Direct Binding of the Ligand PSG17 to CD9 Requires a CD9 Site Essential for Sperm-Egg Fusion

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The function currently attributed to tetraspanins is to organize molecular complexes in the plasma membrane by using multiple *cis*-interactions. Additionally, the tetraspanin CD9 may be a receptor that binds the soluble ligand PSG17, a member of the immunoglobulin superfamily (IgSF)/CEA subfamily. However, previous data are also consistent with the PSG17 receptor being a CD9 *cis*-associated protein. In the current study, CD9 extracellular loop (EC2) specifically bound to PSG17-coated beads, indicating a direct interaction between the two proteins. However, CD9-EC2 did not bind to PSG17-coated beads if the CD9-EC2 had the mutation SFQ (173-175) to AAA, a previously studied mutation in egg CD9 that abolishes sperm-egg fusion. Also, PSG17 bound to 293 T cells transfected with wild-type CD9 but not the mutant CD9. By immunofluorescence, PSG17 bound to wild-type eggs but not to CD9 null eggs. The presence of ~2 μM recombinant PSG17 produced a significant and reversible inhibition (60–80%) of sperm-egg fusion. Thus, we conclude that CD9 is a receptor for PSG17 and when the PSG17 binding site is mutated or occupied, sperm-egg fusion is impaired. These findings suggest that egg CD9 may function in gamete fusion by binding to a sperm IgSF/CEA subfamily member and such proteins have previously been identified on sperm.

INTRODUCTION

Mammalian fertilization involves a series of complex cellular and biochemical processes, culminating in sperm-egg fusion and subsequent formation of an embryo. Despite its biological importance, fusion between the plasma membranes of the sperm and the oocyte is not well understood. Although there is general agreement on the concept that gamete fusion may involve the interaction of multiple complementary molecules on the sperm and the oocyte, only a few potentially relevant proteins have been identified.

A protein found on the mammalian egg surface that is known to be required for gamete fusion is CD9. CD9 belongs to the tetraspanin family of proteins, which are integral membrane proteins with four transmembrane domains and two extracellular domains (one short, one long) (Maecker et al., 1997). In mammals there are >30 tetraspanin family members, implicated in a variety of cellular and physiological processes, such as cell motility, cell aggregation, signaling, and cell fusion (Boucheix and Rubinstein, 2001; Hemler, 2001). Tetraspanins are believed to act as "molecular facilitators," grouping specific cell-surface proteins and thus increasing the formation and stability of functional protein complexes (Maecker et al., 1997). CD9 associates with a great variety of membrane proteins, such as membrane-anchored growth factors, integrins, members of the immunoglobulin superfamily (IgSF), and other tetraspanins (Boucheix and Rubinstein, 2001).

Conclusive evidence for a role of CD9 in gamete fusion was the finding that CD9 knockout females are infertile due to the inability of their oocytes to fuse with sperm (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000), but how CD9 acts in fusion remains unknown. It has been suggested that CD9 acts in sperm-egg fusion via an association with a β1 integrin on the egg that binds to ADAM proteins on the sperm, specifically ADAM 2 (fertilin β) and ADAM 3 (cyritestin) (Chen et al., 1999; Takahashi et al., 2001). However, experiments using gametes deficient for either the sperm ADAMs or the egg integrins, question the validity of this model. Sperm lacking fertilin β, cyritestin, or both proteins are still able to fuse with eggs (Cho et al., 1998; Shamsadin et al., 1999; Nishimura et al., 2001). Furthermore, it was recently shown that oocytes lacking all β1 integrins are fully functional in fusing with sperm in vitro and in vivo. Also function-blocking antibodies against other egg integrins (β3 and αv), when incubated with eggs lacking β1 integrins, do not inhibit sperm-egg fusion (He et al., 2003). These results indicate that none of the integrins known to be present on the oocyte is essential for sperm-egg fusion and egg integrins do not have redundant functions in the fusion process.

Because the proposed mechanism for CD9 function in sperm-egg fusion, acting through integrins, does not agree with these current results, an alternative model is required. Recently, PSG17, a member of the pregnancy-specific glycoprotein (PSG) family, has been suggested to be a CD9-ligand (Waterhouse et al., 2002). Pregnancy-specific glycoproteins

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belong to the carcinoembryonic antigen (CEA) subfamily of the immunoglobulin superfamily (IgSF) (Beauchemin *et al.*, 1999). PSGs are a group of proteins synthesized by the placenta and secreted into the maternal circulation. Several PSGs are known to stimulate secretion of cytokines by macrophages (Wessells *et al.*, 2000; Snyder *et al.*, 2001), and thus PSGs may contribute to preventing fetal rejection by the mother by activating the maternal innate immune system (Sacks *et al.*, 1999). PSG17 binds to macrophages with high affinity and the binding is mediated by CD9 (Waterhouse *et al.*, 2002). However, it is not known whether CD9 is the receptor itself or whether it functions as a coreceptor.

We report here that PSG17 binds directly to CD9 and that CD9 amino acid residue F 174 is essential for this interaction. As a CD9-ligand molecule, PSG17 interactions may give insights into the molecular mechanism underlying the role of CD9 in sperm-egg fusion. We found that PSG17 binds to eggs and inhibits gamete fusion, suggesting that CD9 may function in sperm-egg fusion by interacting with an IgSF protein, possibly a CEA subfamily protein. Thus, these results provide evidence for the potential involvement of an IgSF member in sperm-egg fusion and give rise to new ideas of how CD9 acts in this process.

MATERIALS AND METHODS

Cell Culture

Human embryonic kidney (HEK) 293T cells (Edge Biosystems, Gaithersburg, MD) were cultured in DMEM, 10% fetal bovine serum. BeWo cells (American Type Culture Collection, Manassas, VA) were cultured in F12K medium with 1.5 g/l sodium bicarbonate and 10% fetal bovine serum.

Plasmids

The construction of pGEX-CD9EC2, pCD9-eGFP, and pCD9-F174A-eGFP has been described previously (Zhu *et al.*, 2002). For the expression of glutathione *S*-transferase (GST)-CD9EC2-SFQ to AAA, the mutated template (Zhu *et al.*, 2002) was amplified by polymerase chain reaction and subcloned into *Eco*RI and *Bam*HI restriction sites of pGEX-3× (Amersham Biosciences, Piscataway, NJ). pCD9-SFQ to AAA (173-175)-eGFP was generated after digestion of the cDNA in pBluescript KS (Zhu *et al.*, 2002) with *Sal*I and *Sac*I and subcloned into the same sites in the pIRES2-eGFP vector (BD Biosciences Clontech, Palo Alto, CA).

Pull-Down Assays

The GST-mouse CD9 extracellular loop 2 (EC2) fusion protein was generated as described previously (Zhu *et al.*, 2002). Recombinant PSG17N-Myc-His includes the N-terminal immunoglobulin domain of PSG17 and binds with high affinity to CD9-expressing cells (Waterhouse *et al.*, 2002). Recombinant PSG17N-Myc-His purified as described in Waterhouse *et al.* (2002) was tested for its ability to bind to GST-CD9EC2 with the pull-down polyHis protein: protein interaction kit (Pierce Chemical, Rockford, IL) following the manufacturer's protocol. Briefly, 100 μg of purified PSG17N-Myc-His or two control proteins, recombinant green fluorescent protein (GFP)-His (Upstate Biotechnology, Lake Placid, NY) or CEACAM1a[1-4]-His (Zelus *et al.*, 1998; Beauchemin *et al.*, 1999), were incubated with the immobilized cobalt chelate gel overnight at 4°C. After five washes, 100 μg of GST-CD9EC2 (prey) or the same construct carrying the SFQ (173-175) to AAA mutation were added to the gel and incubated overnight with gentle rocking at 4°C. After seven washes, the proteins were eluted with 150 μl of 290 mM imidazole elution buffer. Twenty-five microliters of the eluted material was loaded on a 4–20% NuPAGE gel (Invitrogen, Carlsbad, CA), and the proteins were detected by immunoblotting with an anti-CD9 mAb, KMC8 (BD Pharmingen, Palo Alto, CA), or an anti-GST monoclonal antibody (mAb) (Santa Cruz Biotechnology, Santa Cruz, CA) followed by horseradish peroxidase-conjugated goat anti-rat antibody or goat anti-mouse antibody and the Super Signal chemiluminescent detection system (Pierce Chemical).

Detection of PSG17N-Myc-His Binding to Transfected 293T Cells by Enzyme-linked Immunosorbent Assay (ELISA)

The assays were performed as described previously (Waterhouse *et al.*, 2002). Briefly, HEK 293T cells were seeded in poly-L-lysine–coated 96-well plates and were transiently transfected with plasmid DNA by using LipofectAMINE

2000 (Invitrogen). At 48 h posttransfection, the cells were washed with binding buffer containing 0.01% sodium azide after which PSG17N-Myc-His (10 μg/ml) or no ligand was added to each well. After 1-h incubation, the cells were washed five times and binding of PSG17N-Myc-His was detected with horseradish peroxidase-conjugated anti-Myc mAb (Invitrogen) followed by trimethylbenzyl-peroxidase substrate (KPL) and 2 N H_2SO_4. The color change was quantitated at 450 nm in an ELISA reader.

Flow Cytometry

For detection of PSG17N-Myc-His binding to 293T cells transfected with pCD9-eGFP, pCD9-F174A-eGFP, and pCD9-SFQ to AAA (173-175)-eGFP, the cells were sequentially incubated with 10 μg/ml PSG17N-Myc-His, anti-Myc mAb, and phycoerythrine (PE)-labeled rat anti-mouse IgG1 (BD Pharmingen). Expression of wild-type CD9, and the mutated forms of CD9 on the surface of the transfected cells, was confirmed by staining with biotin-labeled anti-CD9 mAb, KMC8, followed by Cy-Chrome-labeled streptavidin (BD Pharmingen).

Gamete Isolation

Mature, cumulus-free oocytes were collected from superovulated 6–8-wk-old ICR female mice as described previously (Yuan *et al.*, 1997). To loosen the zona pellucida (ZP) the oocytes were treated with 30 μg/ml chymotrypsin (Sigma-Aldrich, St. Louis, MO) for 3 min at 37°C and 5% CO_2 in medium M199 (Invitrogen) containing 3.5 mM sodium pyruvate, 1000 IU of penicillin-streptomycin, and 0.3% bovine serum albumin (BSA) (Sigma-Aldrich) (M199*). The zonae pellucidae were then removed mechanically using a narrow bore pipette. The oocytes were washed through three 100-μl drops of fresh M199* and then incubated in M199* at 37°C and 5% CO_2 for 3 h before use. Sperm were collected from the cauda epididymis and vas deferens of 10–12-wk-old ICR males. Sperm were allowed to disperse in a 500-μl drop of M199* containing 3% BSA and then diluted 1:10 in 500 μl of M199* + 3% BSA and capacitated for 2 h at 37°C and 5% CO_2.

Egg Immunofluorescence

Wild-type or CD9-null zona-free oocytes were obtained as described above and incubated in 50 μg/ml PSG17N-Myc-His (~26 kDa) or XylE-His (~35 kDa) in M199* for 30 min at 37°C in a 5% CO_2 incubator. After three washes in M199*, the cells were incubated in anti-Myc antibody (Invitrogen) (10 μg/ml in phosphate-buffered saline [PBS]-0.4% BSA) for 1 h at room temperature. Oocytes were washed through three drops of PBS-0.4% BSA and then exposed to Oregon green-conjugated anti-mouse IgG secondary antibody (Molecular Probes, Eugene, OR) diluted 1:100 in PBS-0.4% BSA for 1 h at room temperature. After washing, oocytes were mounted on glass slides and visualized using a Zeiss Axiophot microscope.

In Vitro Fertilization Assay

Zona-free eggs were loaded with 4',6-diamidino-2-phenylindole dihydrochloride (Polysciences, Warrington, PA) at 10 μg/ml for 15 min at 37°C, 5% CO_2. After washing out excess dye, the eggs were incubated for 30 min at 37°C and 5% CO_2 in M199* alone (control) or M199* containing 50 μg/ml PSG17N-Myc-His (~2 μM). Oocytes were inseminated in droplets containing recombinant protein for the first set of in vitro fertilization experiments, or were washed in M199* alone, by using three successive 7-min incubations in 100-μl M199* drops at 37°C, 5% CO_2 before insemination, for the in vitro fertilization experiments testing the reversibility of the inhibition. Sperm were added at a final concentration of 1–3 × 10⁵ sperm/ml, and gametes were coincubated for 40 min at 37°C, 5% CO_2. The oocytes were then washed to release loosely bound sperm, fixed in 4% paraformaldehyde in PBS for 10 min, and mounted onto microscope slides. Sperm-egg fusion was scored by the fluorescent labeling of sperm nuclei by 4',6-diamidino-2-phenylindole transferred from the preloaded eggs. Two different parameters of fusion were calculated: fertilization rate is the percentage of oocytes with at least one fused sperm, and fertilization index is the mean number of fused sperm per egg.

Sperm Motility Assay

Capacitated sperm were incubated for 30 min at 37°C, 5% CO_2 in M199* alone (control) or containing 50 μg/ml PSG17N-Myc-His. Samples were placed on warm glass slides and analyzed using a light microscope. Cells showing progressive motility were counted as "motile," whereas cells with nonprogressive motility or no motility were considered as "immotile."

RESULTS

Direct Binding of PSG17 to CD9

Previous results demonstrated that PSG17 binds specifically to CD9-expressing cells (Waterhouse *et al.*, 2002), but it has been uncertain whether PSG17 binds to CD9 itself or to a CD9-associated protein. To test whether PSG17 binds di-
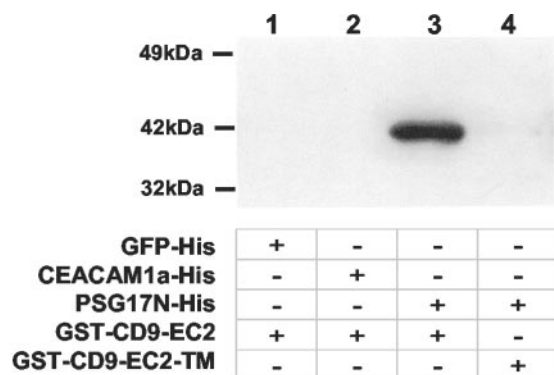
Figure 1. PSG17N binds to the extracellular loop 2 of murine CD9. Cobalt chelate beads were incubated with GFP-His (lane 1), CEACAM1a[1-4]-His (lane 2) or PSG17N-Myc-His (lanes 3 and 4) used as baits. The beads were then incubated with GST-CD9EC2 (lanes 1–3) or the same construct carrying the triple mutation SFQ (173-175) to AAA (GST-CD9EC2-TM) (lane 4). After several washes, the proteins were eluted with a buffer containing 290 mM imidazole, separated on a 4–20% NuPAGE gel, and detected by Western blot by using an anti-CD9 antibody.

Figure 2. Binding of PSG17N-Myc-His to CD9-transfected 293T cells. HEK 293T cells were transfected with empty plasmid (EP), pCD9-eGFP (wild-type CD9:WT-CD9), pCD9-F174A-eGFP (single mutant CD9: SM-CD9), or pCD9-SFQ to AAA (173-175)-eGFP (triple mutant CD9: TM-CD9). Forty-eight hours posttransfection, the cells were incubated with 10 μg/ml PSG17N-Myc-His and bound PSG17N-Myc-His was detected after treatment with HRP-conjugated anti-Myc mAb and TMB/peroxidase substrate. The data are expressed as mean absorbance $\pm$ SE. Each data point represents four identical wells and the experiment was repeated two independent times with similar results.

rectly to CD9, pull-down assays were performed. The recombinant N-terminal Ig domain of PSG17 containing both 6 $\times$ His and Myc epitope tags was bound through its His tag to cobalt beads. Two control proteins were used, eGFP-6 $\times$ His and CEACAM1a[1-4]-6 $\times$ His. CEACAM1a is closely related to PSG17; it is also an IgSF member and, like PSG17, belongs to the CEA subfamily. Recombinant CEACAM1a[1-4] has also been shown to have biological activity (Zelus *et al.*, 1998). The beads with bound protein were incubated with a fusion protein, including GST fused to the CD9 long extracellular loop (EC2) (GST-CD9 EC2). After extensive washing, the presence of CD9 on the beads was detected by Western blot by using an anti-CD9 antibody. GST-CD9 EC2 bound to PSG17-coated beads but not to the GFP-coated beads or to the CEACAM1a[1-4]-coated beads (Figure 1, lanes 1–3), showing that PSG17 binds directly to CD9-EC2.

PSG17 Binds to a Specific Site on CD9

It has been previously shown that amino acid residues 173–175 in CD9 EC2 are critical for CD9 function in sperm-egg fusion. When CD9 EC2 residue F174 is replaced by A, CD9 activity in gamete fusion is greatly reduced; when the three residues (173–175) are altered to AAA, the mutant CD9 has no activity in fusion (Zhu *et al.*, 2002). To ask whether this region of CD9 is also relevant for the binding of PSG17, a GST-CD9 EC2 construct with the triple mutation SFQ to AAA was expressed and used in the pull-down assay. In contrast to the wild-type construct, the mutated CD9 EC2 was not pulled down by PSG17-coupled beads (Figure 1, lane 4), suggesting that amino acid residues 173–175 are directly involved in binding to PSG17.

We also evaluated whether mutations in CD9 would affect PSG17 binding when CD9 was expressed in a cell membrane. We compared by ELISA the binding of PSG17 to HEK 293T cells that had been transfected with either wild-type CD9 or mutant CD9. Two CD9 mutations were used, the triple mutant, residues 173–175 SFQ to AAA, mentioned above, or the single mutant, residue F174 to A. Compared with control cells transfected with empty plasmid, wild-type CD9 transfected cells bound about five times higher levels of
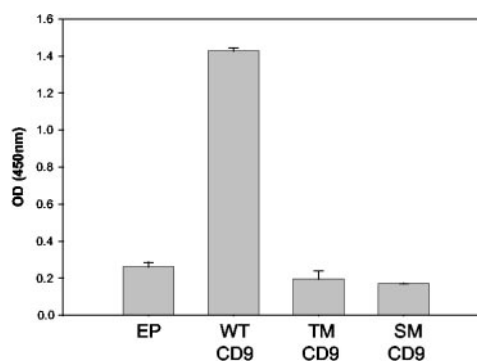
PSG17 (Figure 2). Cells transfected with CD9 with the triple mutation (SFQ 173–175 to AAA) or CD9 with the single mutation (F 174 to A) bound PSG17 at the same level as the control (empty plasmid) transfected cells. Cells transfected with any of the three constructs showed similar surface staining by immunofluorescence by using the anti-CD9 KMC8 antibody (our unpublished data).

Similar results were obtained using fluorescence-activated cell sorting (FACS) analysis of PSG17 binding to CD9-transfected 293T cells. 293T cells were transfected with plasmids coding for wild-type or mutant CD9 fused to enhanced green fluorescent protein (eGFP). Transfected cells were incubated with PSG17-N-Myc-His and stained with an anti-Myc antibody followed by a secondary antibody coupled to PE. Expression levels of the CD9-eGFP fusion proteins were evaluated by the fluorescence of eGFP and PSG17 binding was quantified by PE fluorescence. Results indicated that eGFP and PE-labeled double positive cells constituted 37% of the forward versus side scatter-gated pCD9-eGFP transfected cells (Figure 3). In contrast, only 0.4% of the gated cells were eGFP and PE double positive when cells were transfected with pCD9-F174A-eGFP. Results similar to those with the F174A mutant were obtained upon transfection of cells with CD9-SFQ to AAA (173–175)-eGFP encoding plasmid (our unpublished data). Together, these results show that PSG17 binds to CD9 and that F174 is critical for the interaction with PSG17.

Binding of PSG-17 to Eggs and Inhibition of Sperm-Egg Fusion

Considering the essential role CD9 has in sperm-egg fusion, and in light of the evidence above supporting a direct interaction between PSG-17 and CD9, we tested whether PSG17 binds to eggs and affects gamete fusion. The presence of CD9 on the eggs has been demonstrated (Chen *et al.*, 1999). However, CD9 is known to have multiple *cis*-partners and the complement of CD9 *cis*-partners may be cell type specific, so the accessibility of the PSG17 binding site on the egg could be different from that on macrophages or HEK 293 cells. To
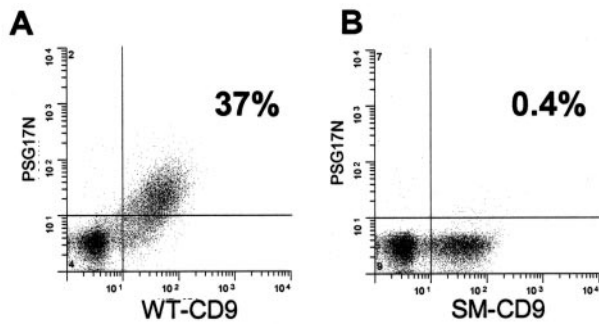
Figure 3. FACS analysis of PSG17N-Myc-His binding to HEK 293T wild-type and mutated CD9-transfected cells. HEK 293T cells were transfected with pCD9-eGFP (wild-type CD9: WT-CD9) (A) or pCD9-F174A-eGFP (single mutant CD9: SM-CD9) (B), after which they were sequentially incubated with 10 μg/ml PSG17N-Myc-His, anti-Myc mAb, and PE-labeled rat anti-mouse IgG1.

explore whether PSG17 is able to bind to eggs, zona-free oocytes were incubated with PSG17N-Myc-His and then examined by indirect immunofluorescence by using an anti-Myc antibody. Oocytes incubated in the presence of a control His-tagged protein (XylE) showed no fluorescence (Figure 4, A and B), whereas oocytes incubated with PSG17 displayed a bright staining (Figure 4, C and D). To confirm that PSG17 binding is CD9-dependent, we examined whether CD9 null eggs were able to bind PSG17 and found no staining in CD9 KO eggs (Figure 4, E and F).

Next, we asked whether binding of PSG17 to CD9 would affect the ability of eggs to fuse with sperm. Zona-free eggs were preincubated with PSG17, and then inseminated with capacitated sperm. After a coincubation period of 40 min, the percentage of fertilized eggs and the mean number of fused sperm per egg were determined. Oocytes preincubated with PSG17 had a significantly lower fertilization rate (58% inhibition) and fertilization index (83% inhibition) than control oocytes (Figure 5). Sperm motility was unaffected by the presence of PSG17 (61% motility with PSG17, 58% motility in the absence of PSG17). Immunofluorescence experiments showed that sperm did not bind PSG17 during coincubation with the eggs, suggesting that the oocyte is the targeted gamete. To study whether the oocyte was blocked in a reversible or irreversible manner, oocytes were incubated with PSG17, washed, and then inseminated with capacitated sperm. Eggs, exposed to PSG17 and then washed, had a fertilization rate and a fertilization index significantly higher than those of the nonwashed oocytes, and not significantly different from the values obtained for control oocytes (Figure 6). These results indicate that the inhibitory effect could be reversed by PSG17 removal and argue against a toxic effect of the protein preparation on the oocytes.

DISCUSSION

Tetraspanins are known to associate with a variety of different molecules, such as membrane-anchored growth factors, integrins, other tetraspanins, and members of the IgSF. A key feature of these reported molecular interactions is that all of them occur in *cis*, i.e., between the tetraspanin and other transmembrane proteins anchored in the same lipid bilayer (Maecker *et al.*, 1997; Boucheix and Rubinstein, 2001). One *trans*-interaction has been reported for a tetraspanin, the association between CD81 and the Hepatitis C virus enve-

Figure 4. Binding of PSG17N-Myc-His to the oocyte. Zona-free eggs from wild-type (A–D) or CD9 knockout animals (E and F) were incubated in 50 μg/ml XylE (a His-tagged control protein) (A and B) or PSG17N-Myc-His (C–F) for 30 min, and stained by indirect immunofluorescence by using an anti-Myc antibody, followed by Oregon Green-conjugated secondary antibody.

lope glycoprotein E2. There is evidence indicating that the tetraspanin CD81 interacts in vitro with the virus protein (Flint *et al.*, 1999), and key amino acids in CD81 for this
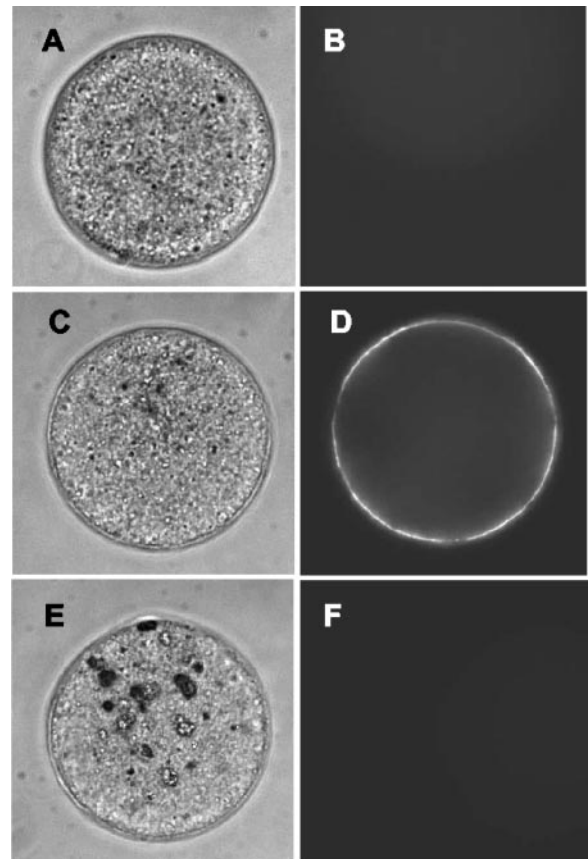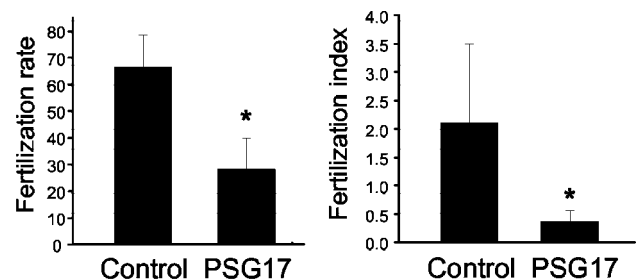

Figure 5. Inhibition of sperm-egg fusion in the presence of PSG17N-Myc-His. Zona-free eggs were incubated for 30 min in medium (Control) or in medium containing PSG17N-Myc-His (50 μg/ml), and then inseminated with capacitated sperm. After coincubating the gametes for 40 min, oocytes were washed and the fertilization rate (number of fertilized eggs/total number of eggs) and the fertilization index (mean number of sperm fused per egg) were scored. Data represents the mean value ± SE from four independent experiments. *$p < 0.05$ compared with control.
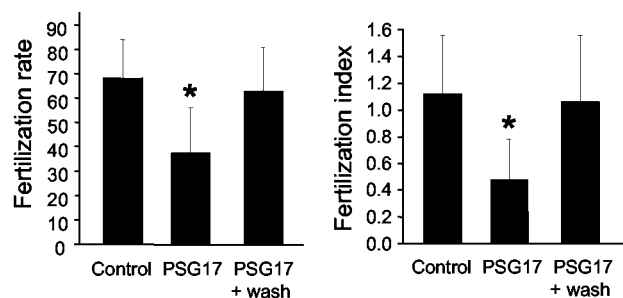
Figure 6. Reversibility of sperm-egg fusion inhibition by PSG17N-Myc-His. Zona-free eggs were incubated for 30 min in medium containing PSG17N-Myc-His (50 μg/ml), washed through 3 drops of medium, and then inseminated with capacitated sperm (PSG17 + wash). Control groups consisted of oocytes incubated in medium (Control), or in the presence of PSG17N-Myc-His without its subsequent removal by washing (PSG17). After coincubating the gametes for 40 min, oocytes were washed and the fertilization rate and the fertilization index were scored. Data represents the mean value ± SE from three independent experiments. *p < 0.05 compared with control and to PSG17 + wash.

interaction have been identified within the EC2 loop (Flint *et al.*, 1999; Drummer *et al.*, 2002). Nevertheless, the interaction between CD81 and E2 does not seem to mediate virus entry (Petracca *et al.*, 2000; Takikawa *et al.*, 2000) and there are other molecules proposed to function as Hepatitis C virus receptors (Germi *et al.*, 2002; Scarselli *et al.*, 2002), so the biological significance of the CD81–E2 interaction is uncertain.

Previous work implicated CD9 in the binding of an external ligand, PSG17, to macrophages (Waterhouse *et al.*, 2002). One of the aims of the present study was to determine whether CD9 mediates the binding of PSG17 directly or, on the contrary, whether it functions in an indirect way, enhancing the binding of PSG17 to a CD9-associated protein. Results of pull-down experiments, carried out with the purified recombinant proteins, indicate PSG17 binds directly to the EC2 loop of CD9, thus confirming that CD9 is the actual receptor for PSG17. Because PSG17 is the first molecule known to be a biological ligand for a tetraspanin, the association between PSG17 and CD9 represents an unexpected, novel interaction in which the tetraspanin is a receptor.

The EC2 loop of tetraspanins, particularly the stretch of residues preceding the most C-terminal cysteine, is a region in which tetraspanin-associated proteins bind (Stipp *et al.*, 2003). This region in CD9 is also the location of CD9 mutations that affect gamete fusion. A striking feature of our data is that the same CD9 mutations that affect CD9 activity in gamete fusion abolish CD9-PSG17 binding. In previous work, we found that CD9 with F (174) mutated to A has greatly reduced activity in gamete fusion and CD9 with SFQ (173–175) mutated to AAA is no longer active in gamete fusion (Zhu *et al.*, 2002). In our current results, use of the F to A or SFQ to AAA mutants indicate that these residues are also essential for CD9 binding to PSG17. A possible trivial explanation for these results could be that misfolding of CD9 is produced by the amino acid changes. However, substantial data indicate that the mutated protein (SFQ to AAA) has correct folding (Zhu *et al.*, 2002), and therefore it is likely that CD9 residues SFQ 173-175 represent part of a PSG17 binding site.

Alternative explanations exist for how soluble PSG17 might inhibit gamete fusion. Because PSG17 binding ini-

tiates signal transduction in macrophages, it is possible that PSG17 binding to egg CD9 produces a similar effect that might trigger egg activation. However, oocytes exposed to PSG17 and then washed, showed a fertilization rate and index similar to control oocytes, indicating that the inhibition is reversible, and therefore arguing against an induction of egg activation by PSG17. Another possibility is that the binding of PSG17 inhibits gamete fusion by displacing a CD9 *cis*-partner from its normal association with CD9. The two most abundant and tightly associated CD9 *cis*-partners in tissue culture cells are both members of the EWI subfamily of the IgSF, EWI-F, and EWI-2 (Charrin *et al.*, 2001; Stipp *et al.*, 2001a,b). Given the fact that CD9 associates in *cis* with these IgSF members and that PSG17 is also an IgSF member, one can speculate that PSG17 disrupts the *cis*-association between CD9 and egg EWI-F or EWI-2. However, the EWI subfamily and the CEA subfamily (of which PSG17 is a member) have relatively little sequence relationship aside from both possessing Ig domains, making this hypothesis less attractive. Further exploration of this possibility will require the identification of CD9-associated proteins in the oocyte and their binding site(s) in CD9.

Another explanation of our findings is that egg CD9 may bind in *trans* to a PSG17-related ligand present on the sperm surface. When the CD9 SFQ site is mutated or already occupied by soluble PSG17, the sperm surface ligand cannot bind to egg CD9 and this essential step in gamete fusion is blocked. Relevant to this interpretation are two separate findings in our previous article (Zhu *et al.*, 2002). One was that soluble CD9-EC2 when preincubated with eggs, inhibits gamete fusion, indicating that CD9-EC2 interacts with another egg surface protein. A second finding was that the SFQ sequence in CD9 is required for fusion, but the issue of SFQ's acting in *trans* or *cis* was not addressed. If CD9 has a *trans*-interaction, we would propose it is in addition to the *cis*-interaction indicated by the previous finding.

Although CD9-EC2 inhibits gamete fusion when preincubated with eggs, it has no effect on fusion when preincubated with sperm. We previously suggested this could mean that egg CD9 does not bind to sperm (Zhu *et al.*, 2002), but other explanations of this result are possible. For instance, a sperm *trans*-ligand for CD9 may be inactive or inaccessible until after initial steps in sperm-egg adhesion occur and CD9 is positioned to interact with the *trans*-ligand. Once these adhesion steps occur, the *trans*-ligand becomes activated or accessible to bind the egg surface CD9 in preference to the soluble CD9-EC2.

A sperm *trans*-ligand for CD9 might be a membrane-associated form of PSG17 or a related CEA member. A CEA protein has been identified on the sperm surface and named "sperad." Sperad, initially called AH-20 (Primakoff and Myles, 1983), has been described in guinea pig sperm (Quill and Garbers, 1996). Relevant to sperad's biological function, monoclonal antibodies G3 and G11 stained the equatorial region of acrosome-reacted guinea pig sperm and were able to completely inhibit the fusion of guinea pig sperm with hamster oocytes (Allen and Green, 1995). Sperad was recently reported to be the protein recognized by antibodies G3 and G11 (Ilayperuma, 2002, 2003). Thus, current findings include 1) CD9 is required for sperm-egg fusion; 2) CD9 binds PSG17, a member of the CEA subfamily, and PSG17 inhibits sperm-egg fusion; and 3) there is a CEA protein on sperm that has been implicated in sperm-egg fusion. Together, these results support the idea that CD9 may function in gamete fusion by binding to a sperm CEA protein.

During recent years models for gamete fusion have focused on an adhesion role of a sperm ADAM(s) binding to an egg integrin(s) and CD9 was implicated as facilitator of this interaction (Takahashi *et al.*, 2001; Evans, 2002). Recent data have raised doubts about the participation of ADAMs and integrins in sperm-egg fusion (Primakoff and Myles, 2002; He *et al.*, 2003), although CD9 is clearly required. Our current findings suggest the participation in gamete fusion of IgSF proteins that bind to CD9. In this study we found that CD9 is a receptor for an IgSF/CEA subfamily ligand, PSG17, which binds to a CD9 site, including residues SFQ 173-175, known to be an active site for gamete fusion. Further work should reveal whether during gamete fusion the egg SFQ site binds an IgSF/CEA ligand on the sperm surface and/or is essential for CD9 *cis*-interactions in the egg plasma membrane.

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