Normal Fertilization Occurs with Eggs Lacking the Integrin α6β1 and Is CD9-dependent

Brent J. Miller,* Elisabeth Georges-Labouesse,§ Paul Primakoff,‡ and Diana G. Myles*

*Department of Molecular and Cellular Biology, University of California Davis, ‡Department of Cell Biology and Human Anatomy, School of Medicine, University of California Davis, Davis, California 95616; §Institut de Génétique et de Biologie Moléculaire et Cellulaire, Centre National de la Recherche Scientifique, Institut National de la Sante et de la Recherche Medicale, ULP, 67404 Illkirch, C.U. de Strasbourg, France

Abstract. Previous results, based on inhibition of fertilization by an anti–α6 integrin mAb (GoH3), suggest that the α6β1 integrin on mouse eggs functions as the receptor for sperm (Almeida, E.A., A.P. Huovila, A.E. Sutherland, L.E. Stephens, P.G. Calarco, L.M. Shaw, A.M. Mercurio, A. Sonnenberg, P. Primakoff, D.G. Myles, and J.M. White. 1995. *Cell.* 81:1095–1104). Because the egg surface tetraspanin CD9 is essential for gamete fusion (Kaji, K., S. Oda, T. Shikano, T. Ohnuki, Y. Uematsu, J. Sakagami, N. Tada, S. Miyazaki, and A. Kudo. 2000. *Nat. Genet.* 24:279–282; Le Naour, F., E. Rubinstein, C. Jasmin, M. Prenant, and C. Boucheix. 2000. *Science.* 287:319–321; Miyado, K., G. Yamada, S. Yamada, H. Hasuwa, Y. Nakamura, F. Ryu, K. Suzuki, K. Kosai, K. Inoue, A. Ogura, M. Okabe, and E. Mekada. 2000. *Science.* 287:321–324) and CD9 is known to associate with integrins, recent models of gamete fusion have posited that egg CD9 acts in association with α6β1 in fusion (Chen, M.S., K.S. Tung, S.A. Coonrod, Y. Takahashi, D. Bigler, A. Chang, Y. Yamashita, P.W. Kincade, J.C. Herr, and J.M. White. 1999. *Proc. Natl. Acad. Sci. USA*. 96:11830–11835; Kaji, K., S. Oda, T. Shikano, T. Ohnuki, Y. Uematsu, J. Sakagami, N. Tada, S. Miyazaki, and A. Kudo. 2000. *Nat. Genet.* 24:279–282; Le Naour, F., E. Rubinstein, C. Jasmin, M. Prenant, and C. Boucheix. 2000. *Science.* 287:319–321; Miyado, K., G. Yamada, S. Yamada, H. Hasuwa, Y. Nakamura, F. Ryu, K. Suzuki, K. Kosai, K. Inoue, A. Ogura, M. Okabe, and E. Mekada. 2000. *Science.* 287:321–324). Using eggs from cultured ovaries of mice lacking the α6 integrin subunit, we found that the fertilization rate, fertilization index, and sperm binding were not impaired compared with wild-type or heterozygous controls. Furthermore, a reexamination of antibody inhibition, using an assay that better simulates in vivo fertilization conditions, revealed no inhibition of fusion by the GoH3 mAb. We also found that an anti-CD9 mAb completely blocks sperm fusion with either wild-type eggs or eggs lacking α6β1. Based on these results, we conclude that the α6β1 integrin is not essential for sperm–egg fusion, and we suggest a new model in which CD9 acts by itself, or interacts with egg protein(s) other than α6β1, to function in sperm–egg fusion.

Key words: sperm–egg fusion • tetraspanin • membrane adhesion • oocyte • ovarian cultare

Introduction

Sperm–egg binding and fusion is critical to the initiation of development in many organisms, but identification of the molecules involved in the adhesion, fusion, and signaling is incomplete. In the present study, we examined the role of molecules on the egg surface that have been implicated in the process of gamete binding and fusion.

On the surface of the mammalian egg, two proteins, the integrin α6β1 and the tetraspanin family member CD9, have been reported to act in sperm–egg binding and fusion. Integrins are transmembrane αβ heterodimers that play crucial roles in cell–cell adhesion, cell–extracellular matrix adhesion, and multiple signaling pathways (Yamada, 1997). The α6β1 integrin was first proposed as the receptor for sperm on mouse eggs by Almeida et al. (1995). Previously, α6β1 had been found to be a laminin receptor whose adhesion activity was blocked by the anti-α6 mAb GoH3 (Sonnenberg et al., 1988). Almeida et al. (1995) reported that GoH3 also inhibited sperm–egg binding and fusion. Subsequently, the role of the α6β1 integrin in sperm–egg binding and fusion was called into question. All the binding and fusion assays of Almeida et al. (1995)

Address correspondence to Dr. Diana G. Myles, Department of Molecular and Cellular Biology, University of California Davis, One Shields Avenue, Davis, CA 96516. Tel.: (530) 752-1553. Fax: (530) 752-7522. E-mail: dgmyles@ucdavis.edu

were done with eggs where the extracellular coat (the zona pellucida) was removed by protease digestion. Evidence suggests that the inhibitory effects of the GoH3 antibody on sperm–egg binding and fusion depends on zona removal techniques (Evans et al., 1997). In addition, GoH3 was found not to inhibit sperm–egg fusion in in vitro fertilization assays using zona intact eggs (Evans, 1999). The conflicting evidence from these different studies has left unclear whether or not α6β1 does have a role in sperm–egg binding and fusion.

Recently, another egg surface protein, CD9, has been discovered to play an essential role in sperm–egg fusion (Chen et al., 1999; Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000). CD9 is a member of the transmembrane 4 superfamily, also called the tetraspanin family. Transmembrane 4 superfamily proteins contain four transmembrane domains, two extracellular loops, one cytoplasmic loop, and cytoplasmic amino and carboxyl termini. Chen et al. (1999) found that anti-CD9 mAbs inhibited sperm–egg binding and fusion. The involvement of CD9 in sperm–egg fusion has been confirmed using CD9 knockout mice. The phenotype of the CD9 mutant mice is restricted to infertility in females, despite the broad tissue distribution of CD9. Eggs from the CD9 knockout mice bind sperm normally, but are severely inhibited in their ability to fuse with sperm (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000). Moreover, the tetraspanins CD9 and CD81 appear to play a role in the fusion of myoblasts to form myotubes during muscle cell differentiation (Tachibana and Hemler, 1999).

Tetraspanins are generally thought to work via lateral interactions with other cell-surface proteins such as CD4, CD8, CD19, CD21, major histocompatibility complexes I & II, integrins, and cytoplasmic signaling molecules (Horejsi and Vlcek, 1991; Wright and Tomlinson, 1994; Carmo and Wright, 1995; Hemler et al., 1996; Berditchevski et al., 1997; Maecker et al., 1997; Yauch et al., 1998). Thus, in membrane fusion, tetraspanins may be best thought of as fusion facilitators rather than receptors, and may enlist/direct other cell-surface molecules to function in the fusion process. One reported exception to this concept is CD81, a receptor for the hepatitis C virus (Pileri et al., 1998).

The physical association of tetraspanins and integrins has been established in various cell types (Hemler, 1998; Porter and Hogg, 1999). In fertilization, it has been suggested that CD9 may act through association with the α6β1 integrin in sperm–egg binding and fusion (Chen et al., 1999; Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000). The data presented here show that gamete binding and fusion can proceed normally in the absence of the α6 integrin, and are consistent with the conclusion that CD9 is essential for sperm–egg fusion, but acts either alone or through interactions with proteins other than α6β1.

Materials and Methods

Genotyping of α6 Knockout Mice

The generation of the total α6 integrin knockout mice (with a deletion of both A and B forms) has been described previously (Georges-Labouesse et al., 1996). The mice lacking the α6 integrin subunit are born alive with severe skin blistering, reminiscent of epidermolysis bullosa (Georges-Labouesse et al., 1996). This condition makes the identity of homozygous knockouts obvious. To confirm the genotypes of homozygotes and littermates, a PCR-based genotyping system was used. Two separate reactions were used to detect the mutant and wild-type alleles. The mutant allele was detected by using the forward primer 5′-GCAGCCACTGTCCCCATG-3′, which is located in the α6 intron upstream from the neomycin cassette insert, and the reverse primer 5′-TCAGAGCAGCCGATTGTC-3′, which is located in the neomycin cassette (GIBCO BRL). These two primers produce an 820-bp band in homozygous and heterozygous mice. The wild-type allele was detected using the forward primer 5′-GTGATAACTCCAGCTTGTGTCAAG-3′ and reverse primer 5′-CCTCTGCAGCGGGAGTGCTTC-3′ (GIBCO BRL). These primers are within the region deleted in the mutant allele and give a product of 500 bp in wild-type mice. The PCR parameters were as follows: 3-min denaturation at 94°C, 34 cycles of amplification at 94°C (for 1 min), 55°C (for 1 min), 72°C (for 1 min), followed by 72°C (for 10 min) using a Robocycler (Stratagene).

Egg Isolation

Because pups died shortly after birth, it was necessary to isolate ovaries within 1–2 h after birth. Ovaries were dissected from newborn wild-type, heterozygous, and homozygous C57/Bl6 pups and placed into minimum essential medium (MEM; GIBCO BRL) at 37°C. They were prepared for grafting by removing the majority of the ovarian bursa. The ovaries were implanted under the kidney capsule of recipient females (Cox et al., 1996). In brief, adult female C57/Bl6 mice were anesthetized, their ovaries removed, and the newborn ovaries were placed under the kidney capsule. The incisions were sutured and the mice revived. The grafted ovaries matured in the recipients for 21–30 d. 2 d before the isolation of the grafted ovaries, recipient mice were injected with 10 IU pregnant mares' serum gonadotropin (Calbiochem or Sigma Chemical Co.). 48 h after the injection, the ovaries were surgically removed. The removed ovaries were placed into medium 199 (M199; GIBCO BRL), supplemented with 3.5 mM sodium pyruvate, 1,000 IU penicillin-streptomycin (GIBCO BRL), and 5% FBS (GIBCO BRL). M199 containing sodium pyruvate and penicillin-streptomycin will be designated as M199*. Germinal vesicle stage oocytes were teased from the ovaries using 26 gauge needles (Becton Dickinson). Granulosa cells were removed from the oocytes by passing the oocytes through a thin bore pipette while transferring through two drops of clean medium. The oocytes were allowed to spontaneously mature for 16–18 h in M199* + 5% FBS at 37°C in 5% CO_2. Mature metaphase II eggs were selected for use in experiments.

In Vitro Sperm–Egg Binding and Fusion Assay with Wild-type and Mutant Oocytes Isolated from Ovaries

Eggs, isolated as described, were treated with 10 μg/ml chymotrypsin in M199* + 0.3% BSA (Sigma Chemical Co.) for 3 min to loosen the zonae pellucidae. The zona pellucida was removed from the eggs because overnight culture can lead to modification (hardening) of the zona and, consequently, a reduction in fertilization rates. The treated eggs were transferred through three clean drops of medium using a narrow bore pipette to mechanically remove the loosened zonae. Zona-free eggs were preloaded with 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI;[1] Polysciences Inc.) at 10 μg/ml in M199* + 0.3% BSA for 15 min at 37°C, 5% CO_2. After DAPI treatment, the eggs were washed through three drops of clean M199* + 0.3% BSA. The eggs were allowed to recover for 3 h before insemination.

Sperm for the in vitro adhesion and fusion assay were isolated from the cauda epididymis and the vas deferens of 10–12-wk-old male C57/Bl6 mice (Harlan Sprague Dawley, Inc.). Dissected cauda and vas deferens were placed into M199* + 3.0% BSA and were stripped of sperm. The sperm were allowed to dissociate for 15 min at 37°C in 5% CO_2. Sperm were capacitated for 3 h in M199* + 3.0% BSA at a 1:10 dilution of the initial sperm suspension. This procedure results in a population of 60–70% acrosome-reacted sperm (Moller et al., 1990).

Sperm and eggs were coincubated for 40 min at 37°C in 5% CO_2. The eggs were scored for binding and fusion. Binding was scored under the light microscope at a magnification of 20. Fusion was scored by the fluorescent labeling of sperm nuclei by DAPI transfer from inside the preloaded eggs. The fertilization index (FI, the mean number of fused sperm per egg), and the fertilization rate (FR, the percentage of eggs fused with at least one sperm) were calculated. To test the effect of the anti-CD9

[1]*Abbreviations used in this paper:* DAPI, 4′,6′-diamidino-2-phenylindole dihydrochloride; FI, fertilization index; FR, fertilization rate.

mAb KMC8.8 on the fertilization of mutant and wild-type eggs; zona-free eggs were preincubated for 30 min with 50 μg/ml KMC8.8 in M199* + 0.3% BSA. After the 30 min preincubation, sperm were added (final concentration 3×10^5 sperm/ml) to the drop containing the eggs and antibody. The gametes were coincubated for 40 min. Binding and fusion were assessed as described above. The control antibodies used were of the same species (rat) and isotype (IgG_{2a},κ) as KMC8.8.

Cumulus-intact Egg Assay for In Vitro Fertilization

To carry out in vitro fertilization assays with cumulus-intact eggs, 6–10-wk-old female ICR mice (Charles River) received pregnant mares' serum gonadotropin and 48 h later human chorionic gonadotropin. 14 h after hCG injection, cumulus masses containing eggs were isolated from the ampulla. The cumulus masses were washed through two 500-μl drops of M199* + 0.3% BSA, and then placed into a 50-μl drop of the same medium. Antibodies (500 μg/ml GoH3 or 50 μg/ml KMC8.8) were added to the drop with cumulus masses and preincubated for 45 min. After preincubation, sperm capacitated for 1.5 h at 37°C, 5% CO_2 in M199* + 3.0% BSA were added to the cumulus masses at a final concentration of $1–5 \times 10^6$ sperm/ml. Gametes were allowed to coincubate overnight. Two cell embryos were counted to assess the FR. To determine if sperm penetrated the zona pellucida, the presence of sperm in the perivitelline space was scored using a Zeiss Axiophot microscope.

Indirect Immunofluorescence with Zona-free Eggs

Zona-free eggs were prepared and allowed to recover as described above. After the recovery period, eggs were incubated with either 50 μg/ml KMC8.8 (PharMingen) or 100 μg/ml GoH3 (PharMingen or Immunotech) for 45 min at 37°C, 5% CO_2. The medium used for the primary antibody incubation was M199* + 0.3% BSA. The eggs were transferred through two 100-μl wash drops containing PBS + 0.1% polyvinyl alcohol (PVA; Sigma Chemical Co.), fixed for 12 min using 4% paraformaldehyde in PBS + 0.1% PVA, and transferred through two wash drops containing M199* + 0.3% BSA. An Oregon green™–conjugated goat anti–rat secondary antibody (Molecular Probes) was used to determine the localization of the primary antibody binding. Staining was visualized using a laser scanning confocal microscope (model LSM 410; Carl Zeiss). The control antibodies used were of the same species and isotype as the primary antibody being tested, unless otherwise noted.

Indirect Immunofluorescence with Cumulus-intact Eggs

Cumulus masses were collected as mentioned above except using M199* + 0.1% PVA. Cumulus masses were washed through three 500-μl drops of fresh medium. Primary antibody staining with either GoH3 (500 μg/ml), KMC8.8 (50 μg/ml), or irrelevant IgG was carried out in 50-μl drops of the same medium for 45 min, followed by washing the cumulus masses through three 500-μl drops of medium. Secondary antibody staining was done with an Oregon green™–conjugated goat anti–rat antibody in a 500-μl drop for 45 min, and cumulus masses were washed through three 500-μl drops. Cumulus-intact eggs were viewed using an LSM 410 confocal microscope.

Calcium Imaging

Zona-free eggs were mixed with 25 μg/ml Oregon green™-BAPTA AM (Molecular Probes) for 1 h at 37°C, 5% CO_2. Once loaded, the eggs were washed through three drops of M199* + 0.3% BSA. Eggs were inseminated on the microscope stage using an open perfusion microincubator (Medical Systems Corp.) to keep the fertilization drop at 37°C. Calcium oscillations were observed using an LSM 410 confocal microscope. Sperm–egg fusion was assessed by DAPI transfer. These techniques are described in detail in Faure et al. (1999).

Results

Eggs from Mice Lacking the α6 Gene Are Morphologically Normal and Do Not Have the α6 Integrin Subunit on the Egg Surface

Mice lacking the α6 gene die shortly after birth. Following previous work of Cox et al. (1996), we developed a method to remove ovaries from wild-type (α6 +/+), heterozygous (α6 +/−), or homozygous (α6 −/−) females at birth, culture these ovaries for 3–4 wk, and recover fertilizable eggs (see Materials and Methods). Eggs from wild-type (α6 +/+) or heterozygous (α6 +/−) ovaries were equivalent to each other in fertilization assays, were used interchangeably, and are referred to as control eggs or $\alpha6^+$ eggs. Eggs from homozygous, mutant (α6−/−) ovaries are termed $\alpha6^-$ eggs. Previous expression studies of the α6 integrin subunit in the developing embryo indicated a potential role in ovarian development (Fröjdman et al., 1995; Zuccotti et al., 1998). After maturation of eggs from α6 (−/−) females, we observed normal egg morphology. Cultured $\alpha6^+$ and $\alpha6^-$ eggs are morphologically similar (Fig. 1, A and B). The number of eggs/female collected from either cultured control (wild-type or heterozygous) ovaries or cultured α6 knockout ovaries were 43 ± 13 and 66 ± 18, respectively (mean ± SEM, $n = 4$). The percentages of cultured control eggs or cultured $\alpha6^-$ eggs that produced first polar bodies after the overnight culture were 75 ± 5% and 76 ± 4%, respectively (mean ± SEM, $n = 4$). These data indicate that the integrin α6β1 does not have a required role in ovary development or oogenesis because apparently normal eggs, capable of fertilization (see below), can be isolated from α6 (−/−) pups via our culture method.

As expected, eggs isolated from α6 (−/−) ovaries do not bind the anti-α6 mAb GoH3 (Fig. 2 B). $\alpha6^+$ eggs, used as controls, showed the typical pattern of GoH3 binding to the microvillus region and no binding over the metaphase plate (Fig. 2 A).

Eggs Lacking the α6 Integrin Subunit Are Fully Functional in Sperm–Egg Binding and Fusion

We directly tested the requirement for α6β1 in sperm–egg plasma membrane binding and fusion by testing the ability of mature eggs lacking the α6 subunit to bind to and fuse with sperm. Immature eggs were collected from transplanted ovaries of wild-type and α6 (−/−) mice, the granulosa cells were removed to induce oocyte maturation, and oocytes were cultured overnight to obtain metaphase II eggs for in vitro fertilization assays. After culturing, the zona pellucida was removed by chymotrypsin, and the eggs were used for in vitro fusion assays. The following three parameters in these assays were measured: (1) FR, the percent of eggs fused with at least one sperm; (2) FI, the total number of fused sperm/total number of eggs; and (3) the mean number of sperm bound to the equator of the egg. There was no reduction in the number of sperm bound or fused with eggs lacking α6 compared with wild-type eggs (Fig. 3). In seven experiments, using a total of 284 eggs, the mean FR for the cultured control eggs was 56% ± 8% (FR ± SEM). The fertilization rate for the $\alpha6^-$ eggs, in parallel experiments, was 59 ± 9%. The FI of $\alpha6^-$ and $\alpha6^+$ eggs were equivalent (Fig. 3 B). In seven experiments, using a total of 230 eggs, the mean FI for both the cultured control eggs and the cultured $\alpha6^-$ eggs was 0.67 ± 0.09 and 0.70 ± 0.1 (FI ± SEM). The mean number of sperm bound to the egg equator was 5.3 ± 2.8 sperm/egg with cultured control eggs and 7.8 ± 2.1 sperm/egg with $\alpha6^-$ eggs. The increased level of sperm binding to $\alpha6^-$ eggs in this assay is not significantly different than control eggs.

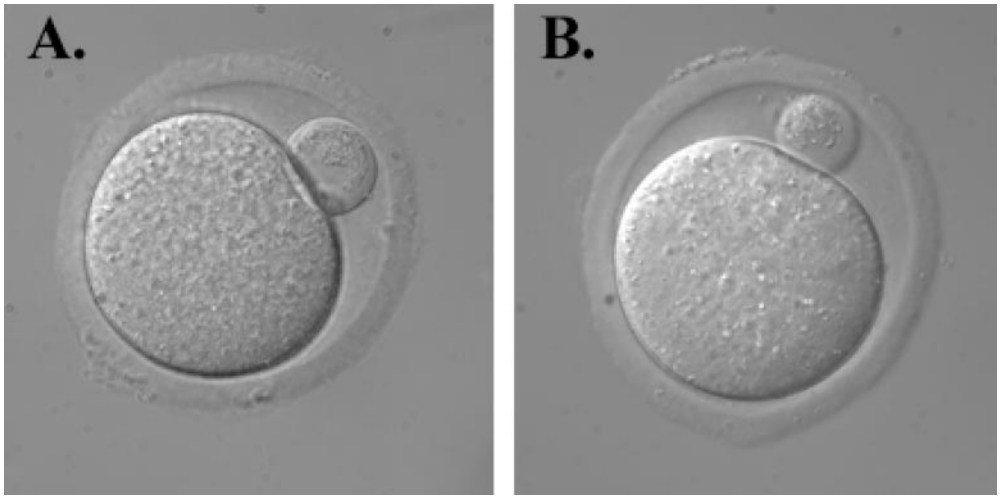

Figure 1. Differential interference contrast micrographs of (A) cultured wild-type egg and (B) α6 knockout egg.

In addition to the in vitro assays carried out using standard procedures, in some experiments we directly observed the interactions of sperm and egg binding and fusion using a microscope equipped with Hoffman modulation optics. The characteristics of sperm binding were similar in assays using $\alpha6^-$ eggs and control eggs. Sperm were bound via both their tip and lateral head regions, presumably corresponding to the inner acrosomal and equatorial regions of the sperm membrane (data not shown).

Sperm Can Initiate Calcium Oscillations in Eggs Lacking the α6 Integrin Subunit

Even though α6β1 was not required for sperm–egg binding or fusion, we considered the possibility that α6β1 was a required component in sperm–egg signaling. A hallmark of early signaling in the egg is the series of transient $[Ca^{2+}]_i$ oscillations that occur directly after sperm binding/fusion. It has been suggested that integrins may participate in initiation and/or propagation of the calcium signal during egg activation (Fenichel and Durand-Clement, 1998). Therefore, we asked if sperm could initiate normal Ca^{2+} oscillations in eggs lacking the α6 integrin subunit. $\alpha6^+$ and $\alpha6^-$ eggs were loaded with a calcium-sensitive dye, Oregon green™ BAPTA-AM, and fertilized under conditions where Ca^{2+} oscillations could be observed. We observed no difference in the calcium oscillations of $\alpha6^-$ eggs as compared with $\alpha6^+$ eggs. Calcium oscillations in the $\alpha6^-$ eggs showed typical time of onset, amplitude, and frequency (Fig. 4; Faure et al., 1999).

The Anti-α6 mAb GoH3 Has No Effect on Sperm Fusion with Cumulus-intact Eggs In Vitro

Our results with the $\alpha6^-$ eggs led us to reexamine the interpretations of previous GoH3 inhibition studies (Almeida et al., 1995; Evans et al., 1997; Evans, 1999). In vivo, the egg is ovulated and fertilized while it is surrounded by its coat (the zona pellucida) and outside the zona, a shell of ~3,000 cumulus cells. The following three types of in vitro fertilization assays have been developed using: (1) zona-free eggs, obtained by removing the cumulus cells by treatment with crude preparations of hyaluronidase and removing the zona pellucida by brief acid treatment or protease digestion; (2) zona-intact eggs, obtained by removing only the cumulus cells by treatment with hyaluronidase; and (3) cumulus-intact eggs, which receive no

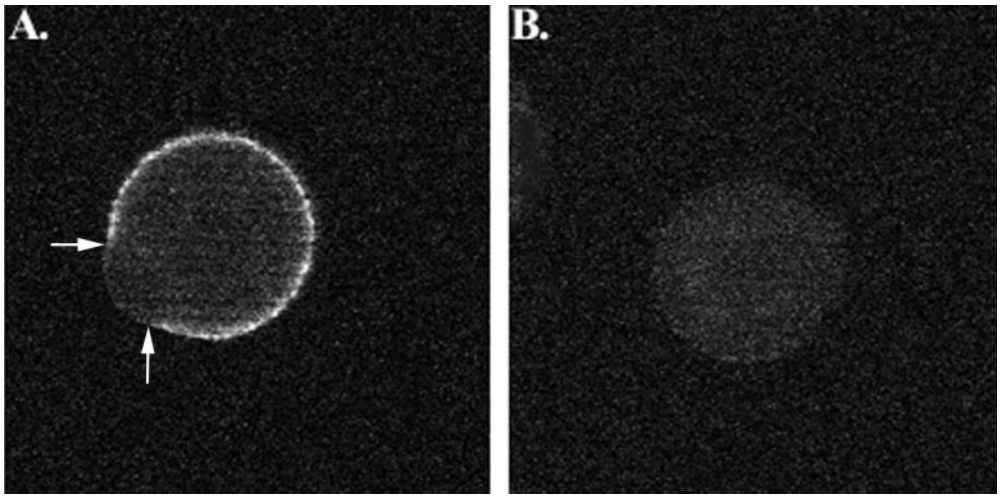

Figure 2. Indirect immunofluorescent staining of zona-free eggs using the anti–α6 integrin mAb GoH3. (A) Cultured $\alpha6^+$ egg; arrows delineate the microvillus region from the area overlying the metaphase plate. (B) A cultured $\alpha6^-$ egg. Eggs incubated with the secondary antibody alone showed no staining.

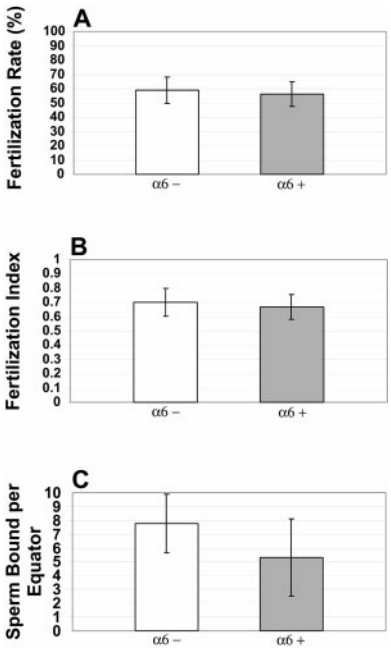

Figure 3. A comparison between $\alpha6^+$ and $\alpha6^-$ eggs of the FR, FI, and number of sperm bound (at the egg equator). The white bars in each panel show results with cultured $\alpha6^-$ eggs, and the gray bars show results with cultured $\alpha6^+$ eggs. The error bars represent the SEM. (A) The fertilization rate, $n = 7{,}284$ eggs. (B) The fertilization index, $n = 7{,}230$ eggs. (C) The number of sperm bound per equator, $n = 4{,}230$ eggs.

treatments. Use of zona-free eggs for sperm–egg fusion assays is common because it removes any possible influence of the zona pellucida and cumulus cells. However, the assay using cumulus-intact eggs most closely resembles in

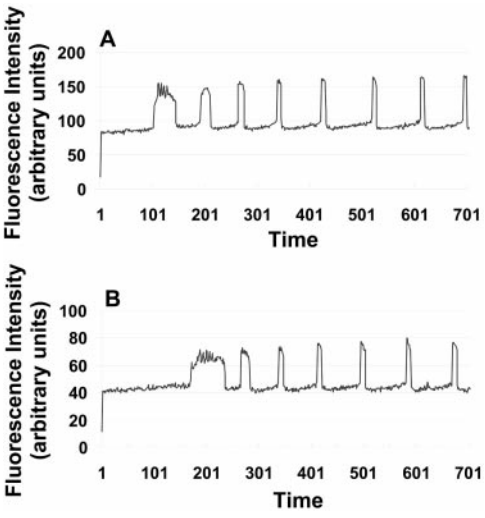

Figure 4. Representative intracellular calcium oscillation patterns. (A) A fertilized $\alpha6^+$ egg and (B) a fertilized $\alpha6^-$ egg. Calcium oscillations were observed in 23/35 $\alpha6^-$ eggs in two independent experiments, and all eggs that showed oscillations had fused sperm. Sperm fusion was determined by DAPI transfer from the egg to the sperm nucleus. The fertilization rates for the two experiments using $\alpha6^-$ eggs were 65 and 66%. The time scale is in seconds/5 and runs from 5 to 3005 s. Insemination is at time zero.

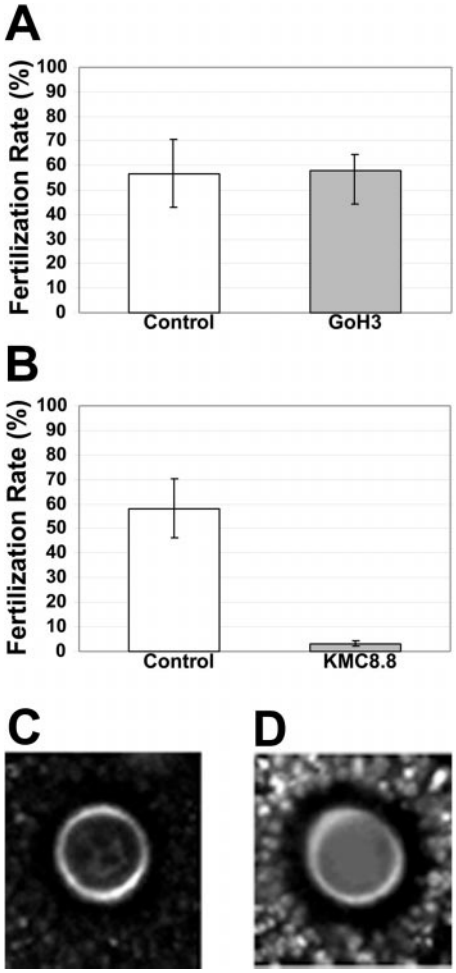

Figure 5. The effects of the anti-α6 mAb GoH3, anti-CD9 mAb KMC8.8, and an irrelevant rat IgG_{2a},κ on sperm fusion with cumulus-intact eggs. (A) The fertilization rate comparison between the control (medium added in lieu of mAb) and GoH3 (500 μg/ml) -treated eggs. (B) The fertilization rate comparison between the control (rat IgG at 50 μg/ml) and KMC8.8 (50 μg/ml) -treated eggs. (C) Immunofluorescent staining of α6 on an unfixed wild-type cumulus-intact egg using the GoH3 mAb (500 μg/ml); 12/14 eggs had the same staining pattern. No primary antibody controls were negative. (D) Immunofluorescent staining of CD9 on an unfixed wild-type cumulus-intact egg using the KMC8.8 mAb (50 μg/ml); 16/16 eggs had the same staining pattern. Cumulus cell membranes are also stained with this antibody. The zona appears dark. The rat IgG control was negative.

vivo fertilization. Because a question had been raised previously by Evans et al. (1997) about the in vitro assay using eggs, where the zona pellucida was removed with chymotrypsin (Almeida et al., 1995), we decided to test GoH3 with cumulus-intact eggs. Wild-type cumulus-intact eggs were preincubated with either GoH3 or control antibodies for 45 min and were inseminated with capacitated sperm. Sperm and eggs were allowed to coincubate overnight, and cultures were checked for two cell embryos 24 h later. The FR of the cumulus-intact eggs incubated with 500 μg/ml of GoH3 or of an irrelevant IgG were equivalent: 57 ± 14% and 58 ± 6%, respectively (FR ± SEM, $n = 4$, Fig. 5 A).

To determine if this assay could accurately measure an-

tibody inhibition, we used the anti-CD9 antibody KMC8.8 as a control. Using cumulus-intact wild-type eggs in the same assay, we measured a 95% inhibition of fertilization in the presence of 50 μg/ml KMC8.8 antibody. The average fertilization rate for control eggs was 58% ± 12%, but for KMC8.8-treated eggs, the fertilization rate was reduced to 3% ± 1% (Fig. 5 B, $n = 4$). Neither the GoH3 nor KMC8.8 antibodies inhibited sperm passage through the cumulus or zona as sperm were observed in the perivitelline space of antibody-treated unfertilized eggs. Diffusion of GoH3 or KMC8.8 to the egg plasma membrane was apparently not impeded in cumulus-intact eggs as shown by indirect immunofluorescence staining of the egg plasma membrane by both antibodies (Fig. 5, C and D).

α6β1 Is Not Required for the Function of CD9 during Sperm–Egg Binding and Fusion

Previous studies have reported that CD9 has a role in sperm–egg fusion (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000) or binding and fusion (Chen et al., 1999). These same studies have suggested that CD9 may function in cooperation with α6β1. To directly test the model that during sperm–egg binding and fusion CD9 functions via associations with α6β1, we tested the effects of the anti-CD9 antibody, KMC8.8, on cultured $\alpha6^+$ and $\alpha6^-$ eggs. Sperm fusion with both $\alpha6^+$ and $\alpha6^-$ eggs was completely inhibited in the presence of 50 μg/ml KMC8.8 (Fig. 6). No apparent decrease in sperm binding was observed in the presence of either the control IgG or the KMC8.8 antibodies (data not shown).

Discussion

Isolation of morphologically normal eggs from ovaries of α6 (−/−) knockout pups allowed us to measure if eggs lacking the α6β1 integrin bind and fuse with sperm. The fertilization rate and index both demonstrate that the α6β1 integrin is not essential for sperm–egg binding and fusion. Furthermore, the inhibition of sperm fusion with $\alpha6^-$ eggs in the presence of an anti-CD9 antibody shows that CD9 acts alone or in association with proteins other than α6β1 to function in gamete binding and fusion.

Earlier evidence suggested the α6β1 integrin, on the egg surface, played a crucial role in sperm binding leading to fusion (Almeida et al., 1995). The primary finding in support of this hypothesis was that in in vitro fertilization assays using zona-free eggs, the anti–α6 integrin mAb GoH3 at 50–400 μg/ml inhibited sperm–egg binding. Furthermore, sperm–egg fusion could be inhibited, presumably by blocking all sperm binding and by incubating eggs with the GoH3 antibody at a concentration of 400 μg/ml.

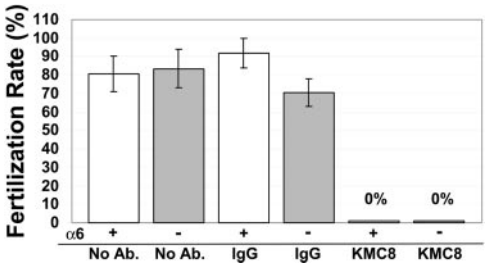

Figure 6. Inhibition of fusion of both $\alpha6^+$ and $\alpha6^-$ eggs by the anti-CD9 mAb (KMC8) at 50 μg/ml. Controls were carried out for both types of eggs with either no antibody or a rat control IgG (50 μg/ml), $n = 3$ for each condition, 181 eggs total.

In light of our current data, we asked why do the α6 knockout results not agree with previous findings implicating α6, specifically GoH3 inhibition (Almeida et al., 1995). Experiments that initially suggested a role for α6β1 during fertilization used a protease (chymotrypsin) treatment to prepare the zona-free eggs for the in vitro fertilization assays (Almeida et al., 1995). The protease treatment may modify some egg plasma membrane proteins, resulting in a loss of function of some protein(s) and/or a modification of critical protein interactions. In either way, chymotrypsin could make the egg susceptible to inhibition by the GoH3 antibody. Evans et al. (1997) have raised the question of whether the technique used to remove the zona may dictate GoH3's effect on binding. The published data are as follows: (1) GoH3 inhibits binding when the protease method is used to remove the zona (Almeida et al., 1995; Evans et al., 1997); (2) GoH3 does not inhibit binding when acid treatment is used to remove the zona (Evans et al., 1997); and (3) GoH3 does not inhibit fertilization of zona-intact eggs (Evans, 1999).

Because fertilization could be inhibited in assays using chymotrypsin-treated eggs, but not acid-treated or zona-intact eggs, we chose to test the ability of the GoH3 mAb to inhibit fusion using cumulus-intact eggs. The assay with cumulus-intact eggs is the closest experimental system to in vivo fertilization as it avoids the use of both crude preparations of hyaluronidase to remove the cumulus cells and chymotrypsin or acid to remove the zona. Data from the assay with cumulus-intact eggs demonstrate that GoH3 has no effect on fertilization under conditions where neither the cumulus cell layer nor the zona pellucida are disrupted. Experiments demonstrating the inhibitory effect of the anti-CD9 antibody in the assay with cumulus-intact eggs show that the assay can detect antibody inhibition, and that the zona does not act as a barrier to antibody diffusion to the plasma membrane. Furthermore, immunofluorescent detection of GoH3 and CD9 on the plasma membrane of cumulus-intact eggs demonstrates that these antibodies can reach the plasma membrane.

Normal sperm fusion with eggs lacking the α6 integrin subunit could mean that another integrin or receptor can substitute for the α6β1 integrin in knockout eggs. However, our results with cumulus-intact eggs and previous results with zona-intact eggs (Evans, 1999) call into question the physiological significance of the inhibitory effects of GoH3 on protease-treated, zona-free eggs (Almeida et al., 1995). In light of the reassessment of inhibitory data with the GoH3 antibody, and the finding that eggs lacking the α6 integrin subunit are not impaired in sperm–egg binding or fusion, we suggest that α6β1 is not required for sperm–egg binding and fusion.

What other egg surface protein might act as a receptor for sperm? One possibility is a different β1 integrin. An anti-β1 integrin polyclonal antibody moderately inhibited sperm–egg binding (Evans et al., 1997) and other α integrin subunits, α2, α3, α5, and αV, known to pair with β1, have been reported to be present on the egg surface

(Tarone et al., 1993; Almeida et al., 1995; Evans et al., 1995). The possibility also exists that a novel integrin or receptor may be present on the surface of the egg and bind sperm.

A new and exciting development is the discovery of a role for the egg surface protein CD9. Anti-CD9 antibodies were reported to inhibit binding and fusion (Chen et al., 1999). With eggs from CD9 −/− females, sperm fusion, but not sperm–egg binding, appeared to be the stage at which the lack of CD9 blocked fertilization (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000). Our anti-CD9 antibody data correlate well with the knockout data as we observed an inhibition of fusion, but no decrease in sperm binding, in the presence of the KMC8.8 antibody with either wild-type or α6 knockout eggs.

A Major Question to Be Answered Is How Does CD9 Function in the Fusion Process

In other systems, CD9 does not appear to function as a receptor itself, but has associations with several other cell-surface molecules (Horejsi and Vlcek, 1991; Wright and Tomlinson, 1994; Carmo and Wright, 1995; Hemler, et al., 1996; Berditchevski et al., 1997; Maecker et al., 1997; Scherberich et al., 1998; Yauch et al., 1998). Because CD9 associates with the α6β1 integrin in other systems (Hemler, 1998) and in mouse eggs (Miyado et al., 2000), the interaction of CD9 and α6β1 has been suggested in models for sperm–egg binding leading to fusion (Chen et al., 1999; Kaji et al., 2000; Le Naour et al., 2000) or sperm–egg fusion (Miyado et al., 2000). In the first model, direct or indirect interaction of CD9 with α6β1 would affect α6β1's ability to bind its sperm surface ligand (Chen et al., 1999; Kaji et al., 2000; Le Naour et al., 2000). In the second model, α6β1 transduces signals to CD9 to initiate/promote fusion (Miyado et al., 2000). Relevant to these hypotheses, our data suggest a different model in which CD9 acts by itself or interacts with egg protein(s) other than α6β1 to function in sperm–egg binding or fusion.

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