

Mediation of sperm–egg fusion: evidence that mouse egg $\alpha_6\beta_1$ integrin is the receptor for sperm fertilin β

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Introduction: A key step leading to fertilization is the binding of sperm to the egg plasma membrane. When a mammalian sperm reaches the egg plasma membrane, fertilin β , an extracellular sperm membrane protein, is believed to bind to an egg plasma membrane receptor triggering fusion. We set out to identify the fertilin β binding partner on the egg plasma membrane.

Results: We synthesized an ^{125}I -labeled peptide with the consensus Asp–Glu–Cys–Asp (DECD) sequence of fertilin β 's disintegrin domain. This peptide contains a benzophenone photoaffinity probe and inhibits sperm–egg fusion. Upon photoactivation in the presence of whole mouse eggs, a single polypeptide was covalently labeled. This polypeptide has been identified by immunoprecipitation as an α_6 integrin complexed with β_1 integrin.

Conclusions: Our experiments establish that small peptides containing the consensus DECD sequence of sperm fertilin β bind specifically to an $\alpha_6\beta_1$ integrin receptor on the egg membrane. We conclude that fertilin β binds directly to the $\alpha_6\beta_1$ integrin on the egg surface and this partnership mediates sperm–egg fusion.

Introduction

Fertilization of a mammalian egg is a multistage process involving cell–matrix and cell–cell interactions that culminates in membrane fusion. First, sperm hydrolyze hyaluronic acid in the cumulus layer in order to penetrate it and reach the zona pellucida, a glycoprotein matrix surrounding the egg. Upon sperm binding to the zona pellucida, the acrosome reaction is initiated, creating a passage through the zona pellucida to the egg plasma membrane. In the final stages of fertilization, sperm bind and fuse with the egg plasma membrane. Although much progress has been made in characterizing the sperm–egg zona pellucida interactions, the molecular players required for sperm binding and fusion with the plasma membrane have not all been identified. In addition, little is known about the signals that initiate embryonic development or the cortical granule reaction that blocks polyspermic fertilization.

A major breakthrough in understanding the molecular basis for sperm binding and fusion to the plasma membrane occurred with the discovery of fertilin. Fertilin is a membrane-bound protein that has been isolated from guinea pig [1] and cow [2] as a complex of fertilin α and fertilin β subunits. Fertilin is present on the equatorial region of the mouse sperm head where initiation of fusion events occurs [3]. Monoclonal antibody PH-30 raised against the fertilin β subunit blocks sperm–egg fusion [1]. Consequently, when the sperm reaches the plasma membrane, fertilin is believed to bind to an egg plasma membrane receptor triggering fusion [4–6].

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Fertilin β is present in all mammalian species screened to date, including humans. The same is true for fertilin α except in humans and gorillas. In humans and gorillas, fertilin α is a pseudogene that does not encode for a full-length protein [7,8]. The domain structures of fertilin α and β have been predicted from their primary amino-acid sequences [9]. Since the discovery of fertilin, a large family of cellular fertilin homologs containing both metalloprotease and disintegrin domains has been identified and designated ADAM (a disintegrin and metalloproteinase) [10] or MDC (metalloprotease/disintegrin/cysteine-rich protein) [11]. The mature forms of fertilin α and fertilin β contain a disintegrin domain, a cysteine-rich region and an EGF-like repeat, in addition to a transmembrane region and a short cytoplasmic tail; the metalloprotease domains have been proteolytically cleaved. Both recombinant extracellular fertilin α and fertilin β bind to the microvillar region of zona-free eggs, where sperm preferentially bind, and inhibit sperm binding [12–14]. In addition, fertilin α contains a putative fusion peptide. Although fertilin α is not expressed in humans or gorillas, other ADAM proteins could substitute as a partner for fertilin β , for example ADAM20 or ADAM21 [15]. In addition, recent studies with fertilin β knockout mice suggest that the role of fertilin α might be different than originally hypothesized, and further experiments are required to determine what that role is [16]. Many of the ADAMs identified to date are present in mammalian male reproductive tissue, although new ADAMs have been found in *Xenopus* testis [17]. ADAMs are also present in somatic tissue. They have diverse roles ranging from myoblast

Figure 1

SVMP P-II disintegrins	
Kistrin [65]	CKFSRAGKICRIPRGD-MPDDRCQTGQSADC
SVMP P-III disintegrins	
Atrolysin [66]	CKFTSAGNVCRPARSECDIAESCTGQSADC
SVMP P-IV disintegrins	
RVVX [67]	CKIKTAGTVCAARDECDVPEHCTGQSABC
ADAM disintegrins	
1 Mouse (fertilin α)	CTFKKKGSLCRPAEDVCDLPEYCDGSTQEC
2 Mouse (fertilin β) [10]	CKLKRKGEVCRLAQDECDVTEYCNGTSEVC
2 Monkey [68]	CLFMSQERVCRPSFDECDLPEYCNGTSASC
2 Guinea pig [9]	CEPKTRKEVCRBESTDECDLPEYCNGSSGAC
2 Human [69]	CLFMSKERMCRPSFECDLPEYCNGSSASC
2 Cow [2]	CAPIPKGHI CRGSTDECDLHEYCNSSAAC
2 Rat [70]	CNLKARGELCRPANQECVTEYCNGTSEVC
2 Rabbit [71]	CTPKERGQSCRPPVGECDLPEYCNGTSALC
3 Mouse (cyritestin) [72]	CTIAERGRRLCRKSKDQCFPEFCNGETEGC
4 Mouse [10]	CKFAPTGTICRDKNGICDLPEYCSGASEHC
5 Mouse (tMDC II) [10]	CTVKMNDVVCRKSVDECDLLEYCNGKDFYC
6 Rat (tMDC IV) [73]	CTYSPSGTLCRPIQNICDLPEYCSGNNIFC
9 Mouse (MDC 9) [74]	CQFLPGGSMCRGKTSSECDVPEYCNGSSQFC
20 Human [15]	CKPLPSGTLCRQVGECDLPEWCNGTSHQC
21 Human [15]	CKFMESGELCRQEVNECDLPEWCNGTSHQC
18 Rat (tMDCIII) [73]	CELSAAGTPCRKAVDPECDPTEYCNGTSSDC
14 <i>C. elegans</i> (AMD-1) [75]	CELRRKAGDTCRSSKSPCDVAEQCDGKSGDC
16 <i>X. laevis</i> (xMDC16) [17]	CKLLPKGTLCRMPKTECDLAEYCDGASNHC

Sequences of the binding-loop regions of representative disintegrins. For the sake of brevity, only a single sequence from each of the SVMP sub-families and nonmammalian ADAM sequences are shown. Also, only mammalian ADAM sequences expressed in testis are shown. They are listed in order of assigned ADAM number with any alternative names in parentheses. The binding loops are blue. The consensus sequences are yellow (SVMP P-II), red (SVMP P-III), and magenta (SVMP P-IV and ADAM). Conserved cysteines are green. The relevant references are indicated.

fusion in mammals [18] to nervous system development in *Drosophila* [19].

Prior to the discovery of fertilin, disintegrin domains were only known to occur as soluble snake toxins in snake venoms [20–22]. These toxins are antagonists of platelet aggregation and bind to integrin receptors, hence their name. There are three classes of toxin that contain disintegrin domains, SVMP-II, SVMP-III and SVMP-IV [20]. All classes of disintegrin domains are rich in cysteine, and the positions of the cysteines are conserved. The tertiary structure of the SVMP-II disintegrins has also been determined [23–25]. The cysteines form a rigid disulfide-linked structure that serves as a framework for presenting a 9–10 amino acid loop that binds to the integrin receptor. The consensus binding sequence of the SVMP-II disintegrins, Arg-Gly-Asp (RGD), is presented at the tip of this poorly structured hairpin loop. SVMP-II disintegrins and small peptides containing the RGD sequence are antagonists of $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ integrins, present on platelets and stromal

cells, respectively, that bind fibrinogen. Comparison of the putative binding loop of the disintegrin domain of fertilin β from different species highlights a consensus (D/E)ECD sequence (hereafter referred to as DECD), rather than RGD (Figure 1). There is no consensus sequence in the analogous region of fertilin α . Fertilin β is therefore more similar to the SVMP-IV class of disintegrins, for which the specific integrin target is not known. Moreover, fertilin is an integral membrane protein, and the SVMPs are not.

Considered together, these similarities and differences between fertilin β and the SVMPs suggested that fertilin β might be a cell-adhesion molecule that binds to an integrin receptor on the egg plasma membrane via its disintegrin domain; fusion might then occur via a second protein-protein interaction [6], or simply as a result of adhesion [26]. In fact, the presence of integrins on the egg plasma membrane is well documented [27–31]. Furthermore, the analogy between RGD disintegrins and DECD disintegrins appears to be correct; small peptides containing part of the consensus DECD sequence of fertilin β from the putative disintegrin-binding loop inhibit sperm-egg fusion and/or binding in mice and guinea pigs [3,27,32–34].

In elegant experiments, Almeida *et al.* [27] have shown that sperm will bind to somatic cells that express both α_6 and β_1 integrins. This binding is inhibited by DECD peptides and anti- α_6 monoclonal antibody (mAb). Furthermore, Evans *et al.* [12,13] have developed an extraordinarily sensitive method for measuring binding of recombinant fertilin to single eggs. Binding of recombinant fertilin β to mouse eggs is reduced by peptides containing the DEC part of the DECD consensus sequence [12], suggesting that the DECD sequence is in the binding loop of fertilin β . Furthermore, recombinant fertilin β with the disintegrin domain deleted does not inhibit sperm-egg binding [14]. Binding of recombinant fertilin β is reduced by anti- β_1 polyclonal antibody. Binding of recombinant fertilin β to mouse eggs is not reduced by anti- α_6 mAb, however. There is agreement therefore that sperm fertilin β binds to a β_1 integrin on the egg surface. The identity of the partner α subunit has not been clearly established. In addition, the above-described experiments do not eliminate the possibility that other extracellular proteins, present both in somatic cells and eggs, are required for binding of fertilin β and sperm to the egg surface integrin.

In our work, we have investigated the cellular target of these DECD peptides, and thus of fertilin β . We wanted to establish if there is a direct binding interaction between sperm fertilin β and egg integrins, or if mediator proteins are required. Using peptide inhibition, photoaffinity labeling and immunoprecipitation, we have shown that DECD peptides bind to egg $\alpha_6\beta_1$ integrin without mediation by other sperm proteins. The DECD peptides represent a new ligand motif for integrins that have a different specificity

than RGD peptides. These results suggest that fertilin β binds directly to the $\alpha_6\beta_1$ integrin on the egg surface and this partnership mediates sperm–egg fusion.

Results and discussion

Design and synthesis of photoaffinity label 1

A number of groups have synthesized peptides that correspond to part of the putative binding loop (Figure 1) of the disintegrin domain of fertilin β , as well as other ADAM proteins present on the sperm surface, for example, ADAM 5 and cyritestin (ADAM 3), and tested them as inhibitors of sperm–egg binding and fusion [3,27,32–34]. Peptides containing the ECD sequence inhibit fusion by 50% at 500 μM [33], as does a peptide with the sequence AQDEC [32]. Dimers of the ECD peptides or cyclic disulfides of CAQDEC do not inhibit fusion, nor does a cyclic peptide containing the sequence ESD. A longer fertilin β sequence CRLAQDEADVTEYC in which the cysteine residue of ECD is replaced with an alanine residue, results in 50% inhibition at 250 μM [27]. These experiments suggest that binding affinity is gained from residues adjoining the consensus sequence, and that the cysteine of ECD is not present in a disulfide. If the DECD sequence is presented in the wrong context, it does not bind to the egg receptor. This is borne out by the result that an ADAM 5 peptide, SVDECDLL, does not inhibit binding or fusion [3]. It is not yet clear whether the variability of the first aspartate residue in the DECD sequence of fertilin β across different species is a result of receptor-specificity differences between species or because that residue is not as important for binding. Cyritestin has a DQCD sequence rather than a DECD sequence and the corresponding binding-loop peptide, SKDQCDFP, shows 50% inhibition at 70–80 μM [3]. The amino-acid sequences of the putative binding loops of fertilin β and cyritestin are significantly different, suggesting that they might have different binding partners on the egg surface. Thus, the minimal peptide required to mimic fertilin β and to inhibit sperm–egg fusion contains the DECD sequence with a reduced thiol. Two flanking amino acids at the amino and carboxyl termini confer specificity.

In order to determine the direct target of the fertilin β disintegrin-binding loop, we synthesized the radioactive photoaffinity probe, **1**, that contains 12 amino acids of the mouse binding loop (Figure 2). We extended the minimal DECD sequence by two additional amino acids from the fertilin β sequence on either side of the DECD to ensure specificity. Two additional amino acids from the fertilin β sequence were included at the amino terminus of AQD-ECDVT, to avoid steric interference at the receptor-binding site upon incorporation of the photoaffinity label, 4-benzoyl-phenylalanine, at the amino terminus. RLAQD-ECDVT was extended by three amino acids at the carboxyl terminus to include the tyrosine residue present in the fertilin β sequence. This tyrosine residue served as a

handle for introduction of an ^{125}I label. An alanine residue was substituted for the cysteine residue at the carboxyl terminus in fertilin β to simplify the synthesis.

We chose 4-benzoyl-phenylalanine as our photoaffinity probe because of the ideal characteristics of the benzophenone moiety [35–37]. Benzophenone is excited to its active ketyl diradical at 350–360 nm. This property allows the probe to be manipulated in ambient light and also minimizes photodamage of proteins. Furthermore, benzophenone is more stable than other photoprobes such as azides or diazo esters. Excited benzophenones react preferentially with unreactive C–H bonds; buffer nucleophiles and solvent water do not interfere with labeling. Benzophenone is a selective photoprobe primarily because hydrogen abstraction must be followed by combination of the ketyl and alkyl radicals formed to achieve labeling. The photoprobe must therefore have a sufficient residence time in the receptor, that is, high affinity, for labeling to occur.

The peptide photoprobe was synthesized using standard solid-phase methods, with Fmoc (N^{α} -9-fluorenylmethylloxycarbonyl) α -amino protection [38,39]. The peptide was cleaved from the resin and deprotected to yield the reduced peptide **2** (Figure 2). The disulfide dimer of **2** was prepared by dimethyl sulfoxide (DMSO)-catalyzed air oxidation to protect the thiol during the iodination reaction and could be stored for extended periods of time. The tyrosine residue in dimer **2** was iodinated using chloramine-T and Na^{125}I to yield dimer **1**, which was reduced to **1**, purified using high performance liquid chromatography (HPLC) and used immediately for photoaffinity labeling experiments (Figure 2). Peptide **3**, which contains the same amino acids as **1** except in a scrambled order, was synthesized and used in photoaffinity labeling experiments as a control.

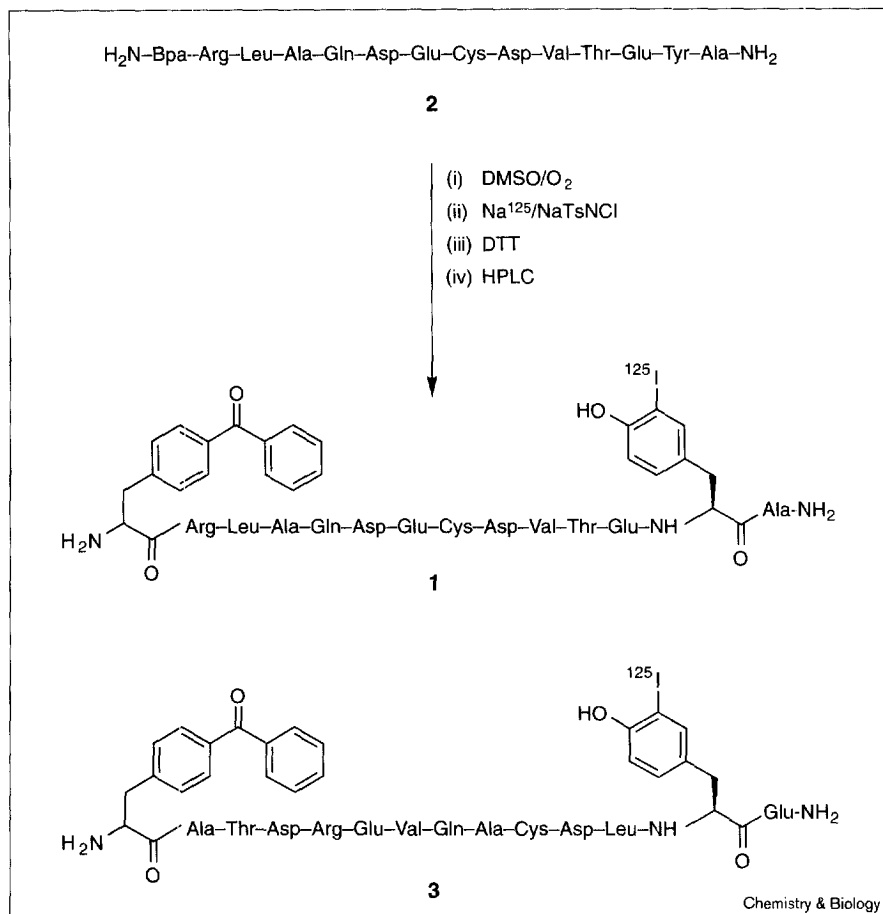
Inhibition of *in vitro* fertilization with DECD peptides

Peptides **2–4** were tested in a mouse *in vitro* fertilization assay (Table 1). Both the fertilization rate (number of eggs fertilized) and fertilization index (number of sperm fused per egg) [34] were determined using zona-free eggs in the absence and the presence of the peptides. As expected, **2** is a good inhibitor of sperm–egg fusion. Lack of inhibition by the scrambled peptide **3** demonstrates that inhibition of fusion is not an artifact arising from addition of benzophenone to the peptide sequence. Inhibition by the linear peptide Ac-ECDA Y -NH $_2$, **4**, has been described previously [33]. This peptide is used as a positive control in the labeling experiments described below. A mimic of the fertilin β disintegrin domain containing a 4-benzoyl-phenylalanine, the DECD peptide **2**, is therefore a good inhibitor of sperm–egg fusion *in vitro*.

Photoaffinity labeling of the fertilin β receptor

After the inhibitory potency of **2** was determined, a photoaffinity labeling experiment was performed using

Figure 2



Structure and synthesis of peptide 1.

Peptides 1 and 3 were a 5:1 mixture of mono- and di-iodinated tyrosine. Please see text for more details.

the radioiodinated form of 1 and the scrambled peptide 3. Peptide 1 was incubated with zona-intact mouse eggs, and the eggs irradiated with near ultraviolet (UV) light to form a covalent bond between peptide and receptor. The

eggs were gently pelleted, washed to remove excess peptide, lysed with sodium dodecyl sulfate (SDS), boiled and analyzed by gel electrophoresis. In a parallel experiment, 1 and excess 4 were incubated with zona-intact eggs, irradiated, lysed and analyzed. The same experiment was performed with peptide 3.

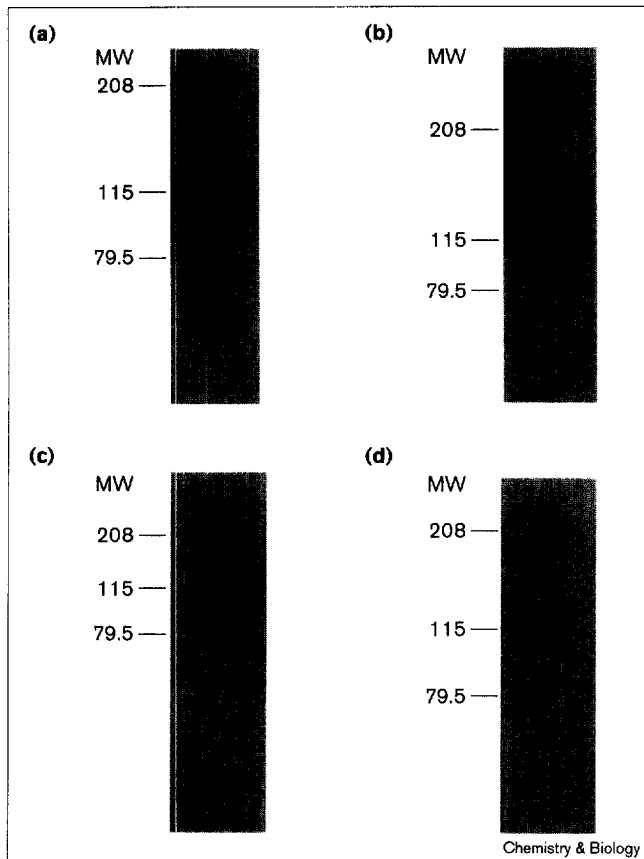
Table 1

Inhibition of *in vitro* fertilization by peptides.

Peptide	Concentration	% Inhibition FR*	% Inhibition FI†
2 [‡]	90 µM	53 ± 10	53 ± 10
2 [‡]	125 µM [§]	70 ± 14	72 ± 14
3	500 µM	9 ± 9	11 ± 9
4 [#]	500 µM	51 ± 17	51 ± 17

The results here represent the average ± s.d. for 4–7 independent experiments and 60–150 eggs per peptide. *The percentage inhibition as measured by number of eggs fertilized (fertilization rate, FR) relative to a nonpeptide control. †The percentage inhibition as measured by the number of sperm fused per egg (fertilization index, FI). ‡The fertilization index and rate for the no peptide control were 0.83 sperm/egg and 72% eggs fertilized, respectively. §This concentration is the highest obtainable due to its solubility. #The sequence of control peptide 4 is Ac-ECDAY-NH₂ [33].

One polypeptide with an apparent molecular weight (M_r) of 115,000 (in its reduced and denatured state) is labeled by 1 (Figure 3a); the nonreduced M_r of this polypeptide is 132,000 (Figure 3b). A second, much less intense band of $M_r = 95,000$ (reduced or nonreduced) is also observed. In the presence of a 125-fold excess of nonradioactive reversible inhibitor 4, no specific labeling of the protein is observed (Figure 3c). Instead, a low-level nonspecific labeling of extracellular egg proteins occurs. Thus, the labeling by 1 is competitive with reversible inhibitors, that is, the incorporation of 4-benzoyl-phenylalanine and iodotyrosine into the DECD peptide does not alter its binding specificity. Furthermore, no labeling by the scrambled peptide 3 was observed (Figure 3d). The presence of the 4-benzoyl-phenylalanine and iodotyrosine is not causing labeling

Figure 3

Autoradiograms of photoaffinity-labeled egg proteins. Approximately 800 zona-intact eggs were used per lane. Bars indicate where prestained molecular weight standards appeared on the gel. Each gel is from a single representative experiment; the labeling reactions were performed three or more times. (a) A 6% reducing SDS-PAGE gel of the labeling reaction performed with **1** (4 μ M). (b) A 6% nonreducing SDS-PAGE gel of the labeling reaction performed with **1** (4 μ M). (c) A 6% reducing SDS-PAGE gel of the labeling reaction performed with **1** (4 μ M) + **4** (500 μ M). (d) A 6% reducing SDS-PAGE gel of the labeling reaction performed with **3** (4 μ M).

artifacts. Sperm were not photoaffinity labeled by **1** (data not shown). These experiments demonstrate that DECD peptides inhibit fertilization by binding specifically to a single extracellular protein on the egg.

Identification of the fertilin β receptor

We performed immunoprecipitations with detergent soluble extracts of zona-intact mouse eggs labeled with **1**. We tested for integrins previously reported to be present in mouse eggs: α_v , β_5 , α_6 , β_1 and β_3 [27–31]. Separate aliquots of labeled egg extract were incubated with each anti-integrin antibody, and antibody–integrin complexes were precipitated with protein G sepharose. The immunoprecipitation pellets and supernatants were analyzed by gel electrophoresis (Figure 4a,b). The quantity of labeled

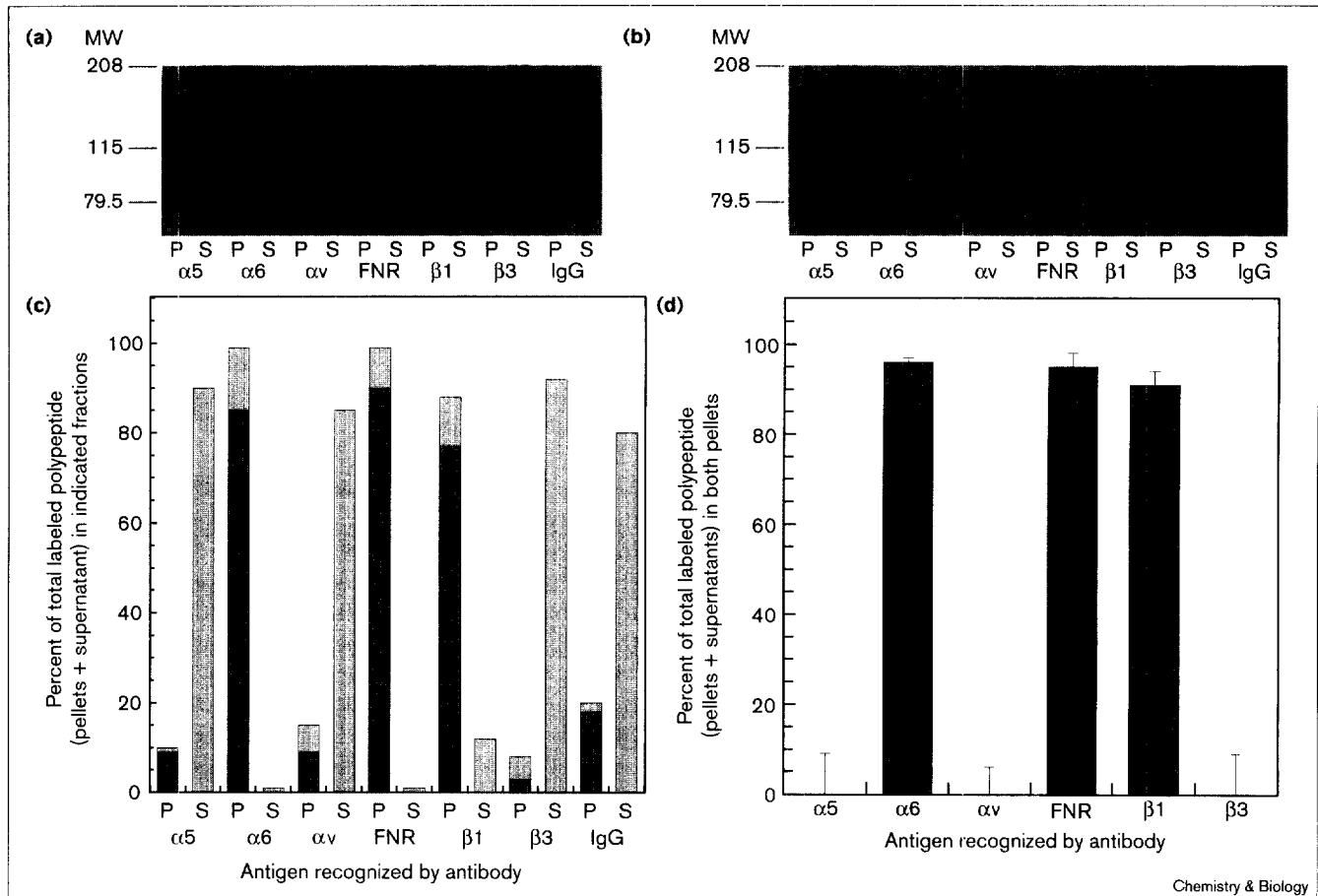
polypeptide in the pellets and supernatants of each immunoprecipitation experiment was determined by phosphorimaging (Figure 4c,d). Both anti- α_6 and anti- β_1 monoclonal, as well as polyclonal anti-FNR ($\alpha_5\beta_1$), antibodies precipitate the labeled protein. Anti- α_v , anti- α_5 , anti- β_3 monoclonal antibodies do not precipitate the labeled protein. The small amount of polypeptide seen in these lanes (~10% of the total labeled polypeptide) is less than is observed in the rabbit IgG serum control lane, and is attributed to nonspecific binding to either the antibody or the protein G sepharose. After two precipitations with either the anti- α_6 or the anti- β_1 antibodies, the polypeptide was completely cleared from the supernatant. The complete precipitation indicated that the band on the gel was not due to labeling of two different polypeptides of the same electrophoretic mobility.

The α_6 and β_1 integrins have very different changes in mobility between reducing and nonreducing gels. Murine α_6 is comprised of a heavy chain ($M_r = 115,000$ – $120,000$) and a light chain that are separated upon reduction, the nonreduced M_r is 130,000–135,000 [40,41]. In contrast, the M_r of murine β_1 increases upon reduction from $M_r = 115,000$ – $120,000$ (nonreduced) to 130,000–135,000 (reduced); presumably reduction of intramolecular disulfides decreases its electrophoretic mobility [40,41]. The decrease in M_r of the **1**-labeled polypeptide from 130,000 to 115,000 upon reduction identifies the **1**-labeled polypeptide as the heavy chain of α_6 (Figure 3a,b). The identity of the $M_r = 95,000$ band is not known, it is probably a proteolytic degradation product of α_6 because the amount present increased with sample handling despite the presence of protease inhibitors. The β_1 polypeptide is not labeled with **1**, but is clearly complexed with the labeled α_6 , because anti- β_1 monoclonal antibodies immunoprecipitate the labeled α_6 .

Both the anti- α_6 and anti- β_1 monoclonal antibodies used are specific for their respective integrin subunits [40,42]. The quantitative precipitation of the polypeptide labeled with **1** by these antibodies eliminates the possibility that other integrin subunits or other proteins not screened in this experiment are labeled (Figure 4). Furthermore, the molecular weights of integrin α chains are not identical. The presence of a single band on the autoradiogram (Figure 3a) strongly suggests that only one type of α chain is labeled, α_6 . Using the specific activity of our photoprobe (2500 Ci/mmol), the amount of radioactivity in the immunoprecipitation pellets, and an estimated labeling efficiency of 20% (average value for benzophenone [37]), we can calculate the concentration of $\alpha_6\beta_1$ on the egg surface. There is approximately 50 attomol of fertilin β receptor on the surface of each egg.

The remarkable specificity of the peptide **1** for $\alpha_6\beta_1$, and the fact that an AQDEC peptide inhibits binding of

Figure 4



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Autoradiograms and quantitation of immunoprecipitation experiments. **(a)** Autoradiograms of a representative immunoprecipitation of labeled polypeptide. Photoaffinity-labeled proteins were immunoprecipitated with anti-integrin antibodies. Each pellet (P) and half of each supernatant (S) was analyzed on a 6% reducing SDS-PAGE gel. Approximately 100 zona-intact eggs were used per lane. Bars indicate where prestained molecular weight standards appeared on the gel. **(b)** The remaining half of each supernatant was immunoprecipitated with the same anti-integrin antibody, and the pellet and supernatant loaded onto a 6% reducing SDS-PAGE gel. The immunoprecipitations were performed at least five times with each antibody. **(c)** The experiment shown in panels (a) and (b) was quantified. For each antibody, the

percentage of labeled polypeptide in the first pellet (P, dark gray bar), second pellet (P, light gray bar), and second supernatant (S, light gray bar), was plotted. Note, half of each supernatant was loaded on the gel in the first immunoprecipitation, and half was used for the second addition of antibody. The raw data were corrected for this split before calculating the percentages. **(d)** Summary of immunoprecipitation experiments. The percentage of labeled polypeptide in the pellet (P) is the sum of the first and second immunoprecipitation pellets (corrected for nonspecific precipitation as determined by the IgG control) divided by the total amount of labeled polypeptide (first pellet, second pellet and second supernatant) for a single antibody. The error bars are \pm s.d. for two to three independent labeling experiments.

recombinant fertilin β to eggs [12], imply that $\alpha_6\beta_1$ is the receptor for fertilin. Furthermore, anti- α_6 mAb GoH3 stains eggs in the microvillar region [27,43]. This location is consistent with the role of $\alpha_6\beta_1$ as a sperm fertilin β receptor that mediates sperm-egg fusion.

$\alpha_6\beta_1$ Integrin mediates sperm-egg binding

Prior studies have addressed the role of various integrins in fertilization. Sperm will bind to cultured somatic cells if they express α_6 and β_1 . In cells missing either α_6 or β_1 , binding of sperm is reduced 5–10-fold and the high level of binding is restored upon transfection of the missing integrin

[27]. This binding is inhibited by the addition of DECD peptides [27]. These experiments therefore suggested that both α_6 and β_1 mediate sperm binding, but did not provide direct evidence that sperm were binding to the $\alpha_6\beta_1$ complex. These experiments did not eliminate the possibility that additional receptors or ligand proteins are required for binding of fertilin β — that is, they did not establish that direct binding of fertilin β to the $\alpha_6\beta_1$ integrin occurs [27]. Our photoaffinity-labeling experiments demonstrate that the disintegrin DECD loop of fertilin β binds directly to $\alpha_6\beta_1$ without an intermediate ligand protein. Furthermore, sperm coreceptors are not required for binding.

Earlier experiments by others to inhibit sperm binding or fusion with antibodies suggested that a β_1 integrin interacts with fertilin β [12,27]. Experiments with anti- α_6 monoclonal antibodies have yielded conflicting results. The identity of $\alpha_6\beta_1$ as a laminin receptor was established using a function-blocking mAb GoH3 [44,45]. This same mAb has been used to characterize sperm–egg interactions. Almeida *et al.* [27] reported that anti- α_6 mAb GoH3 inhibits sperm–egg binding to zona-free mouse eggs in a concentration-dependent manner, although it does not inhibit fusion. Two other groups, however, were unable to inhibit fertilization or sperm–egg binding with GoH3, the same anti- α_6 mAb [12,30]. Evans *et al.* [12] have shown that these differences arise from the different methods (acid versus chymotrypsin treatment) used to remove the zona pellucida from the eggs. Furthermore, they demonstrated that eggs with increased amounts of GoH3 epitope (i.e., chymotrypsin-treated) bind half as many sperm and 30% less recombinant fertilin β than eggs with 2–3-fold less GoH3 epitope (i.e., acid-treated). These data presented an apparent paradox in that eggs with less GoH3 epitope (i.e., α_6) bound more sperm and recombinant fertilin β than eggs with more epitope. They also showed that binding of recombinant fertilin β was not blocked by GoH3 regardless of zona-removal method.

In our labeling experiments, we do not remove the zona pellucida — that is, the eggs are not treated with acid or chymotrypsin before labeling. All the extracellular egg proteins are unmodified and intact. Under these conditions, the GoH3 mAb immunoprecipitates $\alpha_6\beta_1$ that is covalently labeled with a 14 amino-acid DECD peptide (Figure 4). Thus, binding a small fertilin β mimic does not block the GoH3 epitope. Our experiments suggest that the laminin- and DECD-binding sites are not identical. Perhaps chymotrypsin treatment alters the conformation of $\alpha_6\beta_1$ sufficiently such that sperm binding becomes GoH3 sensitive. The conformational changes of integrins are well documented and can be assayed using monoclonal antibodies specific for exposed and unexposed epitopes [46]. Reduced levels of epitope do not necessarily correlate with reduced levels of antigenic protein. Complete resolution of these differences requires epitope mapping of the monoclonal antibodies, and mapping of the DECD- and laminin-binding site(s) and chymotrypsin cleavage site(s) in $\alpha_6\beta_1$.

Previously, $\alpha_6\beta_1$ was believed to bind exclusively to the E8 fragment of A-chain laminin [45,47,48]. Our results provide direct evidence that ligands other than laminin bind to $\alpha_6\beta_1$. $\alpha_6\beta_1$ therefore plays a role in mediating cell–cell interactions, in addition to its previously determined function in cell–matrix attachment. Eggs do not express laminin; it is expressed later during embryogenesis [29]. Laminin is, however, present on the acrosomal surface of sperm [49,50]. $\alpha_6\beta_1$ is also present during

embryogenesis [29]. The ligand specificity of integrins is modulated by a variety of agents and events [51]. These diverse roles might be modulated by variable splicing [52–54]. Splicing in the extracellular domain of $\alpha_7\beta_1$ regulates its affinity for laminin [55]. Although, splicing in the same position of the extracellular ligand-binding domain of $\alpha_6\beta_1$ does not regulate its affinity for laminin [56], it could regulate its affinity for fertilin β .

Although we have provided strong evidence that a fertilin β – $\alpha_6\beta_1$ interaction mediates sperm–egg fusion, the steps after binding remain undefined. It has been proposed that fertilin α contains an amphipathic fusion peptide, similar to viral fusion peptides, and triggers fusion after binding through fertilin β [9]. Recent experiments have shown that the fertilin β peptide, which is cationic, has a β -sheet structure and only fuses vesicles containing anionic lipids [57]. It therefore has a different fusion mechanism than the fusion peptide of HIV2 gp41, a known β -sheet-fusion peptide [58]. Fertilin α is homologous to meltrin α , another ADAM protein implicated in myoblast fusion, offering further indirect evidence that fertilin α is a fusion protein [18]. Sperm bind to somatic cells expressing $\alpha_6\beta_1$, but do not fuse [27], however, suggesting that other egg-specific proteins are required for fusion. This is the case in other types of cell fusion, for example, the recent discovery that chemokine receptors are required as HIV-1 coreceptors in viral fusion [59]. In addition, there are at least ten different ADAM family members [11,60] expressed in mouse testis, any number of which could be required for fusion. Other nonADAM proteins could also be involved. Recent experiments suggest that the sperm proteins cyritestin [3] and fertilin α [13] also bind to receptors on the egg plasma membrane. Furthermore, fertilin $\beta^{-/-}$ sperm from fertilin β knockout mice show reduced levels of fertilin α present in the sperm. Although fertilin $\beta^{-/-}$ sperm binding to the egg is reduced eightfold compared to wild-type sperm *in vitro*, sperm–egg fusion is only reduced 50% [16]. This observation suggests that multiple adhesion interactions occur before and during fusion. Unraveling this complexity is necessary for a complete understanding of the sperm–egg fusion process.

Significance

In mammals, the sperm protein fertilin β is involved in adhesion of a sperm to the egg plasma membrane. Fertilin β is a member of the widely distributed family of extracellular membrane-bound proteins termed ADAMs (a disintegrin and metalloproteinase). We have obtained very strong evidence for the identity of the fertilin β receptor using a receptor-specific photoaffinity label and immunorecognition. The photoaffinity label used was a peptide corresponding to the binding loop of the disintegrin domain of fertilin β that inhibits fertilization *in vitro*. We conclude that the photoaffinity peptide, and therefore, fertilin β , binds directly and with high specificity to $\alpha_6\beta_1$

integrin without mediating proteins. This binding event moderates fusion. Localization of the $\alpha_6\beta_1$ integrin on the microvillar surface of eggs is consistent with its participation in sperm-egg fusion. Our results indicate that ADAM disintegrins and integrins mediate cell-cell interactions. The DECD disintegrin-binding loop represents a new ligand motif for integrins. How widely this type of ligand-receptor pair is utilized remains to be determined. Potentially, ADAM-integrin interactions could modulate functions as diverse as myoblast fusion [18] and neurogenesis [19].

Further work will be required to determine whether fertilin β binding to $\alpha_6\beta_1$ is the only receptor-ligand interaction required for sperm-egg fusion. The successes in developing Arg-Gly-Asp (RGD) antagonists specific for $\alpha_{11b}\beta_3$ [61] or $\alpha_v\beta_3$ [62] suggest that $\alpha_6\beta_1$ is an important target for rational and combinatorial drug design (using the DECD motif) to develop new methods of contraception.

Materials and methods

General

Amino acids were purchased from Advanced Chemtech (Louisville, KY), except 4-benzoyl-phenylalanine (Bpa), which was purchased from Bachem (Philadelphia, PA). Peptide synthesis resin and O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) were purchased from Perseptive Biosystems (Framingham, MA). [251]-Sodium iodide was purchased from Amersham Life Science (Arlington Heights, IL). Solvents were from Fisher Scientific, Inc. (Springfield, NJ). Other reagents were purchased from Sigma-Aldrich Co. (Milwaukee, WI). MALDI-TOF mass spectrometry was carried out using a Bruker Protein TOF instrument at the CASM, SUNY at Stony Brook. The matrix used was α -cyano-4-hydroxycinnamic acid. Mass calibrations were performed using internal standards with appropriate molecular weights. The HRFAB mass spectra were acquired on a 70-SE-4F mass spectrometer in the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois. A Molecular Dynamics 445 PhosphorImager and Imagequant software were used for analysis of 125 I-protein gels.

Synthesis of peptide 1

Peptides were synthesized on a Rainin PS3 synthesizer using Fmoc α -amino protection, *t*-butyl, trityl and 2,2,5,7,8-pentamethylchroman-6-sulfonyl (PMC) sidechain protection, [5-(4-aminomethyl)-1-(3,5-dimethoxyphenoxy)-valerate]-polyethylene glycol-polystyrene (PAL-PEG) resin and HATU activation to yield the carboxy-terminal amide. Amino acids and activator were used in fourfold molar excess with dimethylformamide as solvent. All amino acids were of the L-configuration. The peptides were purified by C18 reversed-phase HPLC using a linear gradient from 5% to 95% 0.1% TFA/CH₃CN in 0.1% TFA/H₂O. The molecular mass of the peptides was confirmed by mass spectrometry.

The UV spectrum of **2** had a λ_{max} = 262 nm as expected for a peptide containing 4-benzoyl-phenylalanine. The pure peptide was analyzed by HRFAB mass spectrometry (MH⁺ calc'd: 1762.7754; found: 1762.7734). Peptide **2** was dimerized in 20% DMSO in air at pH 8, and purified again by C18 HPLC. The dimer (2.8 nmol) was radioiodinated with Na 125 I (2.5 Ci/ μ mol, 2 mCi) and *N*-chloro-*p*-toluenesulfonamide, sodium salt (chloramine-T) (1 nmol) in 50 mM sodium phosphate, pH 6.5 buffer for 1 min. The reaction was quenched with sodium thiosulfate, and then reduced with dithiothreitol (10 mM) for 2 h. The iodinated peptide (2.5 Ci/ μ mol) was purified by HPLC as described above. The identity of the radioiodinated sample was confirmed by coinjection with

authentic cold iodinated peptide that had been prepared in an analogous fashion and analyzed by mass spectrometry. HPLC and mass spectrometry analysis showed that the peptide was a 5:1 mixture of mono- and di-iodinated tyrosine. The mixture was used for photoaffinity labeling experiments. Peptide **3** was prepared in an analogous fashion.

Antibodies

Antibodies were obtained from the following sources: polyclonal rabbit FNR antiserum (versus human whole FNR), Gibco-BRL Life Technologies, Inc. (Gaithersburg, MD); rat mAb 5H10-27 (versus mouse α_6), hamster mAb H9.2B8 (versus mouse α_v), rat mAb GoH3 (versus human α_6), rat mAb KM16 (versus mouse β_1), hamster mAb 2C9.G2 (versus mouse β_3), Pharmingen Incorporated (San Diego, CA).

In vitro fertilization assay

Eggs and sperm were isolated from ICR (Taconic Farms) or CD-1 (Charles River) mice as described in Yuan *et al.* [3]. Zona pellucida were removed by treatment with acid Tyrode's solution [63] for 30 s. Zona-free eggs were loaded with Hoechst 33342 (1 μ g/ml) for 30 min [64] and washed through six 40 μ l drops of M16 medium (0.5% BSA) and recovered at 37°C, 5% CO₂ for 1 h.

Eggs (20–30/40 μ l) were incubated with peptide in M16 medium (3% BSA) for 45 min and then sperm were added (1.0–2.0 \times 10⁴/ml). After 45 min, the eggs were washed in M16 medium (3% BSA) and mounted onto microscope slides. Fusion was scored by fluorescent labeling of sperm nuclei by Hoechst 33342 present in the preloaded eggs. Four to seven experiments per peptide were performed and a total of 60–150 eggs per peptide were used.

Photoaffinity labeling

Zona-intact eggs were prepared as described [3] to remove cumulus cells. They (~800 eggs) were photoaffinity labeled with **1** (4 μ M, 2.5 Ci/ μ mol) for 15 min with a near UV lamp (14 watts, 6 cm, λ_{max} = 350 nm), 4°C in M16 medium (1% polyvinylpyrrolidone (PVP), 100 μ l). In parallel experiments, eggs (~800) were labeled with **1** (4 μ M) and **4** (500 μ M), or **3** (4 μ M, 2.5 Ci/ μ mol). The eggs were gently centrifuged at 2 000 \times g and washed with M16 medium (1% PVP) six times to remove unreacted peptide. The eggs were resuspended in lysis buffer (2% SDS, 40 μ l), boiled for 5–10 min, centrifuged to remove insoluble zona and loaded onto an SDS-PAGE gel. Lysis buffer for reducing gels contained 10% β -mercaptoethanol. The gel was dried, and exposed to X-ray film at –80°C for 5–24 h using two intensifying screens or exposed to a phosphorimaging plate for 24 h. Sperm were treated in an analogous fashion.

Immunoprecipitations

Zona-intact eggs (~1000) were photoaffinity labeled with **1** (4 μ M) and washed as described above. The eggs were resuspended in TBS (800 μ l) containing 0.5% triton X-100, 1 mM CaCl₂, 1 mM MgCl₂, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin and 1 mM phenylmethylsulfonyl fluoride, incubated at 4°C for 20 min, centrifuged to remove insoluble zona and the supernatant divided into 8 aliquots. Each aliquot had between 8000 and 10,000 dpm of 125 I-1-labeled protein. The appropriate primary antibody (5–10 μ g for monoclonals and 0.25–0.5 μ l serum for polyclonals) was added to each aliquot and incubated at 4°C for 4–18 h. Protein G sepharose (50 μ l slurry) was added and the mixture incubated at 4°C for an additional 2 h. The mixtures were centrifuged at 10,000 \times g for 10 min, and the supernatants transferred to fresh tubes. The pellets were washed two times with incubation buffer (150 μ l each) and resuspended in gel loading buffer (2% SDS, 10% β -mercaptoethanol) buffer (40 μ l). Half of each supernatant was concentrated to 20 μ l in Micron-30 microconcentrators (Amicon, Danvers, MA) and mixed with 2 \times gel loading buffer. Both the concentrated supernatant and resuspended pellet were loaded onto a 6% SDS-PAGE gel. The gel was dried, and exposed to a phosphorimaging plate for 24–100 h. The remaining half of each supernatant was treated a second time with the same anti-integrin antibody and processed as described above. The immunoprecipitations were performed at least five times with each antibody.

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