 and 80 μM BAP-a-3s (−60% of control; p < 0.05) (Table II). Such effects were not observed with the mutated control, BAP-a-3m, implying that the effects of BAP-a-3s may be due to the amino acid composition, with four of the eight amino acids being acidic residues.

We also used a different protein binding assay to confirm the effect of BAP-a-3 on recombinant fertilin α binding. This assay utilized fluorescent beads coated with the ligand of interest and then allowed these ligand-coated beads to bind to ZP-free eggs (16). As shown in Fig. 6, incubation of ZP-free eggs with 40 μM BAP-a-3 reduced the binding of recombinant fertilin αD-coated beads (Fig. 6F), as compared with untreated eggs (Fig. 6B) or eggs incubated with control BAP (Fig. 6D). We estimated that the level of bead binding was reduced by ~50%; this is consistent with the results using soluble recombinant fertilin αD detected by the luminometric immunoassay (Table II). BAP-a-3s appeared to have a comparable inhibitory effect (Fig. 6H), whereas the levels of bead binding to BAP-a-3m-treated eggs (Fig. 6J) appeared to be similar to the levels of binding to the control eggs (Fig. 6, B and D), consistent with the results presented in Table II from the luminometric immunoassay for the binding of soluble recombinant fertilin αD.

Effects of Synthetic and BAP-presented a-3 Peptides on the Binding of Recombinant Fertilin β to Eggs.—We also examined whether the a-3 sequence, as a synthetic peptide or a BAP-presented peptide, would inhibit the binding of recombinant fertilin β. The amino acid sequence of peptide a-3 (DLEECDCG) has homology to the sequence of the disintegrin loop of fertilin β (AQDECDVT), including the ECD tripeptide that has been shown to be important for mouse fertilin β function (4, 5). Therefore, we hypothesized that the a-3 sequence could inhibit the binding of recombinant fertilin β because of the presence of the ECD tripeptide.

Peptide a-3, as a synthetic peptide and as a BAP-presented peptide, perturbed the interaction of recombinant fertilin β with eggs (Table III). The levels of binding in the presence of 80 μM of BAP-a-3 and 80 μM of BAP-βDL (positive control, with the sequence AQDECDVT, corresponding to the disintegrin loop of fertilin β) were statistically similar. The scrambled a-3s sequence, as a synthetic peptide (at 500 μM) and as BAP-a-3s (at 80 μM), inhibited recombinant fertilin β binding to eggs to ~52–58% of control levels (Table III). BAP-a-3s had less of an effect when tested at 40 μM (reducing binding to ~77% of control levels). This may indicate that the charge characteristics shared by peptides a-3 and a-3s, both with four of the eight residues being acidic, affected the binding of recombinant fertilin β. In agreement with this, BAP-a-3m, with only two acidic residues, did not inhibit the binding of recombinant fertilin β to eggs (Table III).

We also assessed the effect of BAP-a-3 on bead-immobilized recombinant fertilin β binding to eggs (Fig. 7). BAP-a-3 significantly reduced the binding of recombinant fertilin βD-coated beads (Fig. 7F), as compared with untreated eggs (Fig. 7B) or eggs treated with control BAP (Fig. 7D). BAP-a-3s appeared to have a modest inhibitory effect (Fig. 7H), whereas the levels of bead binding to BAP-a-3m-treated eggs (Fig. 7J) appeared to be

### Table II

Effects of BAP-presented peptides on the binding of recombinant fertilin αD to eggs

| BAP-presented peptide | 20 μM | 40 μM | 80 μM |
|-----------------------|-------|-------|-------|
| a-3 (DLEECDCG)        | 61 ± 3a | 48 ± 3a | 46 ± 5a |
| a-3s (CELDGDCE)       | 109 ± 4b | 58 ± 3a | 60 ± 9b |
| a-3m (DLEAAVCVG)      | 98 ± 7   | 94 ± 4  | 85 ± 7  |
| a-1 (AEDVCVLDLP)      | ND      | 94 ± 19 | ND     |
| a-1s (EPCVDALD)       | ND      | 79 ± 23 | ND     |
| a-1m (AEDVAAALP)      | ND      | 97 ± 11 | ND     |

*a Statistically significantly different (p < 0.05) from levels of protein binding in the presence of control BAP.

### Table III

Effects of peptides on the binding of recombinant fertilin β to eggs

| Peptide (sequence), concentration | Levels of protein binding to control eggsa (%) |
|-----------------------------------|-----------------------------------------------|
| Synthetic a-3 (DLEECDCG), 500 μM | 42 ± 2a, c |
| Synthetic a-3s (CELDGDCE), 500 μM| 52 ± 6b, c |
| BAP-a-3 (DLEECDCG), 40 μM       | 55 ± 5b, c |
| BAP-a-3s (CELDGDCE), 40 μM      | 77 ± 7b, c |
| BAP-a-3m (DLEAAVCVG), 40 μM     | 92 ± 3b, c |
| BAP-βDL (AQDECDVT), 40 μM       | 38 ± 6b, c |
| BAP-a-3 (DLEECDCG), 80 μM       | 49 ± 6b, c |
| BAP-a-3s (CELDGDCE), 80 μM      | 58 ± 1b, c |
| BAP-a-3m (DLEAAVCVG), 80 μM     | 97 ± 7b, c |
| BAP-βDL (AQDECDVT), 80 μM       | 40 ± 4b, c |

*a For experiments with synthetic peptides, the data are expressed as percentages of the level of protein binding in the absence of synthetic peptide.

*b For experiments with BAP-presented peptides, the data are expressed as percentages of the level of protein binding in the presence of control BAP.

The values are expressed as percentages of the level of protein binding in the presence control BAP.

\[
\text{Percent binding} = \frac{\text{Experimental binding}}{\text{Control binding}} \times 100
\]
similar to the levels of binding to the control eggs (Fig. 7, B and D), consistent with the results presented in Table III from the luminometric immunoassay.

Effects of Anti-αα Integrin and Anti-CD9 Antibodies on the Binding of Recombinant Fertilin α to Eggs—Monoclonal antibodies against the αα integrin subunit (GoH3 (16)) and against the tetraspanin protein CD9 (KMC.8 (17) or JF9 (18)) have been reported to inhibit sperm-egg binding during IVF, and there are data that suggest these antibodies can perturb the binding of fertilin β to eggs. Because fertilin α is also a member of the ADAM family, we hypothesized that GoH3 or KMC.8 could perturb the binding of fertilin α. To test this hypothesis, we used the two different binding assays described above to assess the binding of soluble recombinant fertilin αD diluted in the culture medium using the luminometric immunoassay and to assess the binding of recombinant fertilin αD immobilized on fluorescent beads. The results from the two assays were similar with GoH3. GoH3 (anti-αα) did not have an effect on the binding of soluble recombinant fertilin α detected by the luminometric assay (Fig. 8A) and had a slight or negligible inhibitory effect on the binding of recombinant fertilin αD immobilized on fluorescent beads as compared with the control nonimmune IgG (Fig. 8B). Interestingly, however, different results were obtained in the two different assays with KMC.8 (anti-CD9). KMC.8 did not inhibit the binding of soluble recombinant fertilin αD (Fig. 8A) but had an inhibitory effect on the binding of bead-immobilized recombinant fertilin αD (Fig. 8D).

We also examined the effects of GoH3 and KMC.8 on the binding of recombinant fertilin αE to eggs. KMC.8 did not affect the binding of soluble recombinant fertilin αE (data not shown). GoH3 decreased the binding of soluble recombinant fertilin αE very slightly, to ~75% of levels observed to eggs treated with control IgG (data not shown). However, we could not confirm this result using bead-immobilized recombinant fertilin αE. This may be because a modest inhibitory effect (e.g. a 25% decrease, as was observed with the luminometric immunoassay) in the level of bead binding is very difficult to assess confidently.

DISCUSSION

In this study, we have demonstrated that the disintegrin-like domain of mouse fertilin α participates in sperm-egg adhesion during fertilization and have identified a region of this domain that partially mediates this adhesive activity. This region has the amino acid sequence DLEEDCG and is in the N-terminal portion of the disintegrin-like domain (amino acids 416–423, peptide a-3; Fig. 3). We observe that a synthetic peptide with the a-3 sequence DLEEDCG inhibits sperm-egg binding during IVF (Fig. 4) and that a synthetic peptide and a BAP-presented peptide with the a-3 sequence inhibit the binding of recombinant fertilin α to eggs (Figs. 5 and 6 and Table II). Two other candidate peptides, including one corresponding to the putative fertilin α disintegrin loop (peptide a-1), do not have these effects. These data regarding fertilin α differ from those regarding several other ADAMs. Adhesive events mediated by ADAMs such as fertilin β (24–27), cyritestin (8, 28), ADAM9 (6, 29), ADAM15 (31), and ADAM23 (7) are inhibited by peptides corresponding to the sequences in the putative disintegrin loops of these ADAMs. Additional studies of the interactions of fertilin β with eggs (4) and of ADAM15 with CHO cells (30) suggest that the disintegrin loops of these proteins are the key (and perhaps only) adhesion-mediating sequences involved in these particular interactions. Interactions of fertilin α with eggs is instead inhibited by a peptide corresponding to the DLEEDCG region (Fig. 3; which we refer to as the a-3 region, referring to its peptide name and the acronym alternative adhesion-mediating motif in fertilin α) of the fertilin α disintegrin-like domain.
This a-3 region in fertilin α contains an ECD sequence (Fig. 3), a tripeptide that is also found in the putative disintegrin loops of several ADAMs that function as cell adhesion molecules, such as fertilin β (4, 5), ADAM9 (6), and ADAM23 (7). The ECD sequence in the a-3 region of fertilin α is highly conserved in many ADAM family members identified in eight mammalian species and in X. laevis, D. melanogaster, and C. elegans, with 64 of 71 ADAMs having an ECD (42 of 71), or the similar sequences DCD (4 of 71) or QCD (18 of 71) in the region aligning with the fertilin a-3 region. The putative disintegrin loop sequences are similarly conserved, with 49 of 71 ADAMs having an ECD (38 of 71), a DCD (7 of 71), or a QCD (4 of 71) tripeptide. Although it is not known at this time whether the a-3-like regions in other ADAMs play any role in mediating adhesion or how these motifs are presented, the conservation of this sequence in regions aligning with the fertilin a-3 peptide is striking. Nevertheless, it is worth noting that the critical sequence motif of the fertilin α disintegrin-like domain remains to be definitively defined. Mutating the a-3 sequence DLEELDCG to DLEAYVCG (a-3m) abolishes the ability of this sequence to perturb interactions of recombinant fertilin α and β with the egg plasma membrane, although the partial activity of CELGDDCE (a-3s) implies that the amino acid composition or spacing (e.g. alternating acidic residues) could be a factor as well (Tables II and III and Figs. 6 and 7). This differs from what is known about disintegrin loop regions, with ECD motifs in fertilin β (4, 5) and ADAM23 (7) and similar DCD, SCD, or ACD motifs in various forms of ADAM15 and ADAM12 (15) being implicated in adhesive activity. The CD residue pair does not appear to be sufficient to perturb the binding of fertilin α or sperm, because the a-1 and a-2 sequences (AEDVCDLP and DLPEYCDG, respectively) did not have significant inhibitory activity (500 μM of a synthetic peptide or 40 μM of a BAP-presented peptide; Figs. 4 and 5 and Table II).

The DLEELDCG a-3 sequence inhibits the binding of not only recombinant fertilin α but also recombinant fertilin β to eggs. This agrees with previous findings regarding the ECD tripeptide in fertilin β (4, 5) and augments these studies by demonstrating that an ECD tripeptide in the context of a different amino acid sequence (DLEELDCG instead of AQDECDVT) can perturb fertilin β binding to eggs. In addition, the a-3 sequence is the first peptide shown to block two different sperm ligands.

We also present data indicating that two different regions of fertilin α, the disintegrin-like domain and the cysteine-rich domain and/or EGF-like repeat, participate in fertilin α-mediated adhesion of sperm to the egg plasma membrane during fertilization (Fig. 2). This makes fertilin α similar to ADAM12, which also potentially uses multiple domains (the disintegrin-like domain (15) and the cysteine-rich domain (14)) to mediate cell adhesion. To date, three different cysteine-rich domains have been reported to participate in cell adhesion events: those of fertilin α (Refs. 10 and 12 and this study), ADAM12 (14, 32), and atrolysin C, a snake venom metalloprotease that is closely related to ADAM proteins (33). In contrast, the cysteine-rich domain of fertilin β does not appear to have adhesive activity (10). Other ADAM cysteine-rich domains have not been examined to date.

These data suggest that there may be similarities between fertilin α-mediated adhesion and ADAM12-mediated adhesion. Moreover, the utilization of two different regions in fertilin α and ADAM12 is analogous to what is known about fibronectin. Fibronectin supports cell adhesion via the α2β1 integrin through two different domains, the canonical adhesion-mediating RGD tripeptide in the tenth fibronectin type III repeat, and a synergy domain in the ninth repeat, composed of sequence PHSRN and other amino acids (34, 35). Our data suggest that at least two regions of fertilin α are involved in adhesion: the DLEELDCG a-3 region of the disintegrin-like domain and some as yet unidentified portion(s) of the cysteine-rich domain and/or EGF-like repeat. Furthermore, it is possible that the disintegrin loop of fertilin α participates in fertilin α-mediated adhesion, even though we did not observe any inhibition of sperm or recombinant fertilin α binding to eggs with a-1 peptide (Figs. 4 and 5 and Table II), based on what is known about the fibronectin synergy domain. Although the ninth fibronectin type III repeat has no adhesive activity on its own (36) and PHSRN peptides have no inhibitory activity alone or in combination with RGD peptides (34), several residues in this ninth type III repeat play a role in α2β1-mediated cell adhesion to fibronectin (34, 35). BAP-a-3 did not completely inhibit the binding of recombinant fertilin αD even at concentrations up to 80 μM (Table I and Fig. 6); this contrasts findings regarding fertilin β, in which BAP-βDL (corresponding to the fertilin β disintegrin loop) inhibited the binding of recombinant fertilin βD near base-line levels at concentrations of 30–40 μM (4). Thus, the a-3 region and the disintegrin loop and/or some other region(s) in the fertilin α disintegrin-like domain may combine to have synergistic activity in cell adhesion. In the future, it would be interesting to investigate binding affinities of the disintegrin-like and cysteine-rich domains and mutated versions thereof and to perform further structure-function analyses to dissect how this sperm ligand interacts with the egg membrane. Furthermore, because fertilin α can form a heterodimer with fertilin β (23, 37, 38), the face of a fertilin α-fertilin β dimer could present additional combinations of binding sites, all of which could work synergistically to mediate sperm-egg adhesion. Finally, the utilization of two different regions in fertilin α raises the possibility that different receptors on the egg surface may interact with different domains. This may be the case with ADAM12, because the disintegrin-like domain appears to interact with the α2β1 integrin (15) and the cysteine-rich domain appears to interact with a syndecan proteoglycan (14, 32).

With regard to egg binding partners for fertilin α, we present data indicating that an anti-CD9 antibody can perturb the binding of recombinant fertilin αD immobilized on fluorescent beads (Fig. 8B). However, the anti-CD9 antibody had no effect on soluble recombinant fertilin αD (i.e. diluted in culture medium and detected by a quantitative luminometric immunoassay; Fig. 8A). The other antibody we tested, the anti-α2 integrin function-blocking antibody GoH3, did not dramatically affect the binding of recombinant fertilin αD in either binding assay (Fig. 8). These data suggest that the GoH3 antibody does not significantly perturb the ability of fertilin α to interact with the egg membrane and that fertilin α immobilized on small beads interacts with a KMC.8-sensitive binding site, whereas soluble fertilin α interacts with a KMC.8-insensitive site. Although this dissimilarity of the results with the KMC.8 antibody is perplexing, it may in fact be providing some insights into how fertilin α interacts with the egg plasma membrane, particularly in light of data on the molecular, biochemical, and biophysical properties of complex adhesive events, such as adhesion under flow conditions or adhesion during cell motility (39, 40). The data from studies of these systems underscore the fact that adhesion is an integrated process involving multiple binding contacts (i.e. binding sites on individual molecules as well as multiple types of molecules) and is affected by several biochemical and biophysical parameters such as affinities, on and off rates, and ligand density and clustering. In this instance with fertilin α, ligand density is very likely to be affected by immobilization of ligand protein on beads, which could generate a
microenvironment with a relatively high concentration of ligand as compared with free ligand in solution. The presentation of ligand on beads (0.2 μm in diameter) also has the potential to mimic the presentation of fertilin α on the mouse sperm head (~5 μm in length). Therefore, such considerations are especially applicable to sperm-egg interactions (and thus to fertilin α) because gamete adhesion events are likely to show complexities that are somewhat similar to the complexities of adhesion events of motile cells and cells under flow.

Acknowledgments—We thank William Hanna and Bayard Storey for critical reading of the manuscript.

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