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M. S. Chen
University of Virginia

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Evidence that Distinct States of the Integrin $\alpha 6\beta 1$ Interact with Laminin and an ADAM

M.S. Chen,* E.A.C. Almeida,* A.-P.J. Huovila,* Y. Takahashi,* L.M. Shaw,‡ A.M. Mercurio,‡ and J.M. White*

*Department of Cell Biology, University of Virginia, Charlottesville, Virginia 22908; and ‡Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02115

Abstract. Integrins can exist in different functional states with low or high binding capacity for particular ligands. We previously provided evidence that the integrin $\alpha 6\beta 1$, on mouse eggs and on $\alpha 6$ -transfected cells, interacted with the disintegrin domain of the sperm surface protein ADAM 2 (fertilin β). In the present study we tested the hypothesis that different states of $\alpha 6\beta 1$ interact with fertilin and laminin, an extracellular matrix ligand for $\alpha 6\beta 1$. Using $\alpha 6$ -transfected cells we found that treatments (e.g., with phorbol myristate acetate or $MnCl_2$) that increased adhesion to laminin inhibited sperm binding. Conversely, treatments that inhibited laminin adhesion increased sperm binding. Next, we compared the ability of fluorescent beads coated with either fertilin β or with the laminin E8 fragment to bind to eggs. In Ca^{2+} -containing media, fertilin

β beads bound to eggs via an interaction mediated by the disintegrin loop of fertilin β and by the $\alpha 6$ integrin subunit. In Ca^{2+} -containing media, laminin E8 beads did not bind to eggs. Treatment of eggs with phorbol myristate acetate or with the actin disrupting agent, latrunculin A, inhibited fertilin bead binding, but did not induce laminin E8 bead binding. Treatment of eggs with Mn^{2+} dramatically increased laminin E8 bead binding, and inhibited fertilin bead binding. Our results provide the first evidence that different states of an integrin ($\alpha 6\beta 1$) can interact with an extracellular matrix ligand (laminin) or a membrane-anchored cell surface ligand (ADAM 2).

Key words: integrin • ADAM • cell adhesion • “avidity/affinity modulation” • fertilin β

INTEGRINS are transmembrane heterodimers that act as receptors for extracellular matrix components and membrane-anchored cell surface coreceptors (Hynes and Lander, 1992; Clark and Brugge, 1995). Integrins can be activated to bind extracellular ligands by intracellular signals. Such inside-out signaling is thought to involve changes in the conformation and/or aggregation state of the integrin (Faull and Ginsberg, 1995; Brown and Hogg, 1996; Bazzoni and Hemler, 1998; Hato et al., 1998). Inside-out signaling can be invoked experimentally by activating the protein kinase C pathway with phorbol esters (Strulovic et al., 1991) or by coexpressing certain integrin-associated proteins or oncogenes (Fenczik et al., 1997; Hughes et al., 1997; Hemler, 1998; Porter and Hogg, 1998). Inte-

grins can also be activated by several extracellular mechanisms including: varying divalent cations, applying certain ligands or antibodies, and controlling whether or not the α subunit is proteolytically processed (Delwel et al., 1996). Whether activated by intracellular or extracellular means, what has been observed to date is activation of an integrin from a low to a high binding state for a particular ligand(s), or differential binding of specific integrin antibodies to the low and high binding states (Diamond and Springer, 1994; van Kooyk et al., 1994; Faull and Ginsberg, 1995).

Fertilin α and β are sperm surface glycoproteins that have been implicated in binding and fusion to the egg plasma membrane (Blobel et al., 1992; Bigler et al., 1997; Cho et al., 1998). Fertilin α and β (ADAM 1 and 2) are also the prototypes of the ADAM¹ family of cell surface proteins (Wolfsberg and White, 1996; Black and White, 1998). ADAMs have been referred to also as MDCs, cellu-

The first two authors contributed equally.

Address correspondence to Dr. Judith M. White, University of Virginia, Department of Cell Biology, Health Sciences Center Box 439, Charlottesville, VA 22908. Tel.: 804-924-2593. Fax: 804-982-3912. E-mail: jw7g@virginia.edu

Present addresses: Dr. A.-P.J. Huovila, Inst. Medical Technology, Univ. Tampere, Tampere, Finland FIN-33101; Dr. E.A.C. Almeida, Dept. Stomatology, Univ. California, San Francisco, CA 94143-0512.

1. *Abbreviations used in this paper:* ADAM, a disintegrin and metalloprotease; ECM, extracellular matrix; ENV, envelope; TE, embryo culture medium.

lar disintegrins, metalloprotease-disintegrins, and disintegrin-metalloproteases. A table of the ADAMs is available at <http://www.med.virginia.edu/~jag6n/whitelab.html>. Like their closest relatives, the snake venom metalloproteases (SVMs), ADAMs contain disintegrin and metalloprotease domains. Several snake venom disintegrins have been shown to interact with integrins including α IIb β 3 and α 2 β 1 (DeLuca et al., 1995; Jia et al., 1996; Kamiguti et al., 1996). Fertilin α and β are proteolytically processed during sperm maturation such that on mature fertilization competent sperm (the prodomain and metalloprotease domain are removed) each subunit begins with its disintegrin domain (Lum and Blobel, 1997; Waters and White, 1997; Bigler et al., 1997). The disintegrin domain of fertilin β has been implicated as a key, albeit not exclusive, participant in sperm-egg binding based on the inhibitory action of peptide analogues of the disintegrin loop (Myles et al., 1994; Almeida et al., 1995; Evans et al., 1995; Gichuhi et al., 1997), antibodies against the disintegrin loop (Yuan et al., 1997), and recombinant proteins containing the disintegrin domain (Evans et al., 1997; Bigler, D., M.S. Chen, Y. Takahashi, E.A.C. Almeida, and J.M. White, manuscript in preparation). Further studies have demonstrated that the integrin α 6, presumably as the α 6 β 1 complex (Almeida et al., 1995), is involved in fertilin-mediated sperm binding. Antibodies to the α 6 subunit bind to zona-free mouse eggs and inhibit sperm binding. In addition, cells that express the α 6 and β 1 integrin subunits bind more sperm than their nonexpressing counterparts (Almeida et al., 1995; Chen, M.S., and J.M. White, unpublished data). α 6 β 1 is a well characterized laminin receptor (Mercurio and Shaw, 1991; Ekblom, 1996). Therefore, it may participate in both cell-cell interactions, via the disintegrin domain of an ADAM, as well as cell-extracellular matrix (ECM) interactions, by engaging laminin.

Like many integrins, α 6 β 1 on cultured cells appears to rest in a basal state for adhering to laminin. The ability of α 6 β 1 to bind laminin can be stimulated two- to fivefold by treating cells with phorbol esters or by adding Mn^{2+} to the extracellular medium. Activation occurs without a change in the amount of α 6 at the cell surface, suggesting that modulation is caused by changes in either the conformation (affinity) and/or aggregation (avidity) state of the integrin complex (Shaw et al., 1993; Shaw and Mercurio, 1993). These observations suggest, as with α IIb β 3 and other integrins, that adhesion of α 6 β 1 to laminin is responsive to physiological stimulation. The major goal of this study was to determine how agents that modulate the ability of α 6 β 1 to interact with laminin affect binding of sperm and, more specifically, the ADAM protein, fertilin β .

Materials and Methods

Egg Isolation

Mature oocytes were collected from 8–12-wk-old ICR female mice (Harlan Sprague Dawley Inc.) as described previously (Almeida et al., 1995). To prepare zona-free eggs, egg masses were incubated with 3 mg/ml hyaluronidase (Sigma Chemical Co.) in embryo culture medium (TE) (Spindle, 1980) for 5–10 min at 37°C to dissociate cumulus cells, and washed through three 200- μ l drops of fresh TE. Eggs were further treated with 10 μ g/ml of α -chymotrypsin (Sigma Chemical Co.) in TE for 3 min at 37°C to soften the zona pellucida. When the perivitelline space was visibly en-

larged, eggs were immediately washed through two 200- μ l drops of fresh TE. The loosened zonae were mechanically removed by gently passing eggs through a glass pipette \sim 100 μ m in diameter. Eggs were placed in TE, overlaid with light mineral oil, and incubated for 1 h at 37°C in a 5% CO₂ incubator before use. Alternatively, where indicated, egg masses were collected in M199 culture medium (Gibco BRL) supplemented with 0.4% BSA (fraction V, fatty acid free; Sigma Chemical Co.), 3.5 mM pyruvate (Gibco BRL), 100 U/ml penicillin G sodium (Gibco BRL), and 100 μ g/ml streptomycin sulfate (Gibco BRL). The egg masses were then incubated with 0.6 mg/ml hyaluronidase for 3 min, washed through three 100- μ l drops of fresh M199, treated for 1 min in acidic Tyrode's solution, pH 2.5 (Hogan et al., 1994), washed through three 100- μ l drops of fresh M199, and incubated in M199 for 3 h at 37°C in a 5% CO₂ incubator before use. We have found that a 3-h resting period after a brief incubation in acidic Tyrode's solution is necessary for the recovery of egg surface proteins, including the α 6 integrin subunit, and for recovery of optimal fertilization competence (Takahashi et al., 1995). Experiments using eggs treated either with chymotrypsin followed by mechanical shearing and a 1-h recovery, or with acidic Tyrode's solution (followed by a 3-h recovery) to remove the zona pellucida gave similar results.

Sperm Isolation and Solubilization

Sperm were isolated from the cauda epididymis and vas deferens of ICR retired male breeders (Harlan Sprague Dawley Inc.; Hilltop) as described previously (Almeida et al., 1995). For the majority of experiments sperm were capacitated for 2 h by incubation in TE/3% BSA in a CO₂ incubator at 37°C. At this time \sim 50% of the sperm had undergone the acrosome reaction. To prepare sperm lysates for coating fluorescent beads, sperm were washed twice with Dulbecco's phosphate-buffered saline (PBS; Gibco BRL) and resuspended at 5×10^6 sperm in 0.25 ml lysis buffer (CHAPS/gelsolin) at 4°C. CHAPS/gelsolin contains 1.5% CHAPS (Sigma Chemical Co.), 10 mM Tris (pH 7.4), 50 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 5 mM phenylmethylsulfonyl fluoride, 5 μ g/ml pepstatin A, 10 μ g/ml leupeptin, 20 μ g/ml aprotinin, 50 μ g/ml antipain, 2 mM benzamide, 50 μ g/ml soybean trypsin inhibitor, 2.5 mM iodoacetamide, and 50 μ g/ml gelsolin (gift of Dr. P. Janmey; Cytoskeleton). The sperm suspension was passed four times through a syringe with a 27-gauge needle and incubated for 4–18 h at 4°C. Lysates were centrifuged at 14,000 rpm for 10 min at 4°C, and the supernatant was passed through an 0.2- μ m syringe filter. For most experiments the pellet was reextracted with another 0.25 ml of CHAPS/gelsolin, passed four times through a syringe, and incubated for 4 h at 4°C. This lysate was collected, clarified by centrifugation (as above), and pooled with the original extract.

Antibodies

Antibodies were obtained from the following sources: rat anti- α 6 mAb GoH3 (Immunotech) and rat anti- α 6 mAb J1b5 (Dr. C. Damsky). A polyclonal rabbit antiserum against fertilin β was raised against a peptide analogue of its cytoplasmic domain. The polyclonal rabbit antiserum (anti-envelope [ENV]) against the cytoplasmic domain of the Avian Leukosis and Sarcoma Virus subtype C envelope glycoprotein was described previously (Gilbert et al., 1994). Polyclonal antibodies were purified on a column of the immunizing peptide coupled to a gel (SulfoLink Coupling Gel; Pierce Chemical Co.). Antibodies were eluted with 100 mM glycine, pH 2.5, immediately neutralized with 1 M Tris-HCl, and dialyzed extensively with PBS.

Peptides

14-mer peptides corresponding to the sequence of the predicted binding loop of the disintegrin domain of mouse fertilin β , as well as a scrambled fertilin β sequence, were synthesized on a peptide synthesizer (Symphony; Protein Technologies) and purified by HPLC. The following sequences were used: fertilin β , CRLAQDEADVTEYC; and scrambled fertilin β , CETADYQRVECLDC. Peptides were amidated at the COOH terminus and acetylated at the NH₂ terminus. The two terminal cysteine residues were protected with acetoamidomethyl groups. Peptides were dissolved in DMSO to a concentration of 25 mM, and diluted to 250 μ M in egg medium immediately before use.

Preparation of Protein-coated Fluorescent Beads

0.2 μ m yellow-green or crimson fluorescent sulfate-derivatized latex beads (Molecular Probes, Inc.) were coated with fertilin β or laminin E8

as follows. To prepare fertilin β -coated beads, beads from 10 μ l of a 2% bead suspension were incubated with 10 μ l of anti-fertilin β cytoplasmic tail antibody (0.42 mg/ml) for 4 h at 4°C on an orbital platform mixer (Clay Adams). Beads were washed twice with PBS, and incubated overnight with sperm lysates prepared as described above. To prepare beads coated with laminin E8 (gift of Dr. P. Yurchenco), beads were incubated with a solution of laminin E8 (1 mg/ml in PBS) for 2 to 3 h at 4°C on an orbital platform mixer. Fertilin- or laminin E8-coated beads were washed twice with PBS, quenched for 1 h with 0.2 mg/ml goat anti-rabbit IgG (Sigma Chemical Co.), washed twice with PBS, and resuspended to 0.2% in PBS. Beads were used on the day of preparation and were sonicated in a water bath sonicator (Laboratory Supplies) three times for 5 s each at 4°C immediately before use.

Bead-Egg Binding Assay

20–40 zona-free eggs, prepared as described above, were pretreated for 15 min with experimental agents in 20- μ l drops of TE. The divalent cation composition of TE is 2.4 mM Ca^{2+} , 0.47 mM Mg^{2+} . Protein-coated fluorescent beads were added to give a final concentration of 0.02%, and eggs were incubated in a 5% CO_2 incubator. Alternately, where indicated, eggs were pretreated for 15 min with experimental agents, and incubated with beads in Puck's saline A (GIBCO BRL), a buffer that does not contain any divalent cations. Where indicated, eggs were pretreated with latrunculin A (Biomol) for 1 h in M199, washed three times with Puck's saline A, and incubated with beads as described above. The eggs were gently agitated every 15 min. After 1 h at 37°C, the eggs were washed with three 100- μ l drops of fresh medium using an \sim 100 μ m glass pipette. After washes, small drops containing the eggs were placed in 24-well dishes, and overlaid with light mineral oil for imaging by confocal microscopy.

Sperm-Macrophage Binding Assay

α 6B- and mock-transfected P388D1 cells were previously generated by transfection of cells with the pRc/CMV vector containing the human α 6B cDNA or vector alone; the vector alone contains the neomycin resistance gene (Shaw et al., 1993). Cells transfected with the vector alone are referred to in this paper as "mock-transfected." Subconfluent monolayers of α 6B- or mock-transfected P388D1 macrophages were grown in RPMI 1640 (Gibco BRL) supplemented with 15% heat-inactivated fetal bovine serum (HyClone Laboratories Inc.), 2 mM glutamine (Gibco BRL), 25 mM Hepes, pH 7.4, 100 U/ml penicillin G sodium, 100 μ g/ml streptomycin sulfate, and 0.3 mg/ml Geneticin (G418 sulfate; Gibco BRL). Cells were detached from culture dishes by scraping and resuspended in RPMI to a concentration of 10^5 cells/ml. 5×10^4 cells were plated in 24- or 48-well tissue culture dishes (Falcon Plastics) for 3 h at 37°C in a 5% CO_2 incubator. The cells were washed twice with TE/3% BSA and pretreated with experimental agents for 15 min at 37°C. Capacitated sperm were added to a final concentration of 5×10^6 sperm/ml, and incubated for 1 h at 37°C with gentle shaking at 15-min intervals in a 5% CO_2 incubator in the presence of experimental agents. Cells were washed once with TE/3% BSA, once with PBS, and fixed with 1.6% paraformaldehyde in PBS. The cultures were observed with a phase-contrast microscope and the number of sperm bound per cell was counted. Five fields per well of duplicate wells were analyzed for each condition. Each field contained \sim 100 cells. Cation dependence experiments were performed in Puck's saline A instead of RPMI. Cells from the same starting suspension were used for parallel sperm-macrophage binding assays and macrophage-laminin adhesion assays.

Macrophage-Laminin Adhesion Assay

Laminin adhesion assays were performed as described previously (Shaw et al., 1993) and in parallel with sperm-macrophage binding assays. Briefly, 96-well plates (Costar Plastics) were coated for 14–18 h with 20 μ g/ml EHS laminin-1 (Sigma Chemical Co.) in Ca^{2+} -, Mg^{2+} -free PBS (Gibco BRL), and washed three times with PBS before use. Subconfluent monolayers of transfected P388D1 macrophages were washed once with RPMI, detached from culture dishes by scraping, and resuspended in RPMI to a concentration of 5 – 10×10^5 cells/ml. Cell suspensions were added to the laminin-1-coated wells, and incubated for 1 h in the presence of experimental agents as indicated. Cells were washed twice with RPMI, fixed with methanol for 15 min at 25°C, stained with 0.2% crystal violet in 2% ethanol for 15 min, washed several times with water, and solubilized with 1% SDS for 1 h. Optical density was measured at 595 nm using a microplate reader (Molecular Devices Corp.). Quadruplicate samples were

analyzed for each data point. Cation dependence experiments were performed in Puck's saline A instead of RPMI.

Sperm-Egg Binding Assay

Zona-free eggs were placed in 100- μ l drops of TE under mineral oil or, where indicated, in drops of Puck's saline A supplemented with the indicated amount of CaCl_2 . Eggs were pretreated with experimental agents for 15–30 min before sperm addition. Capacitated sperm were added to give a final concentration of $\sim 5 \times 10^5$ sperm/ml, and incubated for 1 h at 37°C in a 5% CO_2 incubator. Eggs were washed three times in fresh medium, fixed in 4% glutaraldehyde, and mounted on glass slides for observation by phase-contrast microscopy. 30–40 eggs were analyzed per condition and the average number of sperm bound per egg was determined.

Western Blotting, Biotinylation, and Immunoprecipitation of Sperm Proteins

For Western blots, sperm from the caput or cauda epididymis were isolated in M199 containing 0.1% polyvinyl alcohol (Sigma Chemical Co.) and, unless indicated, lysed in CHAPS/gelsolin as described above. The lysate was incubated with 50 μ l of a 50% slurry of concanavalin A (ConA) agarose beads (Vector Laboratories Inc.) for 1 h at 4°C. The beads were washed twice with PBS, resuspended in 15 μ l of 2 \times SDS gel sample buffer (250 mM Tris-HCl, pH 7, 12.5% sucrose, 8% SDS, 20 mM EDTA, 0.2 mg/ml bromophenol blue, and where indicated, 100 mM dithiothreitol), incubated at 95°C for 5 min, and then subjected to 10% polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose (Schleicher and Schuell Inc.) and the membrane was incubated with blocking buffer (1 M glucose, 10% glycerol, 3% BSA, 1% milk, and 0.5% Tween 20 in PBS, pH 7.4) for 30 min. The nitrocellulose was then incubated with 5 μ g/ml purified anti-fertilin β cytoplasmic tail antibody in TBST (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20 [Sigma Chemical Co.]) for 14 h at 4°C followed by incubation with goat anti-rabbit IgG coupled to horseradish peroxidase (Jackson ImmunoResearch Laboratories Inc.) in TBST for 1 h. Horseradish peroxidase was detected by enhanced chemiluminescence.

For immunoprecipitation of fertilin β from solubilized sperm, \sim 1 million capacitated sperm from the cauda epididymis were incubated with 1 mg/ml EZ-Link sulfo-NHS-LC-biotin (Pierce Chemical Co.) for 15 min at 25°C, washed twice with PBS, and lysed in CHAPS/gelsolin as described above. Supernatants were precleared for 1 h at 4°C with 10 μ l of protein A agarose beads (Boehringer Mannheim Corp.) coupled with preimmune IgG (3 μ l serum/10 μ l beads) for both the anti-fertilin β , and anti-ENV antibodies. Supernatants were divided and immunoprecipitated for 1 h at 4°C with 5 μ l of protein A agarose beads coupled with either anti-fertilin β or anti-ENV IgGs (0.2 μ g/ μ l beads). Beads were divided and washed either twice with PBS, or seven times with RIPA buffer (10 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 150 mM NaCl). Beads were resuspended in 15 μ l of 2 \times SDS gel sample buffer, incubated at 95°C for 5 min, and subjected to nonreducing 10% polyacrylamide gel electrophoresis. Samples were transferred to nitrocellulose, and biotinylated proteins were detected (Vectastain ABC Elite; Vector Laboratories Inc.) by enhanced chemiluminescence.

Staining of Zona-free Eggs with FITC-Phalloidin

Zona-free eggs were prepared using acidic Tyrode's solution as described above and allowed to recover for 3 h at 37°C. Where indicated, eggs were treated for 1 h with 20 μ g/ml latrunculin A in M199. Eggs were fixed with 1% paraformaldehyde (Electron Microscopy Sciences), and 0.1% polyvinyl alcohol (Sigma Chemical Co.), in PBS for 30 min at 25°C. Eggs were washed three times with blocking buffer (20 mM glycine and 2% BSA in PBS) and permeabilized with 2% Triton X-100 (Sigma Chemical Co.) in blocking buffer for 1 h at 25°C. Eggs were washed three times with blocking buffer, and incubated with 100 nM FITC-phalloidin (Sigma Chemical Co.) in blocking buffer for 30 min at 25°C. Eggs were washed four times with blocking buffer and mounted onto slides for analysis by confocal microscopy.

Results

We previously provided evidence that the disintegrin domain of fertilin β (ADAM 2) can interact with the integrin

$\alpha 6\beta 1$. An anti- $\alpha 6$ mAb (GoH3) as well as peptide analogues of the fertilin β disintegrin loop specifically inhibited sperm-egg binding. Sperm bound more extensively to cells that expressed $\alpha 6\beta 1$ than to their nonexpressing counterparts. This binding was specifically inhibited by GoH3 and by a fertilin β disintegrin loop peptide analogue (Almeida et al., 1995). Since $\alpha 6\beta 1$ is a well characterized laminin receptor whose apparent avidity for laminin can be regulated experimentally, we compared the effects of agents that modify the apparent avidity of $\alpha 6\beta 1$ for laminin for their effects on binding of the ADAM protein, fertilin β . We first compared the effects of integrin avidity/affinity modulators on sperm and laminin binding to $\alpha 6$ -transfected cells. We also compared their effects on the ability of eggs to bind beads coated with either fertilin or laminin E8.

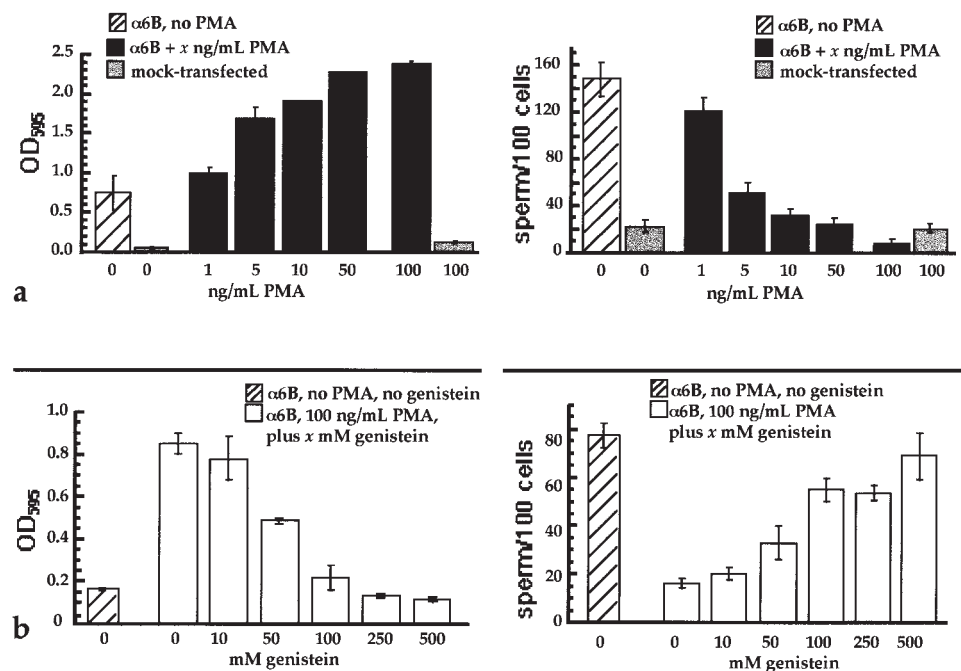
Inverse Effects of Integrin Avidity/Affinity Modulators on Binding of Sperm and Laminin

When transfected with the $\alpha 6$ subunit cDNA P388D1 mouse macrophages express $\alpha 6$ in complex with a protein the size of the $\beta 1$ integrin subunit on the cell surface. No protein the size of the $\beta 4$ subunit, the only other known $\alpha 6$ binding partner (Sonnenberg et al., 1987; Hemler et al., 1989), was seen (Shaw et al., 1993). As shown previously, (Shaw et al., 1993) and in Fig. 1 a, left, $\alpha 6$ -transfected macrophages adhered to laminin to a greater extent than their mock-transfected counterparts. This adhesion could be stimulated by treatment with PMA. As shown previously (Almeida et al., 1995) and in Fig. 1 a, right, $\alpha 6$ -transfected macrophages bound more sperm than their mock-transfected counterparts. However, in contrast to its enhancing effect on laminin adhesion, PMA inhibited sperm binding to parallel

cultures of $\alpha 6$ -transfected macrophages (Fig. 1 a, right). As observed previously (Shaw, L., and A. Mercurio, unpublished data), genistein, a tyrosine kinase inhibitor, reversed the PMA stimulation of laminin adhesion to $\alpha 6$ -transfected macrophages (Fig. 1 b, left). In contrast, genistein restored sperm binding to parallel cultures of PMA-treated $\alpha 6$ -transfected macrophages (Fig. 1 b, right).

Inclusion of Mn^{2+} in the extracellular medium has also been shown to modulate integrin function. As shown previously (Shaw et al., 1993) and in Fig. 2 a, left, Mn^{2+} stimulated the adhesion of $\alpha 6$ -transfected macrophages to laminin. In contrast, Mn^{2+} inhibited sperm binding to $\alpha 6$ -transfected macrophages (Fig. 2 a, right). The number of sperm bound at 0.01 mM Mn^{2+} was less than at 0.05 mM Mn^{2+} , in accordance with previous observations that some amount of a divalent cation is required for sperm binding to eggs (Yanagimachi, 1978; Evans et al., 1995). When $\alpha 6$ -transfected macrophages were incubated in medium containing 0.5 mM Mn^{2+} and increasing amounts of Ca^{2+} , their ability to adhere to laminin decreased (Shaw and Mercurio, 1994) (Fig. 2 b, left). Conversely, adding increasing amounts of Ca^{2+} to medium containing 0.5 mM Mn^{2+} increased sperm binding to $\alpha 6$ -transfected macrophages (Fig. 2 b, right). Adhesion of mock-transfected cells to laminin was not increased in the presence of Mn^{2+} (Shaw et al., 1993; Chen, M., and J. White, unpublished data).

Collectively, the results presented in Figs. 1 and 2 indicate that PMA and Mn^{2+} stimulate laminin adhesion to $\alpha 6$ -transfected macrophages, whereas PMA and Mn^{2+} inhibit sperm binding to these cells. Reciprocally, whereas genistein and Ca^{2+} inhibit the stimulatory effects of PMA and Mn^{2+} , respectively, on laminin adhesion, they restore sperm binding to PMA- and Mn^{2+} -treated cultures of $\alpha 6$ -



each condition. PMA (100 ng/ml) induced a (a, left) 3.2-fold and (b, left) 4.8-fold increase in laminin binding, and a (a, right) 6-fold and (b, right) 4.6-fold decrease in sperm binding, respectively. Values indicate average \pm standard error (SE).

Figure 1. Comparison of the effects of PMA and genistein on laminin and sperm binding to $\alpha 6$ -transfected P388D1 cells. (Left) Laminin adhesion and (right) sperm binding assays were performed in parallel. (a) $\alpha 6\beta$ -transfected cells were either untreated (hatched bars) or treated with the indicated amount of PMA (black bars). Mock-transfected P388D1 cells (gray bars) were either untreated (0) or treated with 100 ng/ml PMA (100). (b) $\alpha 6\beta$ -transfected cells were either maintained in normal media (no PMA, no genistein; hatched bars) or pretreated with 100 ng/ml PMA and increasing concentrations of genistein (white bars). Laminin adhesion assays were performed in quadruplicate. Sperm binding assays were conducted in duplicate wells, and ~ 500 cells were counted for

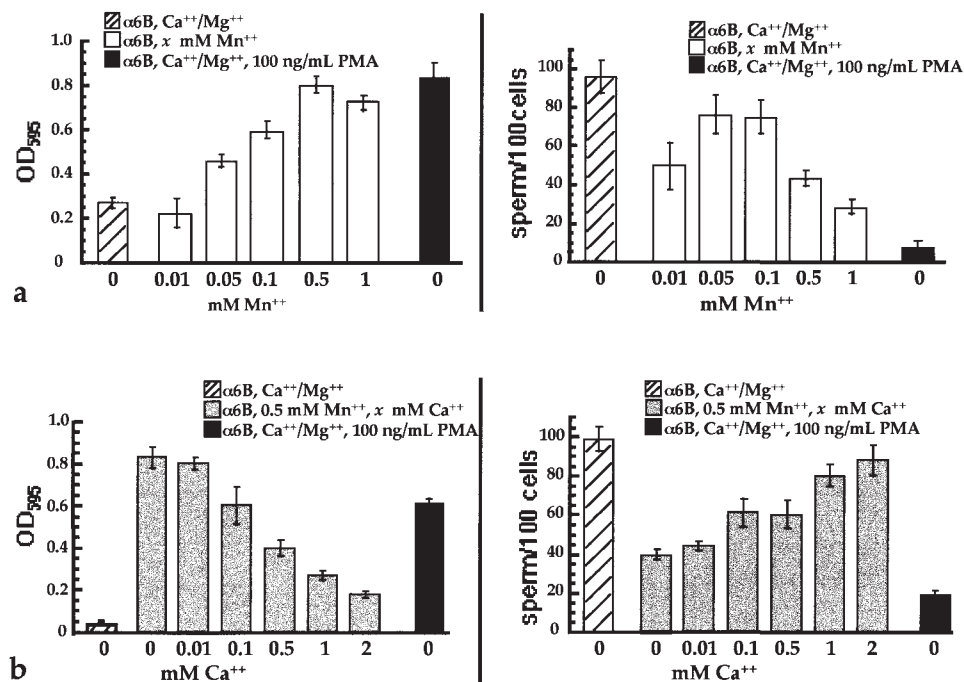


Figure 2. Comparison of the effects of Mn^{2+} and Ca^{2+} on laminin and sperm binding to $\alpha 6$ -transfected P388D1 cells. (Left) Laminin adhesion and (right) sperm binding assays were performed in parallel on $\alpha 6$ -transfected P388D1 cells. (a) (Open bars) Cells were incubated in Puck's saline A supplemented with increasing concentrations of $MnCl_2$. (b) (Gray bars) Cells were incubated in Puck's saline A containing 0.5 mM $MnCl_2$ and increasing concentrations of $CaCl_2$. Binding in Puck's saline A containing 1.8 mM $CaCl_2$ and 0.8 mM $MgCl_2$ in the absence (hatched bars; $\alpha 6B$, Ca^{2+}/Mg^{2+}) and presence (black bars; $\alpha 6B$, Ca^{2+}/Mg^{2+} , 100 ng/ml PMA) of 100 ng/ml PMA are shown for comparison. Data analyses are as described in the legend to Fig. 1.

transfected macrophages. Neither PMA nor Mn^{2+} visibly affected sperm motility (Chen, M., E. Almeida, and J. White, unpublished data).

We next tested the effects of PMA and Mn^{2+} on sperm binding to zona-free mouse eggs. As seen in Fig. 3 a, PMA inhibited sperm binding. Under the conditions of this experiment, we did not see evidence of egg activation, for example cortical granule exocytosis (Almeida, E., and J. White, unpublished data). Addition of Mn^{2+} to our basic egg medium (that contains 2.4 mM Ca^{2+}) inhibited sperm binding (Fig. 3 b). When eggs were placed in a medium free of divalent cations and supplemented with $CaCl_2$, we found that Ca^{2+} supported sperm binding. This finding corroborates previous observations that Ca^{2+} (~1.8 mM) is needed for optimal sperm binding (Fig. 3 c; for reviews see Yanagimachi, 1978; Fraser, 1994). We were unable to test the effect of genistein on sperm-egg binding because at the concentrations used for the $\alpha 6$ -expressing cells (Fig. 1 b, right), the eggs lysed.

Binding of Fertilin-coated Beads to Eggs

We next further probed the molecular basis for the apparent inverse effects of integrin avidity/affinity modulators on $\alpha 6$ -mediated binding of sperm and laminin. Since our hypothesis is that fertilin β (ADAM 2) is responsible, at least in part, for sperm binding to $\alpha 6\beta 1$ on eggs and on $\alpha 6$ -transfected cells, our goal was to compare the ability of mouse eggs to bind fertilin β and laminin. For this purpose we chose to use fluorescent beads coated with either fertilin β or laminin.

We first had to establish a method to solubilize fertilin β from mature (fertilization-competent) sperm using a non-denaturing detergent. Fertilin β (the ~57-kD form) from mature fertilization-competent sperm, harvested from the cauda epididymis, is highly resistant to solubilization with

nonionic detergents (Fig. 4 a, lane 2; Huovila, A., E. Almeida, and J. White, unpublished data), while proteolytically processed fertilin β (and its larger precursors) from immature sperm, harvested from the testis, the caput epididymis (Fig. 4 a, lane 1) or to a lesser extent, the corpus epididymis, are readily solubilized in a variety of non-ionic detergents (Huovila, A., E. Almeida, M. Chen, and J. White, unpublished data). Recent evidence suggests that there are alternate forms of fertilin β on mouse sperm (Huovila, A., I. Kärkkäinen, C. Rea, and J. White, unpublished data). The cytoplasmic tail antibody used in this paper specifically recognizes the ~57-kD form. Proteolytically processed fertilin β (~57 kD) from mature cauda epididymal sperm was not solubilized in our lysis buffer containing the zwitterionic detergent, CHAPS (Fig. 4 a, lane 2). However, inclusion of gelsolin, an actin severing protein, permitted us to extract fertilin β (~57 kD) in the CHAPS-containing lysis buffer (Fig. 4 a, lane 3). Fertilin β (~57 kD) from mature sperm, solubilized in CHAPS/gelsolin (Fig. 4 a, lane 3), comigrated on a Western blot (~57 kD) with fertilin from caput epididymal sperm solubilized in the absence of gelsolin (Fig. 4 a, lane 1). The fact that gelsolin is required to solubilize fertilin β (~57 kD) from mature, but not from immature, sperm suggests that it is somehow associated with the actin cytoskeleton of mature sperm. These observations may be related to the developmentally regulated posterior head localization of fertilin β in mature fertilization-competent sperm (Blobel et al., 1990; Phelps et al., 1990; Hunnicutt et al., 1997).

We next assessed the ability of a fertilin β cytoplasmic tail antibody to immunoprecipitate fertilin β from samples of cell-surface biotinylated sperm solubilized in CHAPS/gelsolin. The antibody precipitated a biotinylated protein of ~57 kD (Fig. 4 b, lanes 1 and 3) that comigrated with a band recognized by the same antibody on a Western blot of nonbiotinylated sperm (Fig. 4 b, lane 7). This band was

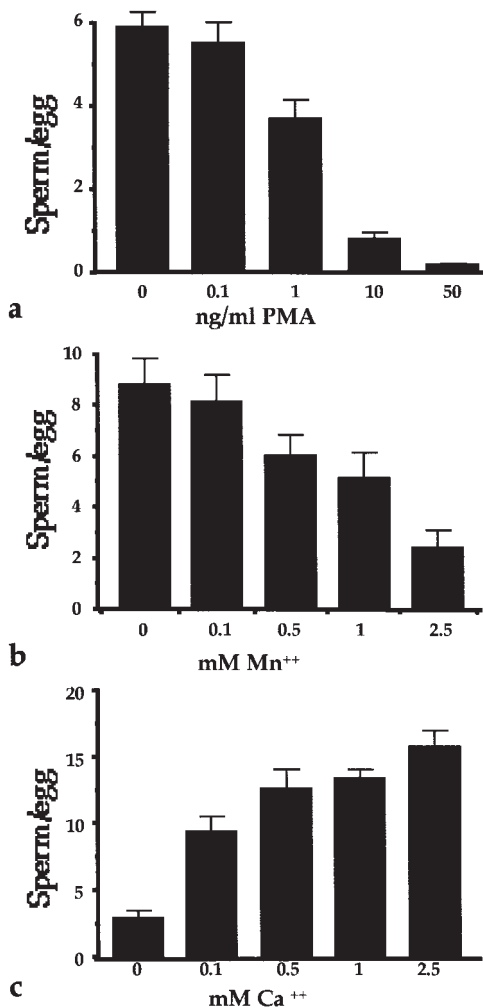


Figure 3. Effects of PMA, Mn²⁺, and Ca²⁺ on sperm binding to zona-free eggs. (a and b) Eggs were placed in drops of TE (that contains 2.4 mM CaCl₂ and 0.47 mM MgCl₂), containing increasing concentrations of (a) PMA or (b) MnCl₂ for 15 min, and incubated with capacitated sperm. (c) Eggs were pretreated in drops of Puck's saline A supplemented with increasing concentrations of CaCl₂, and incubated with capacitated sperm. After 1 h at 37°C, eggs were washed, fixed, and analyzed for sperm binding by phase-contrast microscopy. Approximately 30 eggs were used per condition. Values indicate average ± SE.

not immunoprecipitated with a control antibody against the cytoplasmic tail of the Avian Leukosis and Sarcoma Virus envelope glycoprotein, anti-ENV (Fig. 4 b, lanes 2 and 4). It was also not detected in lanes containing anti-fertilin β or anti-ENV antibodies alone (Fig. 4 b, lanes 5 and 6). When the fertilin β and control immunoprecipitates were washed with PBS (Fig. 4 b, lanes 1 and 2) as opposed to the SDS-containing buffer, RIPA (Fig. 4 b, lanes 3 and 4), an additional band was detected (~37 kD), but it was not specific to the fertilin β immunoprecipitation. These findings suggest that fertilin β is the major sperm surface protein immunoprecipitated with our anti-fertilin β cytoplasmic tail antibody.

We asked whether fluorescent beads coated with fertilin β could bind specifically to eggs. To do this, fluorescent

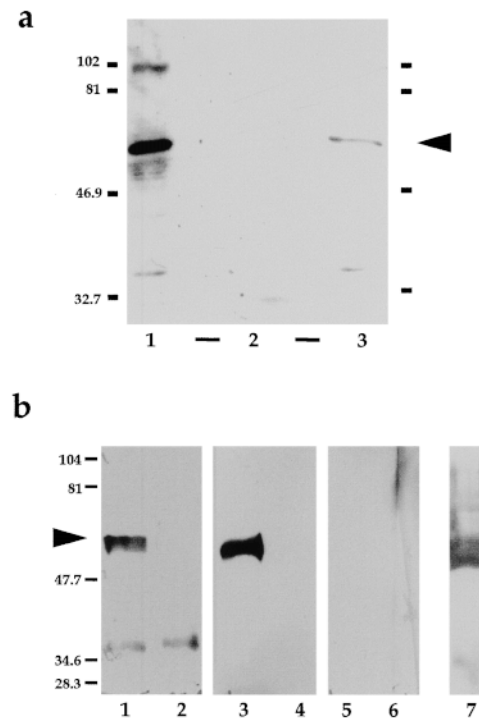
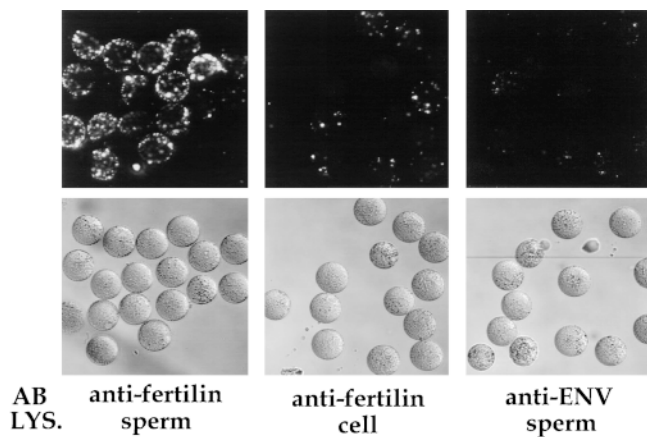


Figure 4. Solubilization and immunoprecipitation of fertilin β. (a) Sperm from the caput (lane 1) or cauda (lanes 2 and 3) epididymis were recovered and solubilized in CHAPS lysis buffer in the absence (lane 1 and 2) or presence (lane 3) of gelsolin. Solubilized proteins were precipitated with ConA as described in Materials and Methods and boiled in 2× SDS gel sample buffer. Samples were subjected to 10% SDS-PAGE under reducing conditions, transferred to nitrocellulose, and blotted with an antibody to the cytoplasmic tail of fertilin β. The arrow indicates the major fertilin β band recognized on mature sperm (mol. mass = ~57 kD). The higher molecular mass band seen in lane 1 (mol. mass = ~100 kD) represents the full-length fertilin β precursor. — indicates blank lanes. (b) Biotinylated sperm from the cauda epididymis were solubilized in CHAPS/gelsolin, as described in Materials and Methods. Cleared lysates were immunoprecipitated with protein A agarose beads precoupled with an antibody against the fertilin β cytoplasmic tail (lanes 1 and 3) or a control antibody, anti-ENV (lanes 2 and 4), for 1 h at 4°C. Samples were then divided in half and washed either twice with PBS (lanes 1 and 2) or seven times with RIPA buffer (lanes 3 and 4). 1 μg anti-fertilin β (lane 5) or anti-ENV (lane 6) IgGs were precoupled to 5 μl protein A agarose beads, and the beads were washed twice with PBS. All samples were subjected to 10% SDS-PAGE under nonreducing conditions, transferred to nitrocellulose, and blotted with Vectastain Elite ABC reagent as described in Materials and Methods. For the sample shown in lane 7, sperm from the cauda epididymis were solubilized by direct boiling in 2× SDS gel sample buffer, subjected to 10% SDS-PAGE under nonreducing conditions, transferred to nitrocellulose, and blotted with an antibody to the cytoplasmic tail of fertilin β. The arrow indicates the fertilin β band which corresponds to the ~57-kD mature fertilin lacking the prodomain and the metalloprotease domain.

latex beads were coated with affinity-purified fertilin β-cytoplasmic tail antibody and incubated with a CHAPS/gelsolin lysate from mature capacitated mouse sperm. After washing, the beads were incubated with zona-free



AB
LYS. **anti-fertilin sperm** **anti-fertilin cell** **anti-ENV sperm**

Figure 5. Binding of fertilin-coated beads to zona-free eggs. Yellow-green fluorescent latex beads were coated with an antibody (AB) against the fertilin β -cytoplasmic tail (anti-fertilin) or with a control antibody (anti-ENV). Beads were then incubated in either a sperm (sperm) or a macrophage (cell) lysate (LYS.). After the beads were quenched (with anti-rabbit IgG), washed, and sonicated, they were incubated with eggs for 1 h in TE as described in Materials and Methods. The eggs were washed and analyzed by confocal microscopy. Paired fluorescence and phase-contrast images from a representative experiment are shown.

mouse eggs. As seen in Fig. 5 (left), fertilin β -coated beads bound to eggs. If the fluorescent beads coated with the fertilin β -cytoplasmic tail antibody were incubated in a somatic cell lysate instead of a sperm lysate, only a low level of binding was observed (Fig. 5, middle). If the fluorescent beads were coated with an irrelevant polyclonal anti-cytoplasmic tail antibody, anti-ENV, and then in a sperm lysate, only a low level of binding was observed (Fig. 5, right). These data indicate that fertilin β -coated beads bind to zona-free mouse eggs and that this binding depends on fertilin β .

We next explored the molecular basis for the fertilin β -bead binding to eggs. As seen in Fig. 6, a fertilin β peptide analogue inhibited binding of fertilin β -coated beads to eggs (Fig. 6, β) whereas a scrambled fertilin β disintegrin loop peptide analogue did not (Fig. 6, β_{scr}). We next tested the effects of GoH3, a function-blocking anti- $\alpha 6$

mAb (Sonnenberg et al., 1988) and J1B5, a non-function-blocking anti- $\alpha 6$ mAb (Damsky et al., 1992). As seen in Fig. 6, GoH3 decreased fertilin bead-binding to eggs (compare GoH3 with Ct). In contrast, J1B5 did not inhibit binding of fertilin β -coated beads. In fact, J1B5 appeared to enhance the binding of fertilin-coated beads (Fig. 6, J1B5). The enhancing effect of J1B5 was seen in replicate experiments. We have recently observed similar inhibitory and stimulatory effects of GoH3 and J1B5, respectively, on the binding of fluorescent beads coated with a recombinant fertilin β disintegrin domain expressed in insect cells (Bigler, D., M.S. Chen, Y. Takahashi, E.A.C. Almeida, and J.M. White, manuscript in preparation). Collectively, the results presented in Fig. 6 indicate that binding of fertilin β -coated beads to eggs is mediated, at least in part, by the disintegrin loop of fertilin β and by the integrin $\alpha 6$ subunit.

Inverse Effects of Integrin Avidity/Affinity Modulators on Binding of Fertilin- and Laminin E8-coated Beads to Mouse Eggs

We next assessed the effects of integrin avidity/affinity modulators on the binding of fertilin- (Figs. 7 and 10) and laminin E8- (see Figs. 8–10) coated fluorescent beads to mouse eggs. Because beads coated with whole laminin formed large aggregates in suspension, we used the elastase digestion fragment, E8, which contains the major integrin $\alpha 6 \beta 1$ binding domain (Goodman, 1992; Yurchenco and O'Rear, 1994). We first analyzed fertilin- (Fig. 7) and laminin E8- (see Figs. 8 and 9) bead binding individually. We then analyzed fertilin and laminin E8 bead binding in a competition experiment (see Fig. 10). As seen in Fig. 7 a, PMA (middle) and Mn^{2+} (right) inhibited binding of fertilin β -coated beads to mouse eggs (fertilin-bead binding in the presence of Mg^{2+} alone [1 mM] was similar to that seen in the Ca^{2+}/Mg^{2+} control media; Almeida, E., M. Chen, and J. White, unpublished data). As seen in Fig. 7 b, the effect of PMA on fertilin bead binding was dose-dependent.

We next assessed the effects of PMA and Mn^{2+} on the ability of fluorescent beads coated with the laminin E8 fragment to bind to mouse eggs. Although mouse eggs express significant levels of $\alpha 6$ on their surface, presumably

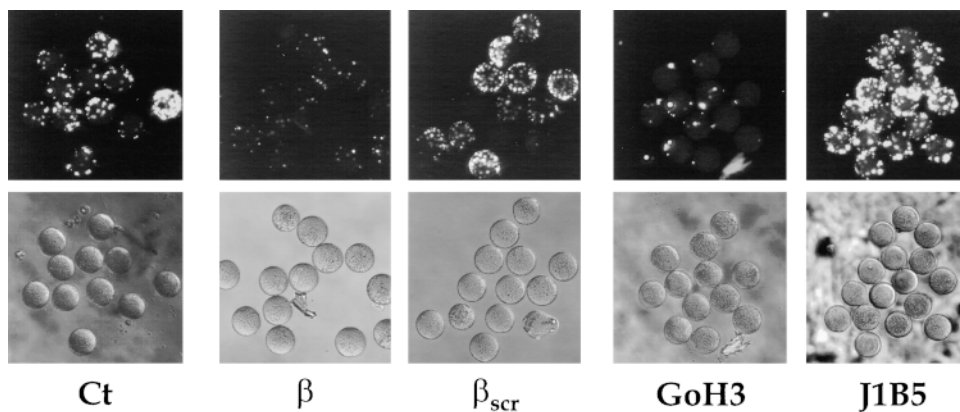


Figure 6. Effects of fertilin β peptide analogues and anti- $\alpha 6$ antibodies on binding of fertilin-coated beads to zona-free eggs. Eggs in TE were either untreated (Ct) or preincubated with 250 μM freshly dissolved peptide analogue (14 residues) corresponding to either the predicted binding domain of fertilin β (β) or a scrambled fertilin β peptide (β_{scr}). In other samples, eggs were preincubated with 100 $\mu g/ml$ of either a function-blocking (GoH3) or a non-function-

blocking (J1B5) anti- $\alpha 6$ mAb. Eggs were assayed for binding of fertilin-coated beads as described in the legend to Fig. 5. Paired fluorescence and phase-contrast images from a representative experiment are shown.

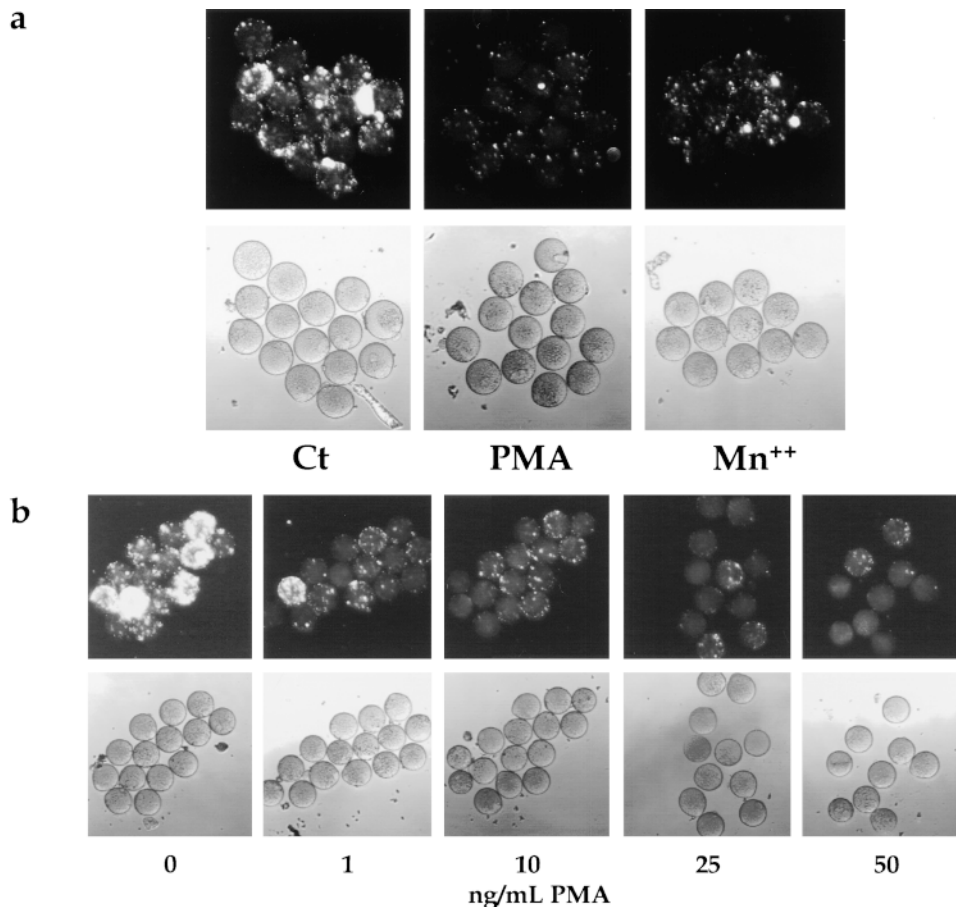


Figure 7. Effects of PMA and Mn^{2+} on binding of fertilin-coated beads to zona-free eggs. Eggs were collected in M199 medium and treated with acidic Tyrode's solution to remove the zonae, and allowed to recover for 3 h at 37°C in M199 as described in Materials and Methods. (a) Eggs were placed in Puck's saline A containing either 1.8 mM $CaCl_2$ and 0.8 mM $MgCl_2$ (Ct), 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$ and 50 ng/ml PMA (PMA), or 1 mM $MnCl_2$ (Mn^{2+}). For the PMA samples, eggs were pretreated with PMA for 10 min. Eggs were assayed for binding of fertilin-coated beads as described in the legend to Fig. 5. (b) Eggs were placed in Puck's saline A containing 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, and the indicated concentration of PMA. Eggs were pretreated with PMA for 10 min and assayed for the binding of fertilin-coated beads as described above. Paired fluorescence and phase-contrast images from representative experiments are shown.

in complex with $\beta 1$ (Tarone et al., 1993; Almeida et al., 1995; Evans et al., 1997; Chen, M., and J. White, unpublished data), the eggs as we isolated and prepared them (in a Ca^{2+} -containing medium) showed virtually no affinity for the laminin E8 beads (Fig. 8 a, left). Treatment with PMA did not induce binding of laminin E8-coated beads (Fig. 8 a, middle). However, Mn^{2+} dramatically increased binding of laminin E8-coated beads (Fig. 8 a, right). The stimulation of laminin E8-bead binding first occurred at ~ 50 – $100 \mu M Mn^{2+}$ and reached a maximum at $200 \mu M Mn^{2+}$ (Fig. 8 b). Binding of laminin E8-coated beads to eggs in the presence of Mn^{2+} was inhibited by GoH3 (Fig. 9, middle) but not by J1B5 (Fig. 9, right). Fluorescent beads coated with the laminin elastase digestion fragment E3, which binds to dystroglycan (Eklom, 1996), did not bind to eggs under any conditions tested (Almeida, E., M. Chen, and J. White, unpublished data).

It has been shown recently that in order for $\alpha 6$ to be switched into its high binding state (for laminin) by PMA, but not by Mn^{2+} , it must be cleaved into its two disulfide-bonded subunits (Delwel et al., 1996). Given the phenotype of laminin E8 binding to eggs (Fig. 8 a), we investigated whether the $\alpha 6$ subunit on the egg is proteolytically processed. We detected significant levels of proteolytically processed $\alpha 6$ on the egg surface. The apparent molecular masses of the two subunits on reducing SDS gels were ~ 120 and ~ 30 kD (Chen, M., and J. White, unpublished

data), in good agreement with the sizes reported for the $\alpha 6$ subunit from somatic cells (Delwel et al., 1996). Therefore, a lack of proteolytic processing does not appear to be responsible for the absence of PMA responsiveness observed in the laminin E8-egg binding assay. The fact that PMA induces adhesion of $\alpha 6$ -expressing somatic cells to laminin but does not induce laminin E8 binding to eggs may be due to either fundamental differences between the $\alpha 6\beta 1$ integrin in eggs versus somatic cells (see Discussion) or simply due to differences between a laminin adhesion assay versus a laminin bead-binding assay.

We next directly compared the ability of mouse eggs to bind fertilin- and laminin E8-coated beads in Ca^{2+} - and Mn^{2+} -containing egg medium. To do this, a mixture of approximately equal numbers of yellow-green beads coated with fertilin β and crimson beads coated with laminin E8 were incubated with eggs in TE medium, which contains 2.4 mM Ca^{2+} , with or without additional Mn^{2+} . Eggs in Ca^{2+} -containing medium bound large numbers of fertilin-coated beads (yellow-green) but virtually no laminin E8-coated beads (crimson) (Fig. 10, left). In striking contrast, eggs in Mn^{2+} -supplemented egg medium bound large numbers of laminin E8-coated beads (crimson), and a significantly reduced number of fertilin-coated beads (yellow-green) (Fig. 10, right).

Integrin function and clustering have been shown to be dependent upon cytoskeletal linkages (Kucik et al., 1996;

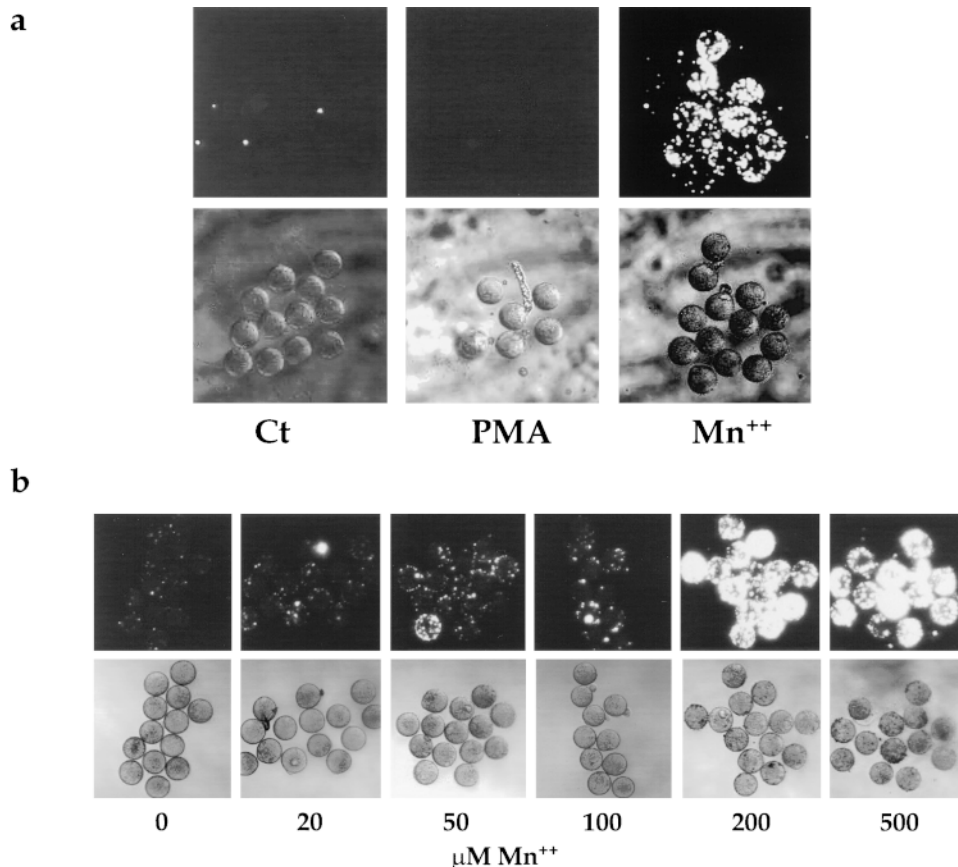


Figure 8. Effects of PMA and Mn^{2+} on binding of laminin E8-coated beads to eggs. Beads were coated with laminin E8 as described in Materials and Methods. Eggs were collected and freed of their zonae pellucidae as described in the legend to Fig. 7 and Materials and Methods. (a) Eggs were pretreated with either 100 ng/ml PMA or 1 mM $MnCl_2$, incubated with laminin E8-coated beads for 1 h at 37°C, and washed and observed with a confocal microscope. (b) Eggs were incubated with laminin E8-coated beads for 1 h at 37°C in Puck's saline A containing increasing amounts of $MnCl_2$ as indicated. Eggs were washed and observed with a confocal microscope. Paired fluorescence and phase-contrast images from representative experiments are shown.

Lub et al., 1997; Yauch et al., 1997). Therefore, we examined the effects of latrunculin, an agent that inhibits actin polymerization and sequesters actin monomers (Spector et al., 1989; Lamaze et al., 1997), on the binding of fertilin β - and laminin E8-coated beads to eggs. Fertilization-competent mouse eggs normally display dense cortical actin, as visualized by phalloidin staining, particularly concentrated over the meiotic spindle (Reima and Lehtonen, 1985; Longo, 1987). Treatment of eggs with latrunculin significantly decreased the amount of polymerized actin stained with phalloidin (Fig. 11, top). Parallel treatment with latrunculin decreased fertilin β bead binding to eggs (Fig. 11, middle), but did not increase laminin E8 bead binding (Fig. 11, bottom).

Discussion

In previous work we presented evidence that the integrin $\alpha 6 \beta 1$, on both eggs and $\alpha 6$ -transfected somatic cells, can interact with the ADAM protein, fertilin β (Almeida et al., 1995). Here we present additional evidence for this interaction. Beads coated with fertilin β captured from a sperm lysate bind to eggs in a manner that is specifically inhibited by both a fertilin β disintegrin loop peptide analogue as well as the anti- $\alpha 6$ mAb, GoH3. Analogous studies using beads coated with fertilin β disintegrin domain-containing constructs made in insect cells (Bigler, D., M.S. Chen, Y. Takahashi, E.A.C. Almeida, and J.M. White, manuscript in preparation) as well as with an ELISA (Huovila, A.,

and J. White, unpublished data) corroborate this conclusion. In a recent study using a fertilin β construct produced in bacteria, Evans et al. (1997) concluded that fertilin β interacts with a $\beta 1$ integrin on the egg but not with $\alpha 6 \beta 1$. The difference in results may stem from the use of recombinant proteins produced in bacterial (Evans et al., 1997) versus eukaryotic (Bigler, D., et al., manuscript in preparation) systems coupled with other technical differences.

Since it appears that $\alpha 6 \beta 1$ can interact with the ADAM protein fertilin β as well as with its well-characterized ECM ligand laminin, we tested the hypothesis that different states of $\alpha 6 \beta 1$ interact with fertilin β and laminin. To do this we compared the effects of agents that modulate laminin adhesion to $\alpha 6 \beta 1$ on the ability of cells transfected with $\alpha 6$ to adhere to laminin or to bind sperm. We tested the ability of eggs to bind beads coated with either fertilin or laminin. Finally, we compared the effect of the actin cytoskeletal-disrupting agent, latrunculin, on fertilin and laminin bead-binding to eggs.

With $\alpha 6$ -transfected cells we found that agents that increase laminin binding (e.g., PMA, Mn^{2+}) inhibit sperm binding. Conversely, agents that inhibit laminin binding enhance sperm binding. In our second set of experiments we found that eggs in Ca^{2+} -containing medium bind beads coated with fertilin, but do not bind beads coated with the laminin E8 fragment. In Mn^{2+} -containing medium, however, eggs show a decided preference for laminin E8-coated beads. PMA treatment of eggs inhibited binding of fertilin-coated beads, but did not induce binding of laminin

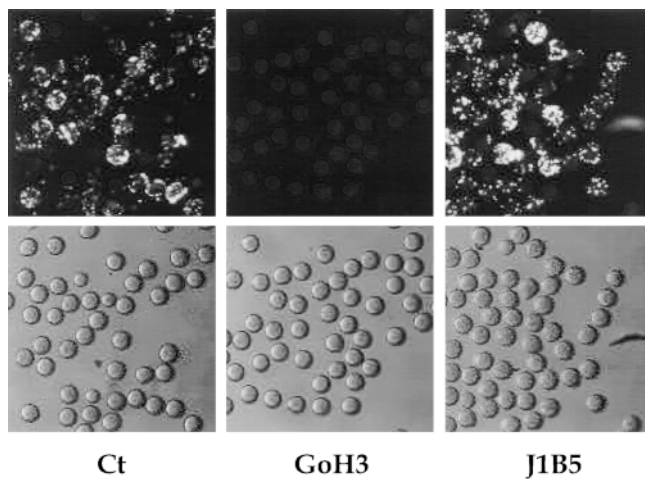


Figure 9. Effect of anti- $\alpha 6$ antibodies on binding of laminin E8-coated beads to eggs. Eggs were pretreated with 100 $\mu\text{g/ml}$ GoH3 or J1B5 in TE in the presence of 1 mM MnCl_2 , incubated for 1 h at 37°C with laminin E8-coated beads, washed, and observed by confocal microscopy. Paired fluorescence and phase-contrast images from a representative experiment are shown.

E8-coated beads. Disruption of the egg actin cytoskeleton with latrunculin also decreased binding of fertilin-coated beads, but did not induce binding of laminin E8-coated beads.

Three State Model for the Egg Integrin $\alpha 6\beta 1$

Our data support the hypothesis that different states of the integrin $\alpha 6\beta 1$ support binding of the ADAM protein fertilin β and the ECM protein laminin. Although previous studies have shown that integrins can be activated from low to high avidity/affinity states for interacting with particular ligands or antibodies (Faull and Ginsberg, 1995; Bazzoni and Hemler, 1998), and although in the case of $\alpha 2\beta 1$ it has been shown that activation of the integrin increases its binding avidity for ECM ligands (collagen and laminin) but not for a non-ECM ligand, echovirus (Bergelson et al., 1993), our findings are the first to suggest that different states of an integrin prefer a cell surface or an ECM ligand. The classical resting state of $\alpha 6\beta 1$ supports binding of fertilin but not laminin. In this respect binding of the disintegrin domain of fertilin β to the resting state of $\alpha 6\beta 1$ resembles the ability of snake disintegrins to bind to the off state of the platelet integrin (Gould et al., 1990; Williams, 1992; Niewiarowski et al., 1994). Conversely, the activated state of $\alpha 6\beta 1$ on the egg strongly supports laminin binding but discourages fertilin binding.

As outlined above, our data suggest that the egg $\alpha 6\beta 1$ integrin can exist in a state, exemplified by native fertilization-competent eggs, that supports binding of the ADAM protein fertilin β but not laminin (Fig. 12, left). It can also exist in a state exemplified by treating eggs with Mn^{2+} that strongly prefers binding laminin and disfavors the ADAM (Fig. 12, right). Our data further suggest that $\alpha 6\beta 1$ on the egg can exist in a third state, exemplified by treating eggs with phorbol esters or latrunculin, with limited affinity for either ligand (Fig. 12, middle). It may be that in the unfer-

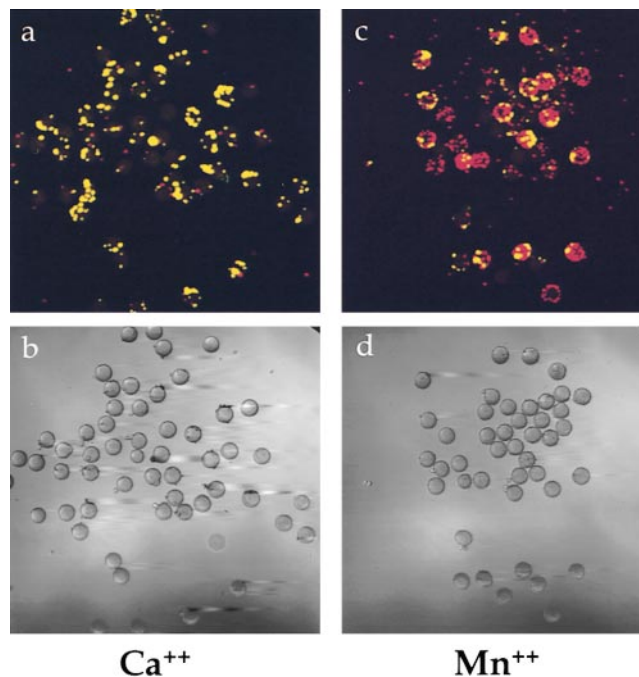
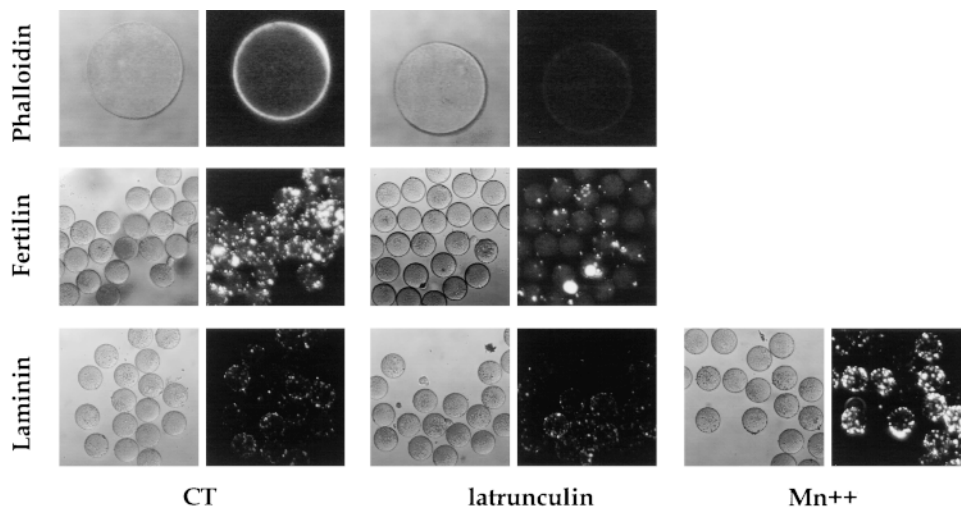


Figure 10. Comparison of the effects of Mn^{2+} and Ca^{2+} on binding of fertilin- and laminin E8-coated beads to zona-free eggs. Eggs were either (a and b) maintained in normal TE medium or (c and d) pretreated for 15 min in TE containing 1 mM MnCl_2 . Eggs were incubated with a 1:1 mixture of crimson beads precoated with the laminin E8 digestion fragment and yellow-green beads coated with fertilin β as described in Materials and Methods. After 1 h at 37°C, the eggs were washed and observed by confocal microscopy. Paired fluorescence (a and c) and phase-contrast (b and d) images from a representative experiment are shown.

tilized egg $\alpha 6\beta 1$ is tethered to the cortical actin cytoskeleton and binds the ADAM protein fertilin β (Fig. 12, left). Release from cytoskeletal anchorage (induced in vitro by treatment with either latrunculin or PMA; Fig. 12, middle) may promote lateral diffusion of the integrin (Kucik et al., 1996; Yauch et al., 1997). Increase in the lateral mobility of $\alpha 6\beta 1$ may in turn disfavor binding between $\alpha 6\beta 1$ and fertilin and may be a prerequisite for interaction between $\alpha 6\beta 1$ and laminin. High avidity adhesion of laminin to $\alpha 6\beta 1$ may require subsequent clustering of $\alpha 6\beta 1$ and/or changes in its conformation (mimicked in vitro by treatment with Mn^{2+} ; Fig. 12, right). It should be noted that in addition to binding different states of the $\alpha 6\beta 1$ integrin, fertilin β and laminin may bind to different sites on the integrin. Approximately 5–10-fold more GoH3 is required to inhibit binding of fertilin β than to inhibit binding of laminin.

Possible Mechanisms for Regulating Different States of the Egg Integrin $\alpha 6\beta 1$

We are currently considering two possibilities for how the integrin $\alpha 6\beta 1$ is kept in an on state for fertilin, and in an off state for laminin in unfertilized eggs. The first is that the $\alpha 6$ or $\beta 1$ subunits in the egg may differ from those expressed in somatic cells. Although we have found both the $\alpha 6A$ and $\alpha 6B$ alternatively spliced cytoplasmic tail iso-



containing 1 mM CaCl_2 (CT and latrunculin) or 0.5 mM MnCl_2 (Mn^{2+}) and incubated with fertilin β - (middle) or laminin E8- (bottom) coated fluorescent beads. Activation of laminin E8 binding by Mn^{2+} is shown for comparison (bottom, far right). After 1 h at 37°C, the eggs were washed and observed by confocal microscopy. Paired fluorescence and phase-contrast images from a representative experiment are shown. Latrunculin did not inhibit binding of laminin E8 beads to Mn^{2+} -treated eggs (Chen, M., and J. White, unpublished data).

forms in the egg, at both the mRNA (Almeida et al., 1995) and protein (Chen, M., Y. Takahashi, and J. White, unpublished data) levels, and although we have detected significant levels of proteolytically processed $\alpha 6$ on the egg surface, it is possible that the $\alpha 6$ or $\beta 1$ subunits on the egg represent alternatively spliced forms of their ectodomains (Delwel et al., 1995; Ziober et al., 1997) or may be differently posttranslationally modified. The second, and our currently favored, possibility is that on the egg the $\alpha 6\beta 1$ integrin is associated with a specific set of integrin-associated proteins that may include both plasma membrane and cytoplasmic proteins (Shattil et al., 1995; Berditchevski et al., 1996; Fenczik et al., 1997; Wei et al., 1997). Such integrin-associated proteins may, in turn, influence how the $\alpha 6\beta 1$ integrin is associated with the actin cytoskeleton (Berditchevski et al., 1996; Hemler, 1998).

Implications for Fertilization and Zygotic Development

Since we have shown that the integrin $\alpha 6\beta 1$ on the egg is in an off state for binding laminin and in an on state for binding fertilin, and we can experimentally induce a switch in its binding state, it is possible that $\alpha 6\beta 1$ exists in alternate binding states during development; for example in oocytes or embryos. We are particularly interested in as-

	Ca^{++}	PMA/ latrunculin	Mn^{++}	
fertilin	+	-	-	<i>Figure 12.</i> Summary of ligand binding states of $\alpha 6\beta 1$ on the egg. Eggs in Ca^{2+} strongly prefer to bind fertilin (left). Eggs in Mn^{2+} strongly prefer to bind laminin (right). PMA- or latrunculin-treated
laminin	-	-	+	

eggs bind significantly less fertilin than untreated eggs, but do not bind laminin (middle). Binding of both fertilin and laminin to eggs is inhibited by GoH3, but not by J1B5.

certaining whether (and when) postfertilization $\alpha 6\beta 1$ may switch to a state that disfavors fertilin and favors laminin. Such a switch (or switches, see Fig. 12) may be relevant to both the noted block to polyspermy that occurs at the egg plasma membrane (Horvath et al., 1993; Maluchnik and Borsuk, 1994) as well as to the noted requirement for $\alpha 6\beta 1$ to support endoderm cell outgrowth on laminin at the time of uterine implantation (Stephens et al., 1993; Sutherland et al., 1993). It is still not clear whether the interaction between fertilin β and $\alpha 6\beta 1$ is required for fertilization *in vivo*, and if so, for what stages of gametogenesis and/or fertilization. For example, in the testes $\alpha 6\beta 1$ is expressed on Sertoli cells at sites devoid of laminin but adjoining developing spermatids (Salanova et al., 1995). Interactions between fertilin β or other sperm ADAMs and $\alpha 6\beta 1$ on Sertoli cells (in an on state for the ADAM) may be involved in Sertoli cell-spermatid interactions. In addition, ADAM- $\alpha 6\beta 1$ interactions could be involved during the long journey that sperm make through the male and female reproductive tracts before reaching the egg (Cho et al., 1998).

Implications for Other ADAM-Integrin Interactions

Evidence has been presented that the ability of an integrin to organize a specific ECM ligand binding site can be developmentally regulated (Ramos and DeSimone, 1996; Martin-Bermudo et al., 1998). Hence, it seems plausible that there might be developmental situations, in addition to fertilization, when cells use differential regulation of an integrin for binding ECM ligands and cell surface coreceptors, such as ADAMs (Wolfsberg and White, 1996). For example, integrin subunits, including $\alpha 6$ and $\beta 1$, are expressed in developing dermal epithelial cells at sites of cell-cell contacts (Hertle et al., 1991) and their coreceptors at these cell contact sites are not known. If their core-

Figure 11. Effect of latrunculin on staining of egg actin with FITC-phalloidin and binding of fertilin- and laminin E8-coated beads to eggs. Eggs were collected in M199 medium and treated with acidic Tyrode's solution to remove the zona and allowed to recover for 3 h at 37°C in M199 as described in Materials and Methods. Where indicated, eggs were pretreated for 1 h at 37°C with 20 $\mu\text{g}/\text{ml}$ latrunculin A in M199. For actin staining (top) eggs were fixed and treated with FITC-phalloidin as described in Materials and Methods. For bead binding (middle and bottom) eggs were washed into Puck's saline A

ceptors are ADAMs, a switch in the affinity of the $\alpha 6$ integrin from a laminin to an ADAM binding mode may be a key step in the switch from a cell-ECM mode, for binding to the basement membrane, to a cell-cell binding mode, upon leaving the basement membrane.

Given the large number of different ADAMs and integrins and their widespread distributions, it is plausible that many more ADAM-integrin interactions exist. The disintegrin domain of ADAM 15 has been reported recently to interact with the integrin $\alpha v \beta 3$ (Zhang et al., 1998). It will be interesting to see whether binding of ADAM 15 and fibronectin are inversely regulated by avidity/affinity modulators as we have seen here for ADAM 2 and laminin interacting with $\alpha 6 \beta 1$. As more ADAM-integrin interactions are identified, it will be interesting to determine their roles in development and their mechanisms of regulation.

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