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Residues SFQ (173-175) in the large extracellular loop of CD9 are required for gamete fusion

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SUMMARY

Gamete fusion is the fundamental first step initiating development of a new organism. Female mice with a gene knockout for the tetraspanin CD9 (CD9 KO mice) produce mature eggs that cannot fuse with sperm. However, nothing is known about how egg surface CD9 functions in the membrane fusion process. We found that constructs including CD9's large extracellular loop significantly inhibited gamete fusion when incubated with eggs but not when incubated with sperm, suggesting that CD9 acts by interaction with other proteins in the egg membrane. We also found that injecting developing CD9 KO oocytes with CD9 mRNA restored fusion competence to the resulting CD9 KO eggs. Injecting mRNA for either mouse CD9 or human CD9, whose large extracellular loops differ in 18 residues, rescued fusion ability of the injected CD9 KO eggs. However, when the injected mouse CD9 mRNA contained a point mutation (F174 to A) the gamete fusion level was reduced fourfold, and a change of three residues (173-175, SFQ to AAA) abolished CD9's activity in gamete fusion. These results suggest that SFQ in the CD9 large extracellular loop may be an active site which associates with and regulates the egg fusion machinery.

Key words: Gamete fusion, CD9, Tetraspanin, mRNA injection, Mutation, Mouse

INTRODUCTION

Sperm-egg binding and fusion initiate the development of a new organism, but the molecular mechanisms of gamete adhesion, gamete membrane fusion and associated signaling are still poorly understood. Recently, one egg surface protein, CD9, was shown to be essential for gamete fusion. The fertility of CD9-deficient female mice is severely reduced because membrane fusion ability is lost in CD9-deficient eggs (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000). CD9 belongs to a family of proteins called tetraspanins, which contain four transmembrane domains, one small extracellular loop (EC1) and one large extracellular loop (EC2), and cytoplasmic amino and carboxyl termini (Boucheix and Rubinstein, 2001). Tetraspanins are reported to function in a variety of cell activities including cell adhesion, motility, proliferation, differentiation and signaling (Maecker et al., 1997; Lagaudriere-Gesbert et al., 1997). Immunoprecipitation and other studies suggest that tetraspanins in the plasma membrane are associated with each other and with several other cell surface molecules, including a subset of β1 integrins and Ig superfamily members, to form a tetraspanin web (Nakamura et al., 1995; Berditchevski et al., 1996; Rubinstein et al., 1996; Serru et al., 1999; Charrin et al., 2001; Stipp et al., 2001; Maecker et al., 1997; Boucheix and Rubinstein, 2001). Tetraspanins may organize specific cell surface molecules to form functional macromolecular complexes on the surface of the cell that express the tetraspanin (Maecker et al., 1997; Boucheix and Rubinstein, 2001). Additionally, it is possible that tetraspanins are ligands for receptors on other cells, as reported for the tetraspanin CD81 (Kelic et al., 2001).

Another egg surface protein, the integrin α6β1, was previously proposed as the receptor for sperm on mouse eggs (Almeida et al., 1995). It was thought that α6β1 binds to the disintegrin domain of fertilin β on the sperm surface (Chen and Sampson, 1999; Chen et al., 1999; Evans, 2001). Because it was found that α6β1 and CD9 could be coimmunoprecipitated from mouse eggs, it was suggested that CD9 plays its crucial role in gamete fusion through involvement in a fertilin β-α6β1/CD9 intergamete complex (Miyado et al., 2000). This view, however, is challenged by two other reports. One shows that fertilin β-deficient sperm fuse with eggs at 50% of the wild-type level (Cho et al., 1998). The other shows that α6β1-deficient eggs fuse with sperm at the same level as the wild-type eggs (Miller et al., 2000). These two reports indicate that there must be other molecules on the cell surface of gametes that can act in sperm-egg fusion.

Thus, how CD9 functions in gamete fusion is still unknown. In the present study, we addressed two questions. Does CD9 work by binding to sperm or to other egg surface proteins? What is the binding site on CD9? Our data suggest that CD9 interacts through its large extracellular loop (EC2) domain with other egg surface proteins (i.e. in *cis*), and that the three residues SFQ (173-175) in EC2 are required for CD9's function in gamete fusion. These findings add to our understanding of the mechanism of the gamete fusion process and emphasize the importance of identifying the egg protein(s) that interact with the SFQ site in CD9.

MATERIALS AND METHODS

Plasmid construction

The plasmid pmCD9 was constructed by inserting the full-length mouse CD9 cDNA (GenBank accession number NM007657) into the *Eco*RI site of the pBluescript vector (Stratagene). The plasmid phCD9 was constructed by inserting the full-length human CD9 cDNA (GenBank accession number NM001769) into the *Eco*RI site of pcDNA3.1 (Invitrogen). pmCD9 was used as the parental plasmid to construct the three-amino-acid-mutant plasmid pmCD9-SFQ(173-175)AAA by PCR. Two overlapping mutated fragments were generated by two sets of primers (forward 5′-GCAGGAATTCCGGCCCTTCTGT-3′ and reverse 5′-AACCgcGgccgcTTCCAAAAGCTGTTTCTTGGG-3′, forward 5′-GGAAgcggcCgcGGTTAAGCCCTGCCCTGAAGC-3′ and reverse 5′-ATCGATAAGCTTGATATCGAATTC-3′, the mutated sequence in lowercase), digested with *Not*I and ligated together to form a single fragment. This fragment was digested with *Eco*RI and ligated back into the parental *Eco*RI-digested pBluescript vector.

The green fluorescent protein (GFP)-tagged chimeric plasmids were constructed as follows. The forward primer 5′-ACGTagatctAGTCACG-ACGTTGTAAAACGACGGCC-3′ (*Bgl*II site in lower case) and the reverse primer 5′-ACGTaagcttGACCATTTCTCGGCTCCTGCGGAT-3′ (*Hin*dIII site in lower case) were used in PCR to make a construct including the T7 promoter and the open reading frame (ORF) of the mouse CD9 cDNA from the plasmid pmCD9. After digestion with *Bgl*II and *Hin*dIII, the PCR product was ligated into the vector pEGFP-N1 (Clontech) to obtain the GFP chimeric plasmid pmCD9-eGFP. This plasmid was used as the parental vector to construct the single-amino-acid-mutant plasmid pmCD9-F174A-eGFP, according to the instruction of the Quick-Change™ mutagenesis kit (Stratagene). The primers used in the mutagenesis were forward 5′-CCAAGAAACAGCTTTTG-GAAAGTGCCCAGGTTAAGCCCTGCCCTGAAGCC-3′ and reverse 5′-GGCTTCAGGGCAGGGCTTAACCTGGGCACTTTCCAAAAG-CTGTTTCTTGG-3′.

All constructs were sequenced to show that undesired mutations had not been introduced during the PCR and cloning steps.

In vitro synthesis of mRNA

The plasmids pmCD9, phCD9, pmCD9-SFQ(173-175)AAA, pmCD9-eGFP and pmCD9-F174A-eGFP were linearized by digestion with appropriate restriction enzymes whose recognition sites are downstream of the ORFs of the corresponding cDNAs. The linearized plasmids were used as templates to synthesize mRNA using the mMessage mMachine ™ capped RNA transcription kit with T7 RNA polymerase (Ambion), according to the manufacturer's protocol. The mRNAs were dissolved in RNase/DNase-free water.

Antibodies

Antibodies used include the anti-mouse CD9 monoclonal antibodies: KMC8 (Pharmingen), 4.1F12, 1.2C4, 1.1F2 and 1.4G1, isolated and characterized in one of our laboratories (C. B. and E. R., unpublished results). The anti-human CD9 monoclonal antibody, ALB-6, has been described (Rendu et al., 1987).

Media for oocyte culture and in vitro fertilization assay

Medium 1: (M199; Gibco BRL), 3.5 mM sodium pyruvate, 1,000 i.u. penicillin-streptomycin, 0.1% polyvinyl alcohol, 2 mM L-glutamine, 0.2 mM 3-isobutyl-1-methylxanthine (IBMX, Calbiochem); Medium 2: 10 mM Hepes (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] in Medium 1; Medium 3: 5% fetal calf serum in Medium 1; Medium 4: Medium 3 without IBMX; Medium 5: Medium 2 without IBMX; Medium 6: 0.3% bovine serum album (BSA) in Medium 1 without IBMX; Medium 7: 3.0% BSA in Medium1 without IBMX.

Oocyte culture and microinjection

Wild-type or CD9-deficient C57BL6 female mice (Le Naour et al., 2000) (6-8 weeks old) were primed with 10 i.u. of pregnant mare serum gonadotropin 48 hours prior to ovary isolation. Isolated ovaries were placed in Medium 1 to collect germinal vesicle (GV)-stage oocytes. After the removal of granulosa cells, the GV-stage oocytes were allowed to recover in Medium 1 at 37°C, 5% CO_2 for 2-3 hours and then loaded into a 25 μl drop of Medium 2 in a 35 mm tissue culture dish (Corning). A 0.5 μl drop of mRNA solution was placed approximately 2 mm away from the drop containing oocytes in Medium 2. Both drops were overlaid with dimethylpolysiloxane (DMPS, 20 centistokes; Sigma) to prevent evaporation. Each oocyte was injected with approximately 30 pg of mRNA. Injections were carried out on a Zeiss Axiovert 135 equipped with a bipolar temperature controller set at 37°C (Medical Systems Co.) Injected oocytes were transferred to Medium 3 for a period of approximately 12 hours at 37°C, 5% CO_2. After incubation, oocytes were allowed to incubate for approximately 16 hours in Medium 4. The metaphase II eggs among the cultured oocytes were selected for use in experiments.

In vitro fertilization assay (IVF)

Eggs, prepared as described above, were treated with acid Tyrodes solution for about 30 seconds to remove the zona pellucida and then allowed to incubate in Medium 6 at 37°C, 5% CO_2 for 3 hours. Sperm for the in vitro adhesion and fusion assay were isolated from the cauda epididymis and the vas deferens of C57BL6 male mice (10-12 weeks old) into Medium 7, and capacitated for 3 hours in Medium 7 at 37°C, 5% CO_2, resulting in a population of 60-70% acrosome-reacted sperm (Moller et al., 1990). The other steps for in vitro fertilization were carried out as previously described (Miller et al., 2000).

Indirect immunofluorescence of eggs expressing human or mouse CD9

After in vitro fertilization, the eggs were fixed in 4% paraformaldehyde and 0.1% PVA in phosphate-buffered saline (PBS, pH 7.4) for 10 minutes at room temperature. After fixation, eggs were incubated with either 10 μg/ml anti-mouse CD9 antibody KMC8 (BD Pharmingen) or 10 μg/ml anti-human CD9 antibody ALB-6 for 1 hour at 37°C, 5% CO_2 in Medium 6, followed by a 30-minute incubation with an appropriate secondary antibody conjugated to Oregon Green™ or Alexa Fluor™ 488 fluorophore (Molecular Probes Inc.). The fluorescence images were acquired with a laser scanning confocal microscope (model LSM 410; Carl Zeiss).

Visualization of CD9-eGFP fusion protein expression using confocal microscopy

For eggs injected with CD9-eGFP or CD9-F174A-eGFP mRNAs, the expression of the eGFP fusion protein on the eggs was observed with a laser scanning confocal microscope (model LSM 410; Carl Zeiss) after the eggs were incubated in Medium 4 for 16 hours and then coincubated with sperm for in vitro fertilization assay.

Preparation of glutathione S-transferase (GST)-mouse CD9 extracellular large loop (EC2) fusion protein and EC2-histidine (HT) fusion protein

The DNA fragment encoding the extracellular loop of mouse CD9 (EC2) was inserted into *Eco*RI and *Bam*HI restriction sites of the PGEX-

3X vector (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The GST-EC2 fusion protein and GST alone were expressed in *E. coli* BL21 cells (Amersham Pharmacia Biotech). The cells were lysed by mild sonication, and GST-EC2 or GST was allowed to bind to a glutathione affinity matrix (Amersham Pharmacia Biotech). The GST-EC2 fusion protein or GST was eluted from the affinity matrix using 10 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0). The eluted proteins were used fresh in the in vitro fertilization assay.

The EC2 DNA fragment was also inserted into the *Nhe*I and *Xho*I restriction sites of the carboxy-terminal hexa-histidine expression vector pET24a (Novagen, Madison, WI, USA). The resulting plasmid, denoted pET24a-CD9EC2/HT, was transformed into *E. coli* BL21/pLysS cells for protein expression. The cells were lysed in a French press and separated into soluble and insoluble fractions by centrifugation. The insoluble portion was resuspended in 6 M guanidine-HCl, and subsequently clarified by both centrifugation and filtration. The fusion protein EC2/HT was purified by nickel-nitrilotriacetic acid metal-affinity chromatography (Qiagen, Chatsworth, CA, USA), followed by reverse-phase HPLC (Waters, Milford, MA, USA) using a Vydac C18 preparative column (Vydac, Hesperia, CA, USA) with a water/acetonitrile gradient of 0.05%/minute in the presence of 0.1% trifluoroacetic acid. Protein peaks were collected, centrifuged under vacuum to remove the acetonitrile, and lyophilized. A single oxidized species was recognized by the monoclonal antibody KMC8. Circular dichroism analysis revealed that this species had significant helical structure and was thermally stable (C. C. L. et al., unpublished results). This fraction of the EC2/HT was resuspended in PBS just before use in the in vitro fertilization assay.

Western blot analysis

Western blot analysis was performed according to the standard method for ECL western blotting detection reagent (Amersham Pharmacia Biotech). Protein samples were analyzed by non-reducing SDS-PAGE. Gels were transferred onto Hybond-C membranes (Amersham Pharmacia Biotech) by electroblotting. Monoclonal antibody KMC8 (BD Pharmingen) against CD9 was used as the primary antibody at a final concentration of 0.5 μg/ml. The blotting signals were developed using the ECL system (Amersham Pharmacia Biotech), using film development times that gave a signal in the linear range of blot intensities.

RESULTS

The fusion proteins GST-EC2 and EC2/HT inhibit mouse sperm-egg fusion

We first asked which region(s) of the CD9 protein is important for its function in gamete fusion. The monoclonal antibody KMC8 to mouse CD9 has been reported to inhibit sperm-egg fusion but not binding (Miller et al., 2000), but the KMC8 epitope is unknown. We expressed the large extracellular loop (EC2) of mouse CD9 as a GST fusion protein (GST-EC2) and this fusion protein bound KMC8 in western blotting (Fig. 1, lane 1), suggesting that a functional site of CD9 may be in EC2. Freshly purified GST-EC2 fusion protein was tested for its effect on gamete fusion. Three parameters were measured: (1) fertilization rate FR = the percentage of eggs that fuse with at least one sperm; (2) fertilization index FI = total number of fused sperm/total number of eggs; (3) the mean number of sperm bound at the equator of the egg. When zona-free eggs were incubated with GST-EC2 for 3 hours before insemination, gamete fusion was inhibited (Table 1). The fertilization rate was reduced from 100% to 60%, and the fertilization index was reduced from 1.5 to 0.6. The average number of sperm bound to the egg plasma membrane was unaffected. When sperm were incubated with GST-EC2 for 3 hours before insemination, no significant inhibitory effect was seen (Table 1).

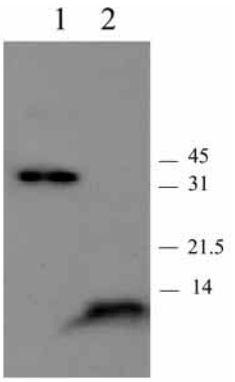

Fig. 1. Western blot of GST-EC2 and EC2/HT. Western blotting was carried out as described in Materials and Methods. Lane 1, 50 ng GST-EC2; lane 2, 10 ng EC2/HT. The molecular mass (kDa) of protein standards is indicated.

To test the effect of EC2 in another kind of construct, EC2 was fused with a histidine tag (HT) and purified by HPLC. EC2/HT binds KMC8 in western blots (Fig. 1, lane 2) and folds properly by biophysical criteria, e.g. it shows expected amounts of helical structure by circular dichroism and has a conformation related to the native form of CD9 on the mouse egg (C. C. L. et al., unpublished results). When zona-free eggs were incubated with the EC2/HT polypeptide for 3 hours before insemination, gamete fusion was inhibited (Table 1). The fertilization rate was reduced from 83% to 43%, and the fertilization index from 0.83 to 0.42. The average number of sperm bound to the egg plasma membrane was not affected (Table 1). When sperm were incubated with EC2/HT for 3

Table 1. The effect of GST-EC2 and EC2/HT on in vitro fertilization

Incubation protein	Pre-incubated gamete	Number of eggs tested	Fertilization rate (%)	Fertilization index	Sperm bound per equator
GST	Egg	78	100±0	1.5±0.15	6.2±0.5
GST-EC2	Egg	86	60±5.1	0.6±0.05	5.7±0.5
GST-EC2	Sperm	63	97±3.0	1.1±0.11	7.1±1.6
Buffer (PBS)	Egg	56	83±3.5	0.83±0.04	4.6±0.3
EC2/HT	Egg	76	43±2.6	0.42±0.02	4.8±0.4
EC2/HT	Sperm	67	75±5.2	0.75±0.05	4.5±0.3

Values are mean±s.e.m.

300 μg/ml of GST-EC2 or 250 μg/ml of EC2/HT were pre-incubated with sperm or with eggs for 3 hours. Then the EC2 construct remained present (undiluted) during a 40 minute insemination period beginning when the other gamete was added. After the 40 minute insemination, fertilization rate, fertilization index and sperm bound per equator were scored (see text).

For eggs pre-incubated with GST-EC2, significant inhibition was seen for both fertilization rate and fertilization index ($P<0.02$).

For sperm pre-incubated with GST-EC2, fertilization rate was unaltered and fertilization index showed a slight, statistically non-significant reduction ($P>0.1$).

For eggs pre-incubated with EC2/HT, significant inhibition was seen for both fertilization rate and fertilization index ($P<0.002$).

For sperm pre-incubated with EC2/HT, fertilization rate and fertilization index showed statistically non-significant reduction ($P>0.2$).

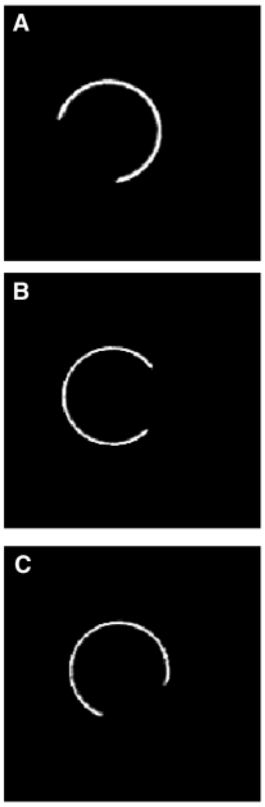

Fig. 2. Immunofluorescent staining of CD9 on the surface of CD9$^{-/-}$ eggs injected with either mouse or human CD9 mRNAs. (A) Immunofluorescent staining using the anti-mouse CD9 antibody KMC8 on wild-type eggs. (B) Immunofluorescent staining using the anti-mouse CD9 antibody KMC8 on CD9$^{-/-}$ oocytes injected with mouse CD9 mRNA. (C) Immunofluorescent staining using the anti-human CD9 antibody ALB6 on CD9$^{-/-}$ oocytes injected with human CD9 mRNA.

hours before insemination, no significant inhibition was observed (Table 1).

In the protocol used in these experiments (see legend of Table 1), the CD9 EC2 construct is preincubated with sperm or with eggs for 3 hours. Then the EC2 construct remains present (undiluted) during the 40 minute insemination period beginning when the other gamete is added. Inhibition occurs when the eggs are incubated with CD9 EC2 for the initial 3 hours plus 40 minutes insemination but not when they are incubated with CD9 EC2 for only the final 40 minute insemination. This indicates that the preincubation time of the CD9 EC2 with the eggs is required for inhibition of gamete fusion. Overall, the results suggest that EC2 is a functional region of the CD9 molecule in gamete fusion, that sperm may lack a counter-receptor for egg CD9, and that the constructs GST-EC2 and EC2/HT may displace endogenous egg CD9 from a complex with other egg molecules, thereby inhibiting sperm-egg fusion.

Injection of CD9 mRNA into CD9-deficient oocytes restores their fusion ability

Given that the CD9 EC2 loop has a role in sperm-egg fusion, we asked what is the functional site within EC2? To address this question, we applied a mouse oocyte expression system (detailed below) using oocytes from CD9 null female mice, along with CD9 mutagenesis and IVF techniques, to investigate the key amino acid residues in EC2 required for CD9's function in sperm-egg fusion.

It has been well demonstrated that mouse oocytes can express functional proteins from injected mRNAs (Paynton et al., 1983; Kola and Sumarsono, 1996; Williams et al., 1998). The mRNAs encoding mouse or human CD9 were synthesized and injected into CD9-deficient oocytes. The injected oocytes were cultured in vitro to the metaphase II stage (see Materials and Methods). After the in vitro culture period, the mean percentages of wild-type (WT) oocytes, KO (CD9$^{-/-}$) oocytes, or injected KO oocytes that produced first polar bodies were in the range 61-78% and were not significantly different among the different groups (data not shown). This result indicates that CD9-deficient GV-stage oocytes, after injection with mRNA, can develop normally to metaphase II.

As expected, KO eggs, injected with CD9 mRNA, express CD9 on the egg surface as seen by immunofluorescent staining using the anti-mouse CD9 monoclonal antibody KMC8 or the anti-human CD9 monoclonal antibody ALB6 (Fig. 2B,C). The localization of staining is similar to that of WT eggs, with CD9 over the microvillar region and absent over the metaphase plate (Fig. 2A). CD9 KO eggs without mRNA injection show no surface staining (data not shown). The absence of CD9 in uninjected CD9 KO eggs and the expression of CD9 in KO eggs injected with CD9 mRNA was also confirmed by western blot (Fig. 6, lanes 1,2).

To test whether or not in ovum expression of the CD9 protein on the egg surface was capable of restoring fusion ability, GV-stage KO oocytes were injected with CD9 mRNAs, allowed to mature to metaphase II, and promptly used for in vitro fertilization assays (see Materials and Methods). The

Table 2. In vitro fertilization assay with CD9$^{-/-}$ oocytes injected with different CD9 mRNAs

Oocyte genotype	Injected mRNA	Number of eggs tested	Fertilization rate (%)	Fertilization index	Sperm bound per equator
(A)					
CD9$^{+/+}$	None	131	83±4.3	0.89±0.07	10.0±1.3
CD9$^{-/-}$	Mouse CD9 (mCD9)	76	46±4.2	0.46±0.04	10.1±2.2
CD9$^{-/-}$	Human CD9 (hCD9)	64	48±3.3	0.58±0.08	12.1±1.0
CD9$^{-/-}$	None	140	0±0	0±0	10.2±1.2
(B)					
CD9$^{+/+}$	None	166	66±7.4	0.83±0.04	14.4±1.7
CD9$^{-/-}$	mCD9-eGFP	92	55±13.0	0.85±0.09	12.3±1.2
CD9$^{-/-}$	mCD9-F174A-eGFP	73	13±4.0	0.20±0.07	12.0±2.1
CD9$^{-/-}$	None	131	1±1.2	0.02±0.01	14.0±1.7
(C)					
CD9$^{+/+}$	None	122	80±3.3	0.81±0.04	8.3±0.8
CD9$^{-/-}$	mCD9	54	48±3.1	0.48±0.03	8.4±1.2
CD9$^{-/-}$	mCD9-SFQ(173-175)AAA	68	1±1.1	0.01±0.01	8.3±1.2
CD9$^{-/-}$	None	78	0±0	0±0	8.6±0.5

Values are mean±s.e.m.

(A) The mean fertilization rate and index for CD9$^{-/-}$ oocytes injected with mouse CD9 mRNA are not significantly different from the values of CD9$^{-/-}$ oocytes injected with human CD9 mRNA (P>0.2).

(B) The mean fertilization rate and index are reduced about fourfold after injection of mCD9-F174A-eGFP mRNA compared to mCD9-eGFP mRNA.

(C) Fertilization is almost abolished after injection of mCD9-SFQ(173-175)AAA mRNA compared to mCD9 mRNA.

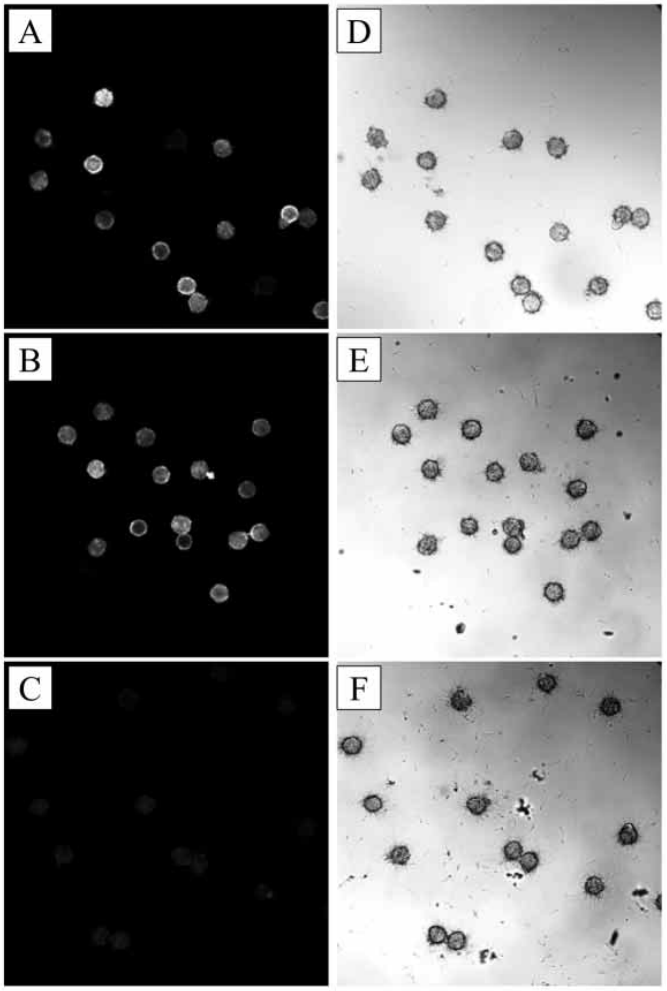

Fig. 3. CD9-eGFP fluorescence observed for CD9$^{-/-}$ oocytes injected with either wild-type or F174A mutant mouse CD9-eGFP mRNA. (A) Fluorescence observed in CD9$^{-/-}$ eggs injected with wild-type CD9-eGFP mRNA. (B) Fluorescence observed in CD9$^{-/-}$ eggs injected with F174A mutant CD9-eGFP mRNA. In A and B, both intracellular and surface fluorescence are observed. (C) Fluorescence observed in uninjected CD9$^{-/-}$ eggs. (D-F) Phase contrast images of A-C, respectively. Fluorescence levels were somewhat variable within each group of eggs injected with the identical amount and type of mRNA.

expression of mouse or human CD9 on the surface of KO eggs rescued sperm-egg fusion (Table 2A). The mean fertilization rate for WT eggs is 83±4.3%; for KO eggs expressing mouse CD9, 46±4.2%, and for KO eggs expressing human CD9, 48±3.3%. No fusion was observed in the KO eggs without mRNA injection. The fertilization index mirrors the fertilization rate (Table 2). Sperm-egg binding is not significantly different in the four groups tested (Table 2).

Reduced fusion ability of CD9-deficient oocytes injected with the CD9-F174A-eGFP mutant mRNA

To hypothesise about which CD9 EC2 residues might be critical for gamete fusion, we considered the structure of the related human tetraspanin CD81 EC2, which has been solved by X-ray crystallographic methods (Kitakodora et al., 2001). The CD81 EC2 structure reveals a ‘head domain’, delineated

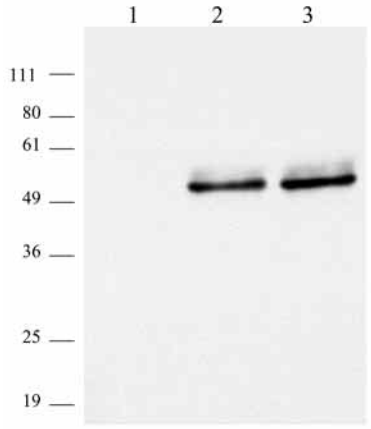

Fig. 4. Western blot assay of CD9-eGFP expression in CD9$^{-/-}$ eggs injected with either wild-type or F174A mutant mouse CD9-eGFP mRNA. The injected eggs were allowed to develop to the M-II phase, then were lysed in non-reducing SDS-sample buffer. Western blots were carried out as described in Materials and Methods. Each lane contains 20 eggs. Lane 1, CD9$^{-/-}$ eggs; lane 2, CD9$^{-/-}$ eggs injected with wild-type CD9-eGFP mRNA; lane 3, CD9$^{-/-}$ eggs injected with mutant CD9- F174A-eGFP mRNA. The molecular mass (kDa) of protein standards is indicated.

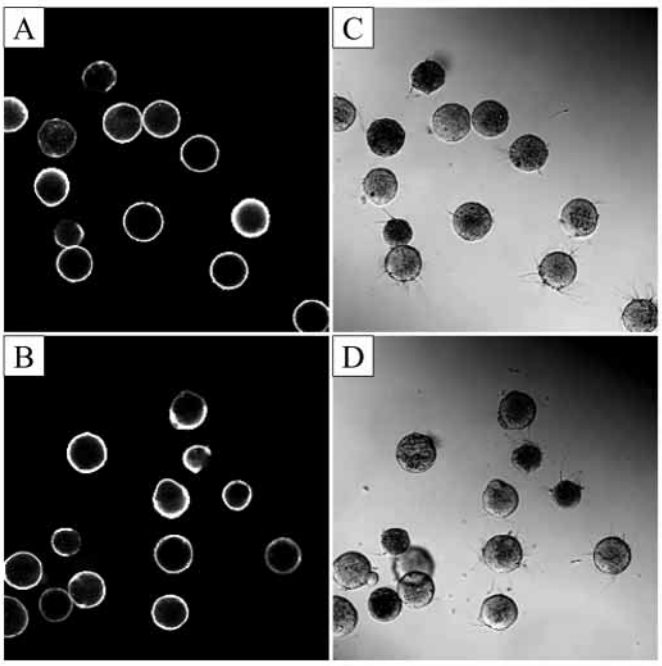

Fig. 5. Immunofluorescent staining of CD9 on the surface of CD9$^{-/-}$ eggs injected with either wild-type or SFQ(173-175)AAA mutant mouse CD9 mRNA. After in vitro fertilization, eggs were fixed and washed as described in Materials and Methods. The fixed eggs were incubated with anti-mouse CD9 antibody KMC8 as the primary antibody, followed by a goat anti-rat IgG (H+L) conjugated with Alexa Fluor^{TM} 488 as the secondary antibody. Immunofluorescent staining was obtained with a laser scanning confocal microscope (Carl Zeiss, LSM 410). (A) Immunofluorescent staining on CD9$^{-/-}$ eggs injected with wild-type mouse CD9 mRNA. (B) Immunofluorescent staining on CD9$^{-/-}$ eggs injected with SFQ-to-AAA mutated mouse CD9 mRNA. (C,D) Phase contrast images of A and B, respectively. Fluorescence levels were somewhat variable within each group of eggs injected with the identical amount and type of mRNA.

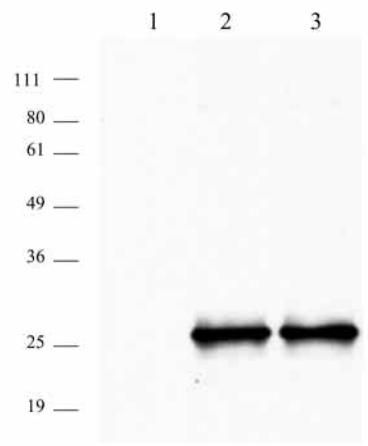

Fig. 6. Western blot assay of CD9 expression in CD9$^{-/-}$ eggs injected with either wild-type or mutant CD9-SFQ(173-175)AAA mRNA. The injected eggs were allowed to develop to the M-II phase, then were lysed in non-reducing SDS-sample buffer. Western blots were carried out as described in Materials and Methods. Each lane contains 20 eggs. Lane 1, CD9$^{-/-}$ eggs; lane 2, CD9$^{-/-}$ eggs injected with wild-type CD9 mRNA; lane 3, CD9$^{-/-}$ eggs injected with mutant CD9-SFQ(173-175)AAA mRNA. The molecular mass (kDa) of protein standards is indicated.

by disulfide bond patterning conserved in all tetraspanin family members. This head domain contains regions of the tetraspanin molecule that can associate with other surface proteins. In its head domain CD81 EC2 has a phenylalanine residue F186 in a solvent-exposed, low polarity patch. F186 is required for CD81 binding with hepatitis C virus (Higginbottom et al., 2000). CD9 F174 is present in a corresponding head domain region and we mutated CD9 F174 to alanine to determine if there would be an effect on sperm-egg fusion.

To allow direct observation of CD9 protein expression, eGFP was fused at the carboxyl terminus of the F174A mutant or wild-type CD9. The wild-type CD9-eGFP or mutant CD9-F174A-eGFP mRNAs were synthesized and injected into CD9-deficient oocytes (see Materials and Methods). In CD9-deficient oocytes, levels of expression detected by immunofluorescence and by western blot are very similar for CD9-eGFP and CD9-F174A-eGFP (Fig. 3A,B, Fig. 4, lanes 2,3). However, the F174A mutant is crippled in its ability to rescue sperm-egg fusion. The mean fertilization rate and fertilization index are reduced about fourfold after injection of mutant mRNA compared to wild-type mRNA (Table 2B). Sperm binding, measured by the number of sperm bound/egg equator, is not significantly different in any of the experimental groups (Table 2).

Lack of fusion ability of CD9-deficient oocytes injected with the CD9-SFQ(173-175)AAA mRNA

The above result indicates that residue F174 may be part of a required site necessary for CD9 function in sperm-egg fusion. To test this, we further mutated the three CD9 amino acid residues SFQ (173-175) to AAA, and determined the effect of injecting this mutant mRNA on the fusion ability of CD9-deficient oocytes. In CD9-deficient oocytes, levels of expression detected by immunofluorescence and by western blot are very similar for CD9 and CD9-SFQ(173-175)AAA (Fig. 5A,B, Fig. 6, lanes 2,3). The SFQ (173-175) to AAA mutation essentially abolished the ability of CD9 to rescue the fusion competence of KO eggs (Table 2). While the mean fertilization rate is 48±3.1% for KO eggs injected with wild-type CD9 mRNA, it is only 1±1.1% for KO eggs injected with mutant CD9-SFQ(173-175)AAA mRNA (Table 2C). Similarly, the mean fertilization index is 0.48±0.03 for KO eggs injected with wild-type CD9 mRNA, but 0.01±0.01 for KO eggs injected with mutant CD9-SFQ(173-175)AAA mRNA (Table 2). Sperm binding, as measured by the number of sperm bound/egg equator, is statistically equivalent for all experimental groups (Table 2).

DISCUSSION

Previous work demonstrated that egg surface CD9 is essential for sperm-egg fusion (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000), but nothing is known about how CD9 acts in this system. Egg CD9 could bind in *trans* to a counter-receptor on sperm as suggested for CD81 on astrocytes interacting with neurons (Kelic et al., 2001). Alternatively, CD9 may function by *cis* interactions on the egg surface with other egg surface molecules. One egg protein suggested to interact in a physiologically significant way with CD9 is the integrin $\alpha6\beta1$ (Chen et al., 1999; Miyado et al., 2000). However, eggs lacking $\alpha6\beta1$ fuse normally with sperm, so a role for this integrin in gamete fusion is in doubt (Miller et al., 2000).

The anti-CD9 monoclonal antibody KMC8 blocks sperm-egg fusion (Chen et al., 1999; Miller et al., 2000), and we found that KMC8 binds to constructs containing CD9 EC2, suggesting a role for EC2 in the fusion process. The EC2 constructs, preincubated with sperm, had no significant effect on fertilization rate or index, suggesting that either the constructs lack biological activity or that sperm do not have a receptor for CD9. The EC2 constructs when preincubated with eggs reduce fertilization rate and index, showing that the constructs do have biological activity. The data therefore suggest that CD9 acts in *cis* at the egg surface and that sperm lack a receptor acting as a *trans* partner for CD9.

Why do the EC2 constructs inhibit sperm-egg fusion when preincubated with eggs? The simplest interpretation is that the exogenously added EC2 can compete with endogenous CD9 for association with some key egg surface molecule(s). But the exogenous EC2, having associated and displaced endogenous CD9, cannot function to promote gamete fusion. The inability of the exogenous EC2 to exercise all CD9 functions may reflect the need for other CD9 regions for full function.

To allow further dissection of the basis of CD9 function, we tested if the fusion ability of CD9 KO oocytes could be restored after the injection of CD9 message. We found a high level of rescue of fusion ability by CD9 message injection. In the current experiments uninjected KO eggs usually did not fuse, and their fertilization rate was 0% (Table 2), though in one test uninjected KO eggs had a fertilization rate of 2%. KO eggs injected with wild-type CD9 mRNA fused at rates of 46-55%, far above the rate for uninjected KO eggs, but usually a little lower than the rate for uninjected wild-type eggs (66-83%).

We found that injection of KO eggs with wild-type mouse CD9 mRNA and wild-type human CD9 mRNA gave equivalent levels of rescue of fusion. Sequence comparison of EC2 of mouse and human CD9 shows they differ by 18 residues, including nine non-conservative substitutions, indicating that certain alterations of amino acids in the mouse EC2 structure are compatible with retention of full function. This result reveals the position of various EC2 residues where specific amino acid substitutions are not deleterious and serves as a control for mutations that do reduce function.

We found that the fertilization index obtained with CD9 null oocytes injected with mutant CD9-F174A-eGFP mRNA was reduced fourfold compared to wild-type CD9-eGFP mRNA. This result indicates that F174 plays a significant role in CD9 function in sperm-egg fusion. It also suggests that F174 might be part of a required site necessary for CD9 function. Further experiments showed that a change of three residues SFQ (173-175) to AAA essentially abolished the ability of CD9 message to restore fusion competence to CD9 KO eggs. This loss of activity is probably not due to misfolding of the mutant protein, and this conclusion is supported by three types of evidence (our unpublished data). (1) Several anti-mouse CD9 monoclonal antibodies including KMC8, 4.1F12, 1.2C4, 1.1F2 and 1.4G1 bind to the SFQ-to-AAA mutant as well as to wild-type CD9; (2) after expression in tissue culture cells, CD9 with the SFQ-to-AAA mutation associates as well as does wild-type CD9 with CD9 P-1, the major *cis* partner of CD9 in various cell types (Charrin et al., 2001; Stipp et al., 2001); (3) EC2 containing the SFQ-to-AAA mutation folds properly by biophysical criteria, e.g. it shows amounts of helical structure equivalent to wild-type by circular dichroism. Therefore, the absence of fusion activity in the SFQ-to-AAA mutant suggests that SFQ in the CD9 EC2 loop comprises a functional site, which may associate with and regulate the egg fusion machinery.

One reason to posit a close interaction of CD9 with an egg protein(s) acting in fusion is that in the in vitro fusion assay with CD9-null eggs, the number of sperm bound to the egg plasma membrane is the same as wild type (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000). In all the experiments here, addition of EC2 constructs and the mutations in CD9 did not affect the number of sperm bound. These results suggest that CD9 may interact with the egg fusion machinery downstream of sperm adhesion.

A possible model to explain our findings is that CD9 is associated with an egg fusion protein. The process of sperm binding to the egg plasma membrane triggers and promotes the formation and stability of an association between the SFQ site of CD9 and the egg fusion protein. The egg fusion protein, upon associating with CD9, changes conformation and specifically interacts with a sperm fusion protein. Thus, relying on a portion of CD9's association repertoire, an inter-gamete functional fusion machinery composed of CD9, the egg fusion protein and the sperm fusion protein, is established and promotes membrane fusion.

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REFERENCES

Almeida, E. A., Huovila, A. P., Sutherland, A. E., Stephens, L. E., Calarco, P. G., Shaw, L. M., Mercurio, A. M., Sonnenberg, A., Primakoff, P., Myles, D. G. and White, J. M. (1995). Mouse egg integrin alpha 6 beta 1 functions as a sperm receptor. *Cell* **81**, 1095-1104.

Berditchevski, F., Zutter, M. M. and Hemler, M. E. (1996). Characterization of novel complexes on the cell surface between integrins and proteins with 4 transmembrane domains (TM4 proteins). *Mol. Biol. Cell* **7**, 193-207.

Boucheix, C. and Rubinstein, E. (2001). Tetraspanins. *Cell Mol. Life Sci.* **58**, 1189-1205.

Charrin, S., Le Naour, F., Oualid, M., Billard, M., Faure, G., Hanash, S. M., Boucheix, C. and Rubinstein, E. (2001). The major CD9 and CD81 molecular partner. Identification and characterization of the complexes. *J. Biol. Chem.* **276**, 14329-14337.

Chen, H. and Sampson, N. S. (1999). Mediation of sperm-egg fusion: evidence that mouse egg alpha6beta1 integrin is the receptor for sperm fertilin beta. *Chem. Biol.* **6**, 1-10.

Chen, M. S., Tung, K. S., Coonrod, S. A., Takahashi, Y., Bigler, D., Chang, A., Yamashita, Y., Kincade, P. W., Herr, J. C. and White, J. M. (1999). Role of the integrin-associated protein CD9 in binding between sperm ADAM 2 and the egg integrin alpha6beta1: implications for murine fertilization. *Proc. Natl. Acad. Sci. USA* **96**, 11830-11835.

Cho, C., Bunch, D. O., Faure, J. E., Goulding, E. H., Eddy, E. M., Primakoff, P. and Myles, D. G. (1998). Fertilization defects in sperm from mice lacking fertilin beta. *Science* **281**, 1857-1859.

Evans, J. P. (2001). Fertilin beta and other ADAMs as integrin ligands: insights into cell adhesion and fertilization. *BioEssays* **23**, 628-639.

Higginbottom, A., Quinn, E. R., Kuo, C. C., Flint, M., Wilson, L. H., Bianchi, E., Nicosia, A., Monk, P. N., McKeating, J. A. and Levy, S. (2000). Identification of amino acid residues in CD81 critical for interaction with hepatitis C virus envelope glycoprotein E2. *J. Virol.* **74**, 3642-3649.

Kaji, K., Oda, S., Shikano, T., Ohnuki, T., Uematsu, Y., Sakagami, J., Tada, N., Miyazaki, S. and Kudo, A. (2000). The gamete fusion process is defective in eggs of Cd9-deficient mice. *Nat. Genet.* **24**, 279-282.

Kelic, S., Levy, S., Suarez, C. and Weinstein, D. E. (2001). CD81 regulates neuron-induced astrocyte cell-cycle exit. *Mol. Cell Neurosci.* **17**, 551-560.

Kitadokoro, K., Bordo, D., Galli, G., Petracca, R., Falugi, F., Abrignani, S., Grandi, G. and Bolognesi, M. (2001). CD81 extracellular domain 3D structure: insight into the tetraspanin superfamily structural motifs. *EMBO J.* **20**, 12-18.

Kola, I. and Sumarsono, S. H. (1996). Microinjection of in vitro transcribed RNA and antisense oligonucleotides in mouse oocytes and early embryos to study the gain- and loss-of-function of genes. *Mol. Biotechnol.* **6**, 191-199.

Lagaudriere-Gesbert, C., Le Naour, F., Lebel-Binay, S., Billard, M., Lemichez, E., Boquet, P., Boucheix, C., Conjeaud, H. and Rubinstein, E. (1997). Functional analysis of four tetraspans, CD9, CD53, CD81, and CD82, suggests a common role in costimulation, cell adhesion, and migration: only CD9 upregulates HB-EGF activity. *Cell Immunol.* **182**, 105-112.

Le Naour, F., Rubinstein, E., Jasmin, C., Prenant, M. and Boucheix, C. (2000). Severely reduced female fertility in CD9-deficient mice. *Science* **287**, 319-321.

Maecker, H. T., Todd, S. C. and Levy, S. (1997). The tetraspanin superfamily: molecular facilitators. *FASEB J.* **11**, 428-442.

Miller, B. J., Georges-Labouesse, E., Primakoff, P. and Myles, D. G. (2000). Normal fertilization occurs with eggs lacking the integrin alpha6beta1 and is CD9-dependent. *J. Cell Biol.* **149**, 1289-1296.

Miyado, K., Yamada, G., Yamada, S., Hasuwa, H., Nakamura, Y., Ryu, F., Suzuki, K., Kosai, K., Inoue, K., Ogura, A., Okabe, M. and Mekada, E. (2000). Requirement of CD9 on the egg plasma membrane for fertilization. *Science* **287**, 321-324.

Moller, C. C., Bleil, J. D., Kinloch, R. A. and Wassarman, P. M. (1990). Structural and functional relationships between mouse and hamster zona pellucida glycoproteins. *Dev. Biol.* **137**, 276-286.

Nakamura, K., Iwamoto, R. and Mekada, E. (1995). Membrane-anchored heparin-binding EGF-like growth factor (HB-EGF) and diphtheria toxin receptor-associated protein (DRAP27)/CD9 form a complex with integrin alpha 3 beta 1 at cell-cell contact sites. *J. Cell Biol.* **129**, 1691-1705.

Paynton, B. V., Ebert, K. M. and Brinster, R. L. (1983). Synthesis and secretion of ovalbumin by mouse-growing oocytes following microinjection of chick ovalbumin mRNA. *Exp. Cell Res.* **144**, 214-218.

Rendu, F., Boucheix, C., Lebret, M., Bourdeau, N., Benoit, P., Maclouf, J., Soria, C. and Levy-Toledano, S. (1987). Mechanisms of the mAb ALB6(CD9) induced human platelet activation: comparison with thrombin. *Biochem. Biophys. Res. Commun.* **146**, 1397-1404.

Rubinstein, E., Le Naour, F., Lagaudriere-Gesbert, C., Billard, M., Conjeaud, H. and Boucheix, C. (1996). CD9, CD63, CD81, and CD82 are components of a surface tetraspan network connected to HLA-DR and VLA integrins. *Eur. J Immunol.* **26**, 2657-2665.

Serru, V., Le Naour, F., Billard, M., Azorsa, D. O., Lanza, F., Boucheix, C. and Rubinstein, E. (1999). Selective tetraspan-integrin complexes (CD81/alpha4beta1, CD151/alpha3beta1, CD151/alpha6beta1) under conditions disrupting tetraspan interactions. *Biochem. J.* **340**, 103-111.

Stipp, C. S., Orlicky, D. and Hemler, M. E. (2001). FPRP, a major, highly stoichiometric, highly specific CD81- and CD9-associated protein. *J. Biol. Chem.* **276**, 4853-4862.

Williams, C. J., Mehlmann, L. M., Jaffe, L. A., Kopf, G. S. and Schultz, R. M. (1998). Evidence that Gq family G proteins do not function in mouse egg activation at fertilization. *Dev. Biol.* **198**, 116-127.