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Original Article

Distinct mechanisms underlie sperm-induced and protease-induced oolemma block to sperm penetration

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ABSTRACT Fertilization of a mouse egg results in modification of the cytoplasmatic membrane (oolemma) which makes fusion with additional sperm impossible. CD9 is a transmembrane protein reported to be responsible for gamete fusion. Since the molecular mechanism of zygote membrane modification after fertilization remains unknown, we were interested to check whether lack of CD9 is the reason for non-penetrability of zona-free zygotes. We wanted also to determine the effect of different methods of zona pellucida removal on the presence of CD9 on the surface of unfertilized eggs and their ability to be fertilized afterwards. We demonstrated that CD9 is present on the surface of both zygotes and parthenogenotes. We showed also that the treatment of eggs with pronase completely removes CD9 from the membrane of eggs making them infertile. Eggs treated with chymotrypsin and acid Tyrode still posses CD9 on their surface and remain fertile. The results of our experiments indicate that modification of the zygote oolemma does not involve a lack of CD9. We cannot exclude however, that the amount of CD9 decreases after fertilization. In addition, our studies indicate that the previously reported infertility of eggs treated with different proteases may result from the decrease or removal of CD9 and probably other proteins responsible for gamete fusion from the surface of eggs.

KEY WORDS: *CD9, block to polyspermy, fertilization, proteases, mouse*

Introduction

The mammalian egg develops several mechanisms to prevent polyspermy during fertilization. The most important reaction is the modification of the zona pellucida due to the release of cortical granules (CG) material (Wassarman 1990; Yanagimachi 1994). This polyspermy block develops during the few minutes after egg activation and prevents penetration of the zona pellucida by more than one spermatozoon. Cortical granule exocytosis probably induces also another mechanism of the polyspermy block that acts in the perivitelline space and develops within minutes after fusion of the first spermatozoon with the egg plasma membrane (oolemma) (Sato 1979). Perhaps this second block which depends on CG derived material, is responsible for preventing fusion of a subsequent spermatozoon, which managed to enter the perivitelline space before the full zona block had developed. Such multiple zona penetration is a relatively rare event in the mouse, but occurs on a regular basis during fertilization in the rabbit (Yanagimachi 1994).

Another mechanism of the polyspermy block acts at the level of the oolemma (Wolf 1978). It has been demonstrated that fertilized, zona-free mouse eggs can be effectively reinseminated within 1 hour, but after that time they cannot be penetrated by further spermatozoa (Maluchnik and Borsuk 1994). Contrary to this, artificially activated eggs remain penetrable by sperm many hours after activation, and even the 8-cell parthenogenetic embryos can be penetrated (Maleszewski and Bielak 1993). It is known that artificial activation induces cortical granules release (Gulyas and Yuan 1985) so the oolemma block to sperm penetration seems to be independent of the cortical reaction. Eggs fertilized by ICSI (intracytoplasmatic sperm injection) can fuse with sperm even at the 2-cell stage (Maleszewski *et al.,* 1996). Both artificial activation of eggs and activation induced with ICSI seem to produce different effects to those induced by normal fertilization processes during which sperm-egg membrane fusion takes place. These experiments suggest that fusion of gamete membranes is crucial for developing the oolemma block to sperm entry.

Egg membrane loses fusibility not only after fertilization but also following treatment with proteases (which are often used to remove the zona pellucida). It has been demonstrated that exposure of mechanically denuded eggs to proteolytic enzymes (chymotrypsin,

Abbreviations used in this paper: CG, cortical granule.

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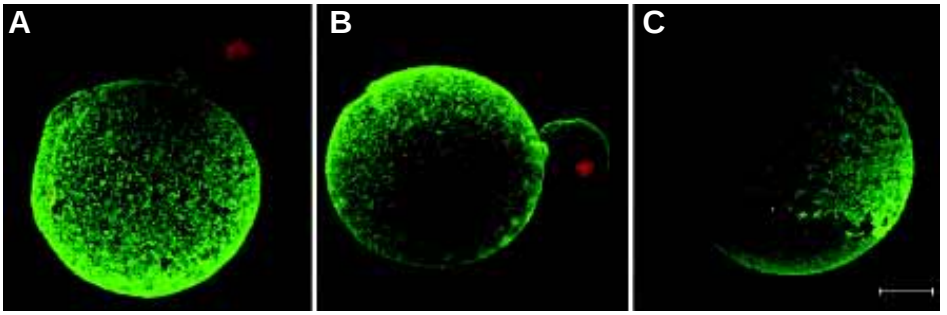

Fig. 1. Confocal laser scanning micrographs of CD9 labeled zygotes, parthenogenotes and unfertilized eggs. *CD9 labeling is visualized in green. Chromatin is stained red (7-aminoactinomycin D dye). Pictures are projections along the Z-axis, made with the LSM 5 Image Browser (version: 3,1,0,99) from stacks. The zona pellucida was removed after a short incubation in acid Tyrode's solution. Cells were incubated 3 h at 37°C and 5% CO_2 for recovery prior to fixation.* **(A)** *CD9 on zygote.* **(B)** *CD9 on parthenogenote.* **(C)** *CD9 on unfertilized egg. Scale bar, 20 μm.*

pronase) reduces sperm penetration markedly (Wolf *et al.,* 1976). Later studies have confirmed that trypsin, chymotrypsin (Boldt *et al.,* 1988; Porter and Calarco 1989) and pronase (Porter and Calarco 1989) treatment decreases both sperm binding and fusion with zona-free mouse eggs.

Recent findings have demonstrated that a crucial role in gamete fusion is played by the integral oolemma protein CD9 which is associated with integrin $\alpha_6\beta_1$ and other membrane proteins (Chen *et al.,* 1999; Miyado *et al.,* 2000). It has been shown that antibodies to CD9 inhibit sperm-egg fusion (Miyado *et al.,* 2000) or binding and fusion (Chen *et al.,* 1999). Moreover, female mice lacking CD9 ($CD9^{-/-}$) are infertile due to the failure of sperm-egg fusion (Le Naour *et al.,* 2000; Miyado *et al.,* 2000; Kaji *et al.,* 2000). Though the molecular mechanism of the sperm-egg interaction is still unclear, the CD9 protein seems to have an essential function in fusion of gametes. However, it is not excluded, that CD9 acts through interactions with integrins, but their role remains controversial (Miller *et al.,* 2000).

The purpose of this study was to investigate whether the loss of oolemma fusibility in zygotes is linked to the loss of CD9 from the plasma membrane. We were also interested in finding out whether various methods of removing zona pellucida affect the CD9 occurrence on the plasma membrane.

Results

CD9 on the Cell Membrane of Zygotes, 1-Cell Parthenogenetic Embryos and Ovulated Eggs

We have shown both in immunofluorescent staining and in western blot analysis that CD9 is present on the surface of zygotes (Fig. 1A; Fig. 2, lane 5). CD9 labeling is also visible on the membranes of parthenogenotes (Fig. 1B; Fig. 2, lane 9) and ovulated eggs in metaphase II (Fig. 1C; Fig. 2, lane 1). Both confocal microscopy and western-blotting did not permit us to find any quantitative differences in the amount of CD9 between these three groups. Lower amount of this protein in zygotes (in comparison to ovulated eggs and parthenogenetic embryos) which can be seen in Fig. 2 (lanes: 1, 5, 9) was not confirmed in following experiments.

The distribution of CD9 on membranes of ovulated metaphase II eggs seems to be polarized. The microvillar area of the egg is labeled more intensively in comparison to the amicrovillar area over the meiotic spindle (Fig. 1C). However it remains unclear whether the difference in labeling reflect difference in distribution of CD9 or it is only the matter of membrane amplification (more epitopes for the anti-CD9 antibody in the microvillar area of the egg).

Effect of Pronase, α-Chymotrypsin and Acid Tyrode on CD9

We have demonstrated that eggs, zygotes and parthenogenotes incubated with pronase completely lose CD9 from the plasma membrane (Fig. 3A; Fig. 2, lanes: 2,6,10). After 5 h incubation following pronase treatment, CD9 does not reappear on the surface of eggs (Fig. 5).

Eggs treated with acid Tyrode's solution (Fig. 3C) or α-chymotrypsin (Fig. 3B) still posses this protein on the surface (they label positively in indirect immunofluorescence). However, the western blotting analysis shows that the amount of CD9 on these eggs is reduced in comparison to untreated, control eggs (Fig. 2, lanes: 1,3,4). Moreover, eggs incubated with acid Tyrode's solution lose more CD9 than eggs treated with α-chymotrypsin. We have obtained similar results for zygotes (Fig. 2, lanes: 7,8) and parthenogenotes (Fig. 2, lanes 11,12). However, there is no visible difference between intact zygotes and those treated with α-chymotrypsin (Fig. 2, lanes 5,7). Treatment with higher concentration of α-chymotrypsin (1000 μg/ml) seems to decrease the amount of CD9 on the surface of ovulated eggs (in comparison to eggs treated with 10 μg/ml of α-chymotrypsin) but it still can be detected in indirect immunofluorescence (data not shown).

The Influence of Pronase, α-Chymotrypsin and Acid Tyrode on Sperm-Egg Fusion

In experiments concerning insemination *in vitro* we have shown that eggs treated with pronase had no sperm nuclei in the cytoplasm. Among 64 eggs we observed only one case of gamete fusion while others eggs remained unfertilized and arrested in metaphase II stage.

Contrary to this, there was 87% of fertilized eggs in the group treated with acid Tyrode solution and 74% in the α-chymotrypsin

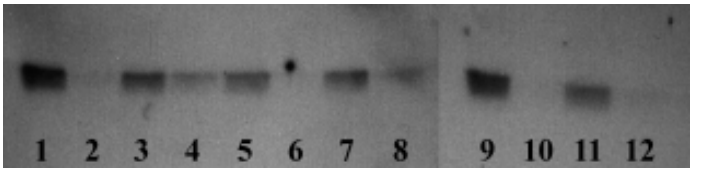

Fig. 2. Immunoblot of unfertilized eggs, zygotes and parthenogenotes treated with four different methods of removal of zona pellucida. *Each lane contains proteins from 100 cells. Lanes 1-4: unfertilized eggs* **(1)** *with zona pellucida;* **(2)** *treated with pronase;* **(3)** *treated with α-chymotrypsin and* **(4)** *treated with acid Tyrode's solution. Lanes 5-8: zygotes* **(5)** *with zona pellucida;* **(6)** *treated with pronase;* **(7)** *treated with α-chymotrypsin and* **(8)** *treated with acid Tyrode's solution. Lanes 9-12: parthenogenotes* **(9)** *with zona pellucida;* **(10)** *treated with pronase;* **(11)** *treated with α-chymotrypsin and* **(12)** *treated with acid Tyrode's solution.*

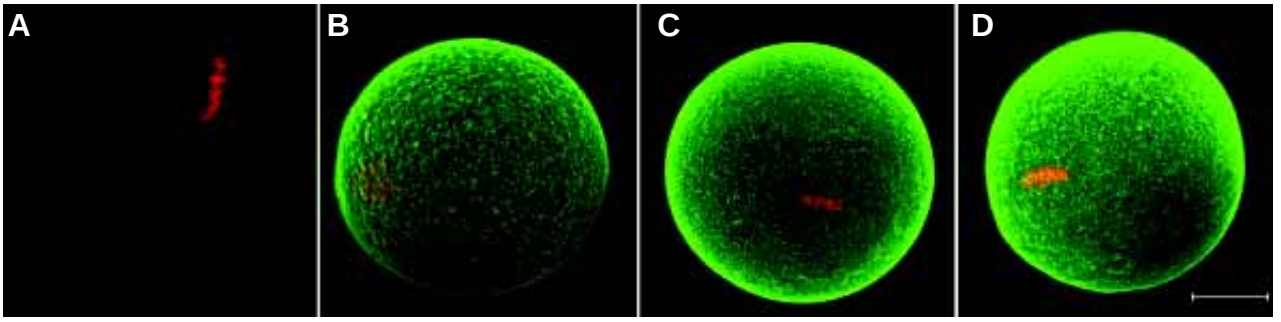

Fig. 3. **Confocal laser scanning micrographs of CD9 labeled unfertilized eggs freed of zona pellucida by means of four different methods.** *CD9 labeling is visualized in green. The position of the metaphase spindle is shown in red or orange (7-aminoactinomycin D dye). Pictures are projections along the Z-axis, made with the LSM 5 Image Browser (version: 3,1,0,99) from stacks. Eggs were fixed immediately after removal of the zona pellucida.* **(A)** *Zona pellucida removed by incubation with pronase.* **(B)** *Zona pellucida removed by incubation with α-chymotrypsin.* **(C)** *Zona pellucida removed with acid Tyrode's solution.* **(D)** *Zona pellucida removed mechanically using a micromanipulator. In the experiments presented in Figs. 1 and 3, control eggs, labeled with secondary antibody alone showed no background staining (not shown). Scale bar, 20 μm.*

treated group. We have observed 1-2 decondensed sperm nuclei inside the fertilized eggs in both the acid Tyrode and the α-chymotrypsin-treated groups. There was no statistical difference in the fertilization rate between them (t-Student test) (Fig. 4).

We did not observe any penetration in eggs treated with pronase and incubated for 5 hours prior to fertilization.

Discussion

The interaction between sperm and egg membranes during fertilization can be divided into two steps. Initially, sperm bind to the surface of the egg, then the spermatozoon and the egg membranes fuse. Sperm binding is necessary for membrane fusion, but the attached sperm may not fuse with the egg. It is still not clear whether plasma membrane block to polyspermy acts on sperm-egg binding or fusion (or both). Horvath and co-workers (1993) suggest that it inhibits sperm binding. Maluchnik and Borsuk (1994) on the contrary, have observed undisturbed sperm attachment and reduced sperm-egg fusion.

If we assume that the oolemma block to polyspermy decreases the level of sperm fusion and has no effect on sperm binding, the results are similar to the fertilization defects in the eggs of CD9-deficient mice. They do not fuse with sperm, although sperm bind normally (Kaji *et al.,* 2000; Miyado *et al.,* 2000). Therefore we have checked whether the lack of CD9 can be responsible for the formation of plasma membrane block to polyspermy in mouse zygotes.

The results of our experiments indicate however, that CD9 is not directly engaged in the mechanism of this block in mice. We have

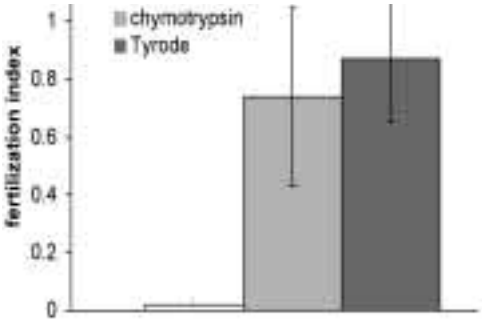

Fig. 4. **The influence of different methods of zona pellucida removal on fertilization rate of ovulated eggs.** *Fertilization index is a proportion of eggs fertilized with one or more spermatozoa to the total number of eggs in the experiment. Data shown are the means ± SD from 4 (α-chymotrypsin and Tyrode) or 3 (pronase) experiments. Each experiment was done on a different day and eggs from several females were pooled for each experiment.*

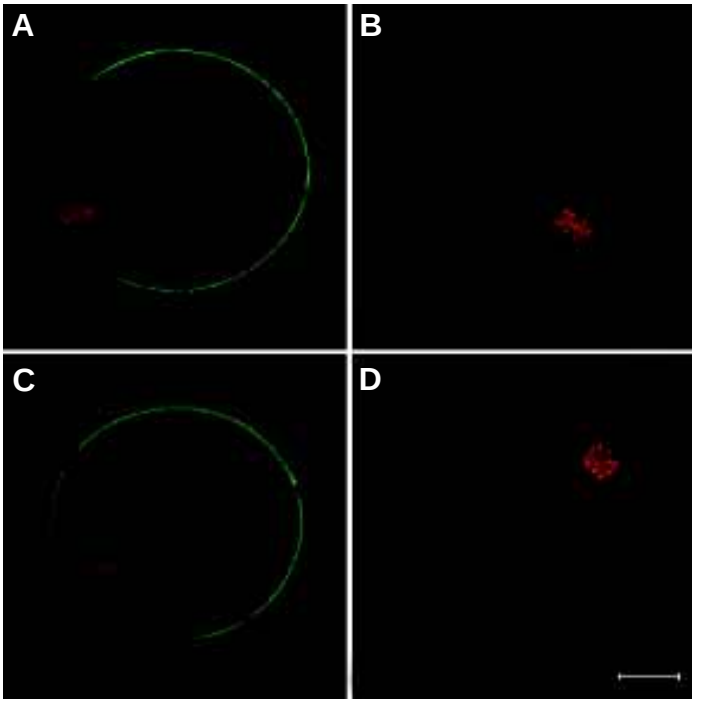

Fig. 5. Lack of recovery of CD9 on pronase-treated ovulated eggs. *CD9 labeling is visualized in green. Chromatin is stained in red (propidium iodide). Pictures are single slices taken at the level of the metaphase spindle. (A,B) Eggs fixed immediately after zona removal.* **(A)** *Acid Tyrode treated egg (positive control).* **(B)** *Pronase treated egg. (C,D) Eggs incubated for 5 h after zona removal.* **(C)** *Acid Tyrode treated egg (positive control).* **(D)** *Pronase treated egg. Experiments were performed twice and representative images from one experiment are presented. Scale bar, 20 μm.*

found this protein on the surface of unfertilized eggs, parthenogenotes and zygotes at the pronuclear stage. It has been confirmed in western-blotting analysis (Fig. 2) and immunofluorescence *in situ* (Fig. 1). We observed that after fertilization the epitope which is recognized by the antibody is still present on membranes of zygotes. We also found that CD9 did not undergo any modifications, which affect its electrophoretic mobility. Thus we suppose that CD9 remains unchanged after fertilization. Interestingly, it turns out that this protein is present both on the cells that develop the plasma membrane block to polyspermy (zygotes) and do not develop it (parthenogenotes). However, these results do not rule out the participation of CD9 protein in the formation of oolemma block to polyspermy. The amount of CD9 on the surface of zygotes could be reduced after fertilization to such a level that is not sufficient for re-fertilization. Moreover some molecular changes like modifications of protein conformation can be undetectable by standard western-blotting procedure or immunofluorescence staining. It is also possible that CD9 is associated with other proteins in the oolemma and these complexes are responsible for both sperm-egg binding and fusion. Miyado and co-workers (2000) have demonstrated that in egg plasma membrane CD9 is bound to integrin α_6. The role of α_6 protein in fertilization has been questioned recently (Miller *et al.,* 2000). However other integrins may be associated with CD9 and take part in this process.

Protease digestion can also change the properties of the oolemma, decreasing the fertilization rate of mouse eggs. Zona-free mouse eggs treated with trypsin and chymotrypsin show reduced sperm binding and sperm penetration levels (Wolf *et al.,* 1976; Boldt *et al.,* 1988; Porter and Calarco 1989). Reduced sperm-egg binding and fusion has also been demonstrated for zona-free mouse eggs incubated with pronase (Wolf *et al.,* 1976; Porter and Calarco 1989). Furthermore, it has been reported that treatment of mouse eggs with trypsin or chymotrypsin causes modifications of surface proteins (Boldt *at al.* 1989; Kellom *et al.,* 1992). These findings reveal that the proteases probably damage sperm binding and fusion sites on the surface of eggs, exerting an inhibitory effect on fertilization.

Our experiments have shown that the surface of eggs treated with pronase is completely devoid of CD9 or at least the external epitope which is recognized by anti-CD9 antibody is lost after pronase treatment. Moreover after pronase treatment eggs were unable to fuse with spermatozoa (Fig. 4). This is consistent with the observed phenotypes of CD9 "knockout" mice (Le Naour *et al.,* 2000; Miyado *et al.,* 2000; Kaji *et al.,* 2000). Eggs derived from CD9-/- females were infertile due to impaired sperm-egg fusion. However, it should be added that pronase is a non-specific protease. It probably destroys other proteins responsible for both sperm-egg binding and gamete fusion. In our study we have demonstrated its effect only on CD9.

When eggs treated with pronase were left for 5 hours after treatment, they did not recover the ability to fuse with spermatozoa. Immunofluorescence staining revealed that CD9 protein did not reappear on the surface of these eggs. It seems that pronase modifies the membrane of egg differently from chymotrypsin since trypsin and chymotrypsin treated eggs recover the fusion ability within few hours after protease treatment (Kellom *et al.,* 1992).

Eggs treated with α-chymotrypsin have not demonstrated a significant loss of CD9 as compared to eggs mechanically denuded (fluorescence staining; Fig. 3 B,D) or intact eggs with the zona pellucida (western-blotting assay; Fig. 2, lanes 1,3). These eggs showed normal fertilizability, with the penetration rate similar to eggs treated with the acid Tyrode (Fig. 4). Apparently, the concentration of α-chymotrypsin, which we used in our experiments, is not significantly destructive to egg membrane proteins taking part in fertilization. Incubation with higher concentration of α-chymotrypsin decreases the amount of CD9 on the surface of ovulated eggs and this seems to be consistent with results of Boldt and coworkers (1988) who have reported that trypsin and chymotrypsin cause a concentration-dependent reductions in both sperm attachment and sperm fusion with mouse eggs.

According to the results discussed above, the oolemma block to polyspermy in zygotes has different mechanism to the block produced by pronase treatment. Clearly, the physiological block to polyspermy does not depend on the total removal of CD9. However we can not exclude that the failure of sperm-egg fusion results from the reduction of the amount or changes in structure of CD9 undetectable by the methods we have used (more detailed studies are in progress). On the other hand the failure of sperm binding and fusion in eggs treated with pronase is probably caused by complete loss of CD9 and other proteins engaged in fertilization.

Materials and Methods

Collection of Ovulated Eggs

Eggs were obtained from the oviducts of F1 (C57Bl/10 x CBA/H) female mice (6-12 weeks old) which were induced to superovulate by i.p. injection of 10 IU pregnant mares' serum gonadotrophin (PMSG; 'Folligon', Intervet) and 10 IU of human chorionic gonadotrophin (hCG; "Chorulon", Intervet), given 48 h apart. Eggs were collected 15-18 h after hCG injection, and were freed from cumulus cells by treatment with 0.1% bovine testicular hyaluronidase (200 IU/mg) in PBS for several minutes. The eggs were rinsed thoroughly in M2 (M-16 medium buffered with HEPES, Sigma; Fulton and Whittingham 1978).

Collection of Zygotes

PMSG-primed F1 females were each housed with 2-3 F1 males after injection of hCG and allowed to mate thereafter. One-cell embryos (zygotes) were collected from oviducts 20-22 h after hCG injection. Zygotes were freed from cumulus cells by treatment with 0.1% bovine testicular hyaluronidase (200 IU/mg) in PBS for 3-5 min and subsequently were rinsed in M2.

Artificial Activation of Eggs

Eggs were artificially activated according to the method of Cuthbertson (1983) by a 8 min. incubation in 8% ethanol solution in M2. After exposure to EtOH, eggs were rinsed thoroughly in M2 and cultured at 37°C under 5 % CO_2 for about 3-5 hours. Eggs were scored for signs of activation and only those with extruded second polar body and a visible pronucleus were selected for experiments.

Removal of Zona Pellucida

Eggs were freed of zona pellucida using four different protocols:
- acid Tyrode's solution (pH 2.5, Nicolson *et al.,* 1975) for ~30 s;
- α-chymotrypsin (30 μg/ml M2) for 10 min;
- pronase (5 mg/ml in Ringer's solution) for 10 min;
- mechanic removal on micromanipulator;

Insemination of Eggs

Spermatozoa from two *caudae epididymideis* of F1 males were suspended at the concentration of approximately 2 x 10^7 sperm/ml in 0.5 ml of fertilization medium (Fraser and Drury 1975) containing 4 mg/ml of bovine serum albumin (BSA, crystalline, Millex). They were incubated for 2.5 h at

37ºC under 5 % CO_2 in air to allow capacitation and spontaneous acrosome reaction. After the first hour of incubation sperm suspension was diluted with fertilization medium in proportion 1:25. Zona-free eggs were transferred into fertilization medium containing 4 mg/ml of BSA. Insemination was carried out at 37˚C by adding 5 µl of preincubated and diluted sperm suspension to 95 µl of fertilization medium containing eggs (final sperm concentration was approximately 4 x 10^4 sperm/ml). After 1 h of incubation with sperm, eggs were washed in M2 and cultured for 2 h at 37˚C under 5 % CO_2 in air. Eggs were stained with Hoechst dye (5 µg/ml, bisbenzimid H 33342, Riedel-de Haën) and mounted in a drop of M2 medium on glass slides. To determine the number of sperm fused with one egg, the number of decondensed sperm nuclei within the eggs was counted.

Indirect Immunofluorescence

Cells were fixed in 4% formaldehyde (20 min at room temperature) then washed twice in PBS and once in PBS with 10mM glycine for 10 min. To block nonspecific sites, cells were incubated for 1 h in PBS with 2% BSA at room temperature. Cells were then incubated with monoclonal anti-mouse CD9 rat antibody (Clone KMC8, PharMingen) (50 µg/ml in PBS with 1% BSA) for 1 h at room temperature and washed 4 times (15 min per wash) in PBS with 1% BSA. Then eggs were labeled with secondary antibody (FITC conjugated mouse anti-rat IgG, Clone MRK-1, PharMingen, 1.25 µg/ml in PBS with 1% BSA) during 1h incubation at room temperature. Cells were washed 3 times (15 min per wash, PBS with 1% BSA) and incubated with propidium iodide (50 µg/ml in PBS) or 7-aminoactinomycin D for 20 min to stain chromatin. After chromatin staining cells were washed for 20 min. in PBS with 1 % BSA and mounted on glass slides in a drop of antifading mounting medium (Citifluor AF1, Citifluor Ltd.). Negative controls were prepared by omitting the first antibody. Samples were examined with confocal laser microscopy (LSM 510 Zeiss).

Western Blotting Analysis of CD9

Eggs, parthenogenotes and zygotes were washed twice in PBS, and proteins were solubilized in Laemmli's buffer containing 5% mercaptoethanol. For each sample 100 eggs, parthenogenotes or zygotes were suspended in 10 µl of Laemmli buffer and boiled for 5 min. Then samples were centrifuged and stored at - 80ºC until use. Each sample was loaded into wells of a 15% SDS-polyacrylamide gel, and electrophoresis was performed using a minigel system (Bio-Rad) prior to transfer to a nitrocellulose membrane (PVDF) with the Hoefer TE22 Mighty Small transfer system. CD9 was immunodetected by incubation of membranes with a monoclonal anti- mouse CD9 rat antibody (KMC8, PharMingen) diluted 1:500 (final concentration 1 µg/ml) for 1 h at room temperature. An anti-rat IgG goat antibody conjugated with peroxidase (Calbiochem) diluted 1:2500 (final concentration 0.4 µg/ml) was used as secondary antibody. Detection was performed by the enhanced chemiluminescence (ECL) technique using ECL Plus reagents according to the manufacturer's instruction (Amersham).

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