Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 311 (2003) 208–214
BBRC
www.elsevier.com/locate/ybbrc

Structural requirements for the inhibitory action of the CD9 large extracellular domain in sperm/oocyte binding and fusion

Adrian Higginbottom,[a] Yuji Takahashi,[b] Laura Bolling,[b] Scott A. Coonrod,[b] Judith M. White,[b] Lynda J. Partridge,[c] and Peter N. Monk[a,*]

[a] *Department of Neurology, University of Sheffield Medical School, Sheffield S10 2RX, UK*
[b] *Department of Cell Biology, University of Virginia Health System, School of Medicine, Charlottesville, VA 22908, USA*
[c] *Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2TN, UK*

Received 26 September 2003

Abstract

CD9 has been shown to be essential for sperm/oocyte fusion in mice, the only non-redundant role found for a member of the tetraspanin family. CD9 can act in *cis*, reconstituting sperm/oocyte fusion when ectopically expressed in oocytes from CD9 null mice, or in *trans*, inhibiting sperm fusion when the large extracellular domain (LED) is added to CD9-positive oocytes as a soluble protein. In contrast to *cis* inhibition, the structural requirements of the *trans* inhibition by soluble CD9 LED are unknown. Here we show that human CD9 LED is as potent an inhibitor as mouse CD9 LED in mouse sperm/oocyte fusion assays and that CD9 LED can also inhibit sperm/oocyte binding. The two disulphide bridges that define membership of the tetraspanin family are critical for structure and function of human CD9 LED and mutation of a pentapeptide sequence in the hypervariable region further defines the critical region for *trans* inhibition.

Keywords: Sperm; Oocyte; Fusion; CD9; Tetraspanins

The tetraspanin family of membrane proteins consists of more than 30 members in mammals [1] and is conserved amongst multicellular organisms, including filamentous fungi [2], *Caenorhabditis elegans* [3], and *Drosophila* [4,5]. Tetraspanins have four transmembrane domains, with short N and C-termini, two short intracellular loops, and a small and a large extracellular domain (LED). The characteristic features of members of this family are 4–6 conserved Cys residues in the LED and polar residues in the transmembrane domains [6]. The functions of the tetraspanins remain largely obscure, although their ubiquitous expression on animal cells suggests a fundamental role in the cell biology of multicellular organisms. However, the only essential role fulfilled by a tetraspanin has recently been shown to be in mammalian fertility [7–11] where the CD9 nullizygous mouse is rendered infertile because sperm are able to bind to, but not fuse with, oocytes. Injecting human or mouse CD9 mRNA to $CD9^{-/-}$ oocytes can restore sperm fusion, indicating that egg development is normal and that CD9 acts at a late stage in the fusion process [12,13]. It has been shown that soluble CD9 LED protein can also inhibit the fusion event at very high concentrations, although there is no apparent binding to the sperm surface [12]. This suggests that CD9 is not a receptor for a sperm surface ligand, but acts on another oocyte molecule to form a fusion-competent structure.

In this report, we have for the first time analysed the structural requirements for the *trans* function of soluble human CD9 LED in mouse sperm/oocyte fusion assays, using LED mutated at a pentapeptide sequence in the putative association site and the Cys residues that characterise the tetraspanin family.

Experimental

Generation of mutant CD9 large extracellular domains. All PCRs were performed using Extensor Hi-Fidelity PCR Master mix (AB

* Corresponding author. Fax: +44-114-226-1201.
E-mail address: p.monk@shef.ac.uk (P.N. Monk).

0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2003.09.196

gene). Full-length human CD9 and CD81 cDNA templates were kind gifts from D. Azorsa and S. Levy, respectively; full-length human CD63 cDNA template was generated as previously described [14]. CD9 $Cys^{152,153,167,181}$ mutants were initially generated as full-length cDNA mutants using the *Muta-Gene Phagemid In Vitro Mutagenesis* kit (Bio-Rad Laboratories). The LED was then produced using the appropriate 5′ and 3′ primers. The Thr^{175}, Phe^{176}, Thr^{177}, Val^{178}, and Lys^{179} mutants were generated by overlap extension PCR. Authenticity of all constructs was verified using the Big Dye terminator sequencing method, analysed on an ABI Prism 373 sequencer.

Expression and purification of soluble tetraspanin extracellular domains. Tetraspanin LED GST fusion proteins were expressed in BL21 codon-plus *Escherichia coli* transfected with the appropriate cDNA cloned into pGEX-KG. Briefly, a single colony overnight culture was used to inoculate a 2 L LB culture flask. Cultures were grown at 37 °C to an OD_{600} of 0.5 before inducing with IPTG to a final concentration of 0.1 mM for 4 h. Cells were pelleted, washed, and lysed with Bugbuster (Novagen) containing a protease inhibitor cocktail. Recombinant protein was purified using a single step affinity chromatography procedure on glutathione beads according to manufacturer's procedure (Amersham Bioscience) and extensively dialysed against PBS, pH 7.2. The concentration of protein was measured using Bradford assay and optical density. Protein purity was assessed using Coomassie and silver staining of SDS–PAGE gels.

Protein analysis. Proteins were separated on 15% SDS–PAGE gels run under non-reducing conditions and analysed by Coomassie staining or Western blot analysis using conformation-sensitive anti-tetraspanin LED mAb (CD9: Alma1, a generous gift of F. Lanza; CD63: H5C6 (Developmental Studies Hybridoma Bank), CD81: 1D6, a generous gift of S. Levy). ELISAs were performed using Maxisorb microtitre plates (Nunc) coated with fusion proteins at 10 μg/ml. After blocking with 5% milk protein conformationally correct LED was detected using appropriate mAb and secondary antibody.

Sperm/oocyte fusion assays. Procedures for mouse in vitro fertilisation are essentially as described [7]. Zona-free oocytes prelabelled with Hoechst dye were preincubated for 30 min with the indicated amounts of GST-LED construct or FAB fragment in a 50 μl drop. Five microlitres of sperm was then added and the samples were incubated for a further 40–50 min in a 37 °C 5% CO_2 incubator. The oocytes were washed, mounted on a slide, and observed by fluorescence microscopy. The average number of sperm bound per oocyte, the average number of decondensed sperm per oocyte (fusion index), and the percentage of oocytes with fused (decondensed) sperm (fusion rate) were calculated. Percentage inhibition was calculated for each sample (10–15 oocytes per sample) relative to values obtained in duplicate samples pretreated with 30 μg/ml GST or 5 μg/ml GST-CD63-LED.

Statistical analysis. Analyses of significance were performed using the GraphPad software package, using unpaired *t* tests with Welch's correction for unequal variance applied where appropriate.

Results and discussion

Mouse and human CD9 large extracellular domains inhibit sperm/oocyte fusion

Female CD9 nullizygous mice are infertile because of a defect in the fusion of sperm with the oocyte [9–11]. Previous publications have demonstrated that transfection of CD9 null oocytes with human or mouse CD9 and CD81 can partially reconstitute the sperm fusion machinery [12,13], and that a soluble form of the mouse CD9 large extracellular domain can inhibit fusion in normal mouse oocytes [12]. We have previously shown that an intact anti-mCD9 mAb, JF9, can inhibit both sperm/oocyte binding and fusion although with different concentration dependencies [15]. Monomeric Fab fragments of JF9 can inhibit only sperm/oocyte fusion, with no significant effect on binding (data not shown), suggesting that inhibition of fusion is largely independent of sperm binding and does not involve CD9 internalisation, steric hindrance or Fc receptor interactions. To further elucidate the role of CD9 in fusion, we tested the activity of mouse (Figs. 1A–C) and human (Figs. 1D–F) CD9 LED domains fused to GST. The binding of sperm was not significantly inhibited by mCD9 LED (Fig. 1A), relative to the control protein GST (30 μg/ml), although there was a clear dose-response to the GST-mCD9 LED. In contrast, the fusion of sperm with oocyte was significantly inhibited, whether measured as fusion index (Fig. 1B) or fusion rate (Fig. 1C). GST control protein at 30 μg/ml had only a very small effect on these parameters, whereas a low dose of GST-mCD9 LED (1 μg/ml) could inhibit 40% of sperm fusion events. Interestingly, this percentage inhibition did not increase at higher doses of mCD9 LED. Human CD9 LED had similar effects in this experimental system. However, sperm binding to oocytes was significantly inhibited by 30 μg/ml hCD9 LED (Fig. 1D). hCD9 gave significant inhibition of fusion at 0.3 μg/ml, with some inhibition noticeable even at 0.1 μg/ml (Figs. 1E and F). This exquisite sensitivity of fusion to inhibition by CD9 LED is in marked contrast to the report of Primakoff and coworkers [12], that showed inhibition only at 250 μg/ml mCD9 LED and may reflect differences in the folding state of the recombinant protein after purification. In additional experiments, the GST was cleaved and removed by affinity chromatography. Interestingly, cleaved hCD9 LED appeared less active (data not shown), suggesting that the GST contributes to the stability of CD9 LED or that GST alone can weakly inhibit fusion. Alternatively, the propensity of GST to form dimers could be driving multimerisation of GST-CD9 LED [16], leading to modulation of inhibitory activity.

Human CD9 and CD81 but not CD63 large extracellular domains can inhibit sperm/oocyte fusion

Since it has been suggested that CD81 may also contribute to the fusion event [13], we tested GST-hCD81 LED and GST-hCD63 LED in the mouse sperm/oocyte fusion system. Human CD63, a largely intracellular protein, was included as a control tetraspanin domain. At 5 μg/ml, the hCD81 and hCD9 LED proteins did not inhibit sperm binding to a greater extent than GST alone (Fig. 2A). However, relative to GST-hCD63 LED, significant inhibition of binding occurred in the presence of GST, GST-hCD81 LED, and GST-hCD9 LED. This is in contrast to a

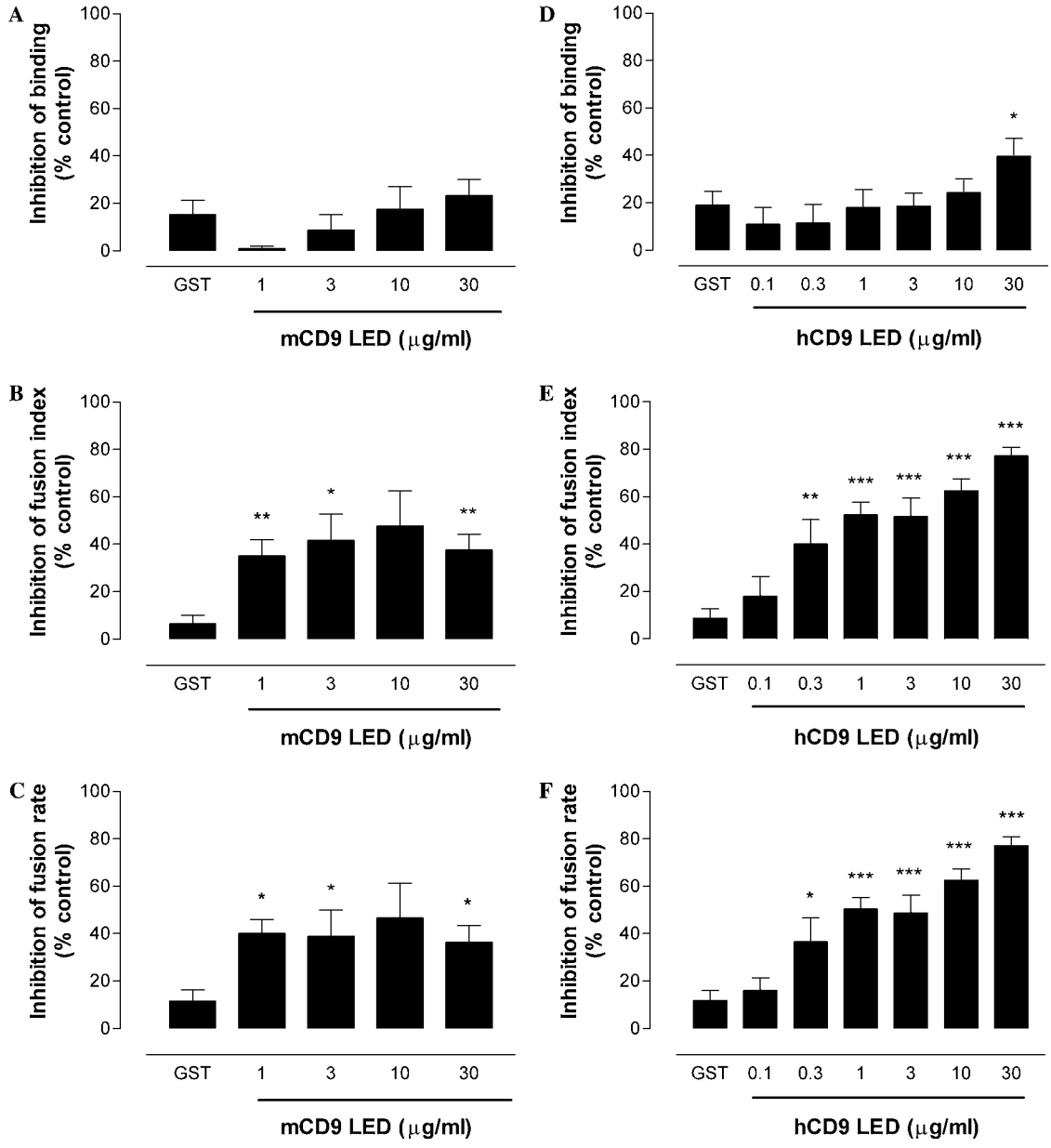

Fig. 1. Effects of mouse and human CD9 large extracellular domain protein on sperm/oocyte binding and fusion. Zona free mouse oocytes (10–15 per sample) prelabelled with Hoechst dye were treated with the indicated amounts of mouse (mCD9 LED) or human (hCD9 LED) CD9 large extracellular domain-GST fusion protein or control (GST) prior to the addition of mouse sperm. By fluorescence microscopy, the mean number of sperm bound per oocyte (A,D), the mean number of oocytes with fused sperm (B,E), and the percentage of oocytes with fused sperm (C,F) were counted. Inhibition was calculated as a percentage of no protein addition values, mean ± SEM of 2–9 separate determinations performed in duplicate. Significantly different from GST control values: $*P < 0.05$; $**P < 0.005$; and $***P < 0.001$; unpaired t test.

previous report on the activity of mCD9 LED [12] in which only small, insignificant effects on sperm binding were noted. This may have been due to the low proportion of active GST-mCD9 LED present and the fact that only GST was used as a control protein. The high activity of GST alone in the binding assay may be a non-specific effect, perhaps due to an exposed hydrophobic surface of GST that is protected in the fusion proteins. Sperm binding is clearly not the rate-limiting step in fertilisation but the biphasic inhibitory effect of hCD9 LED on fusion (Fig. 1E) may be explained by an increased inhibition of sperm binding in conjunction with a direct effect on the fusion process. In fusion assays, only GST-hCD9 LED had a significant inhibitory effect relative to GST GST-hCD63 although GST-hCD81 LED did have a significant effect relative to GST (Fig. 2B). GST-hCD63 LED appears to be a better control protein than GST alone and so was used as control in all subsequent experiments with GST-LED proteins.

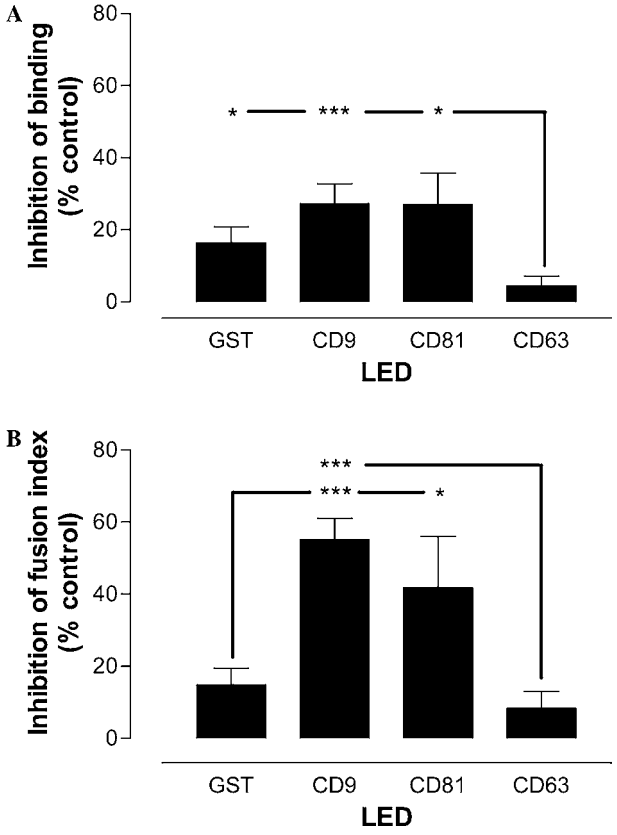

Fig. 2. Effects of human CD9, CD81, and CD63 large extracellular domain protein on sperm/oocyte binding and fusion. Zona free mouse oocytes (10–15 per sample) prelabelled with Hoechst dye were treated with the indicated amounts of human large extracellular domain-GST fusion protein (LED) or control (GST) prior to the addition of mouse sperm. By fluorescence microscopy, the mean number of sperm bound per oocyte (A) and the percentage of oocytes with fused sperm (B) were counted. Inhibition was calculated as a percentage of no protein addition values, mean ± SEM of 4 (CD81, CD63) or 13 (GST, CD9) separate determinations performed in duplicate. Significantly different from either GST or CD63 LED control values: $*P < 0.05$; $***P < 0.001$; unpaired t test with Welch's correction.

Reconstitution studies in CD9-null mouse eggs using mCD9 mRNA show that sites within the hypervariable region of the CD9 LED are involved in sperm/oocyte fusion [12]. However, this has not been previously shown for the inhibitory activity of exogenous CD9 LED protein. To determine the role of the hypervariable region we synthesised peptide analogs of this domain from mCD9 LED between Cys^{167} and Cys^{181}, the analogous region in mCD81 LED and, as a control, mCD63 between Cys^{177} and Cys^{198}. Under oxidising conditions (i.e., when the peptide is likely to be cyclised by formation of a disulphide linkage), the sperm/oocyte fusion index was inhibited by $60.8\% \pm 15.9$, $n = 8$, $36.8\% \pm 17.4$, $n = 6$, and $3.0\% \pm 1.92$, $n = 4$ (mean ± SEM) at 200 μg/ml CD9, CD81, and CD63 peptides, respectively. The CD9 but not the CD81 peptide had a significant inhibitory effect on the fusion index relative to the CD63 peptide (unpaired t test with Welch's correction, $P = 0.0087$ and $P = 0.11$, respectively), confirming the result that CD81 LED is a less potent inhibitor of fusion than CD9 LED.

Cysteines in the CD9 large extracellular domain are critical for structure and function

As soluble hCD9 LED is clearly able to inhibit sperm/oocyte fusion, we exploited this assay system to test the role of the two disulphide bridges. These are purported to be essential for the stability of the LED but that has not so far been examined in CD9. The four Cys residues (Cys^{152}, Cys^{153}, Cys^{167}, and Cys^{181}) were individually mutated to Ala, an amino acid of similar size and polarity to Cys, and the soluble GST LED proteins were expressed and purified. High levels of the mutant proteins were expressed and shown to be of high purity by SDS–PAGE (Fig. 3A, upper panel). The lower bands are typical of GST-tetraspanin LED fusion proteins and are probably cleavage products having lost some or all of the LEDs and so possess no immunoreactivity in Western blots. However, none of the Cys mutants could be detected by Western blot (Fig. 3A, lower panel), although wt hCD9 LED was strongly immunoreactive. It is unlikely that this is due to an increased sensitivity of the epitope to the denaturing conditions of SDS–PAGE, since Cys mutants were also not detected in a non-denaturing ELISA (Fig. 3B), whereas wt hCD9 LED was again strongly immunoreactive. The Cys mutants were tested for inhibitory activity in the sperm/oocyte assay at 5 μg/ml; unfortunately two of the mutants, Cys^{167}Ala and Cys^{181}Ala, formed insoluble precipitates at this concentration and were not studied further. However, as with hCD81 LED [17], we would predict that disulphide links are formed in CD9 LED by Cys^{152}–Cys^{181} and Cys^{153}–Cys^{167}. Thus, the use of mutants Cys^{152}Ala and Cys^{153}Ala allows the assessment of function in the absence of correct disulphide bridge formation. In these experiments, hCD9 LED gave strong inhibition of sperm binding relative to a GST-hCD63 LED control (Fig. 4A). This did not occur with either of the Cys mutants tested which is a clear demonstration that inhibition of binding is highly specific to conformationally correct hCD9 LED. Although wt hCD9 LED was able to inhibit 40% of sperm fusion (Fig. 4B), the Cys mutants were only weakly active, inhibiting fusion by only ~5–10%, similar to that observed with hCD63 LED. In separate experiments, Cys^{153}Ala was used at concentrations of up to 30 μg/ml, but still produced no inhibition (data not shown).

The hypervariable region of the CD9 large extracellular domain is critical for function

The mCD9 LED synthetic peptide sequence contains Phe^{174}, analogous to a key residue in the HCV binding

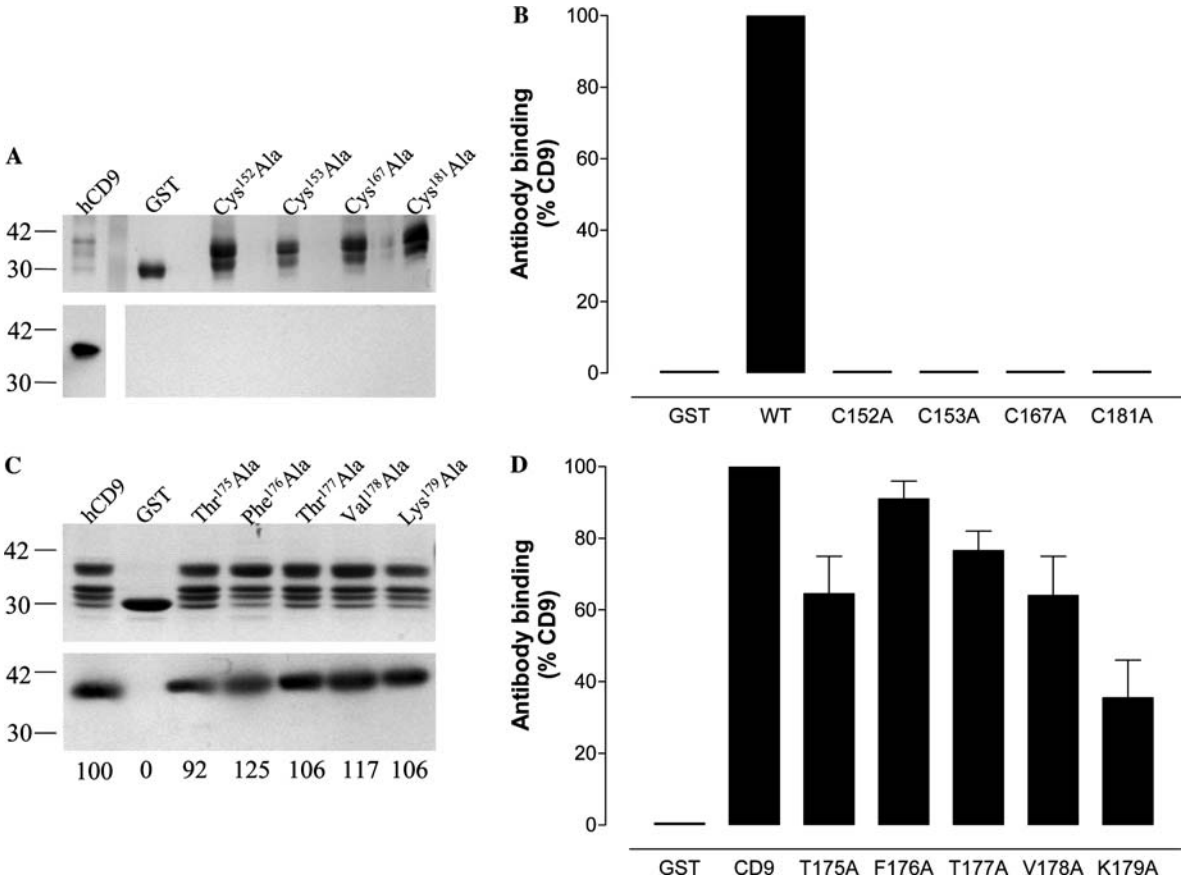

Fig. 3. Expression and antibody recognition of mutant forms of human CD9 large extracellular domain. Following expression and purification, the purity and conformational integrity of wild type and mutant human CD9 large extracellular domains fused to GST were assessed by Western blot (A,C) or ELISA (B,D). After electrophoresis in 15% polyacrylamide gel proteins were stained with Coomassie blue (top panels, A,C) or transferred to nitrocellulose and detected using anti-human CD9 mAb, ALMA1 (lower panels A,C). Although a number of bands are visible on polyacrylamide gel following Coomassie blue staining, only a single band is detected by ALMA1, at the expected molecular weight for GST-CD9 fusion protein (38 kDa). The figures at the bottom of lower panel C show the density of the bands on the Western blot relative to wild type CD9 (= 100). The lines to the left of the gels/blots in A and C show the position of molecular weight markers at 42 and 30 kDa. ELISA was performed using ALMA1 recognition of proteins immobilised on microtitre plates (10 μg/well). The results are presented as a percentage of antibody binding to wild type CD9 large extracellular domain, means ± SEM of two separate determinations performed in duplicate.

site on hCD81 (Phe^{186}) [18]. In reconstitution studies with mCD9 mRNA, mutation of Phe^{174} alone or in conjunction with the adjacent residues Ser^{173} and Gln^{175} prevented the reconstitution of sperm fusion activity in CD9 null eggs [12]. To investigate whether the same residues are important in the inhibition of fusion by soluble LED protein, we made Ala substitutions at the analogous residues in hCD9, Thr^{175}–Phe^{176}–Thr^{177} (Fig. 3). Ala substitutions were also made at the adjacent residues, Val^{178} and Lys^{179}, to allow us to gauge the size of the site involved in the inhibition of fusion. The mutant hCD9 LED proteins were all expressed at equally high levels (Fig. 3C top panel) and all were highly reactive with anti-hCD9 mAb ALMA 1, suggesting that all were properly folded (Fig. 3C, lower panel). The degree of correct folding was also demonstrated by ELISA, using the ratio of anti-GST antibody binding and anti-hCD9 antibody binding to show the relative levels of conformational epitope per unit total protein (Fig. 3D). Relative to GST-hCD63 LED, hCD9 LED caused 20% inhibition of sperm binding at 5 μg/ml (Fig. 4C) but the five mutant forms had variable effects. No reduction in the inhibition of binding was observed with Thr^{175}Ala nor Phe^{176}Ala whereas Thr^{177}Ala, Val^{178}Ala, and Lys^{179}Ala reduced the inhibitory effect by $\geqslant$50% (Fig. 4C). In the sperm/oocyte fusion assay, three of the five mutants (Thr^{175}Ala, Phe^{176}Ala, and Val^{178}Ala) were significantly less inhibitory than wt hCD9 LED (Fig. 4D). In contrast, inhibition of fusion by Thr^{177}Ala and Lys^{179}Ala was not significantly different to wt hCD9. It is important to note that the degree of inhibition of fusion does not correlate with the degree of anti-hCD9 antibody recognition, a measure of correct folding (Fig. 3D). Thus, it is likely that loss of inhibitory activity by mutant CD9 LED is due to specific changes in amino acid side-chain rather than a global change in LED conformation. Neither Thr^{177}, which is poorly conserved between species, nor Lys^{179},

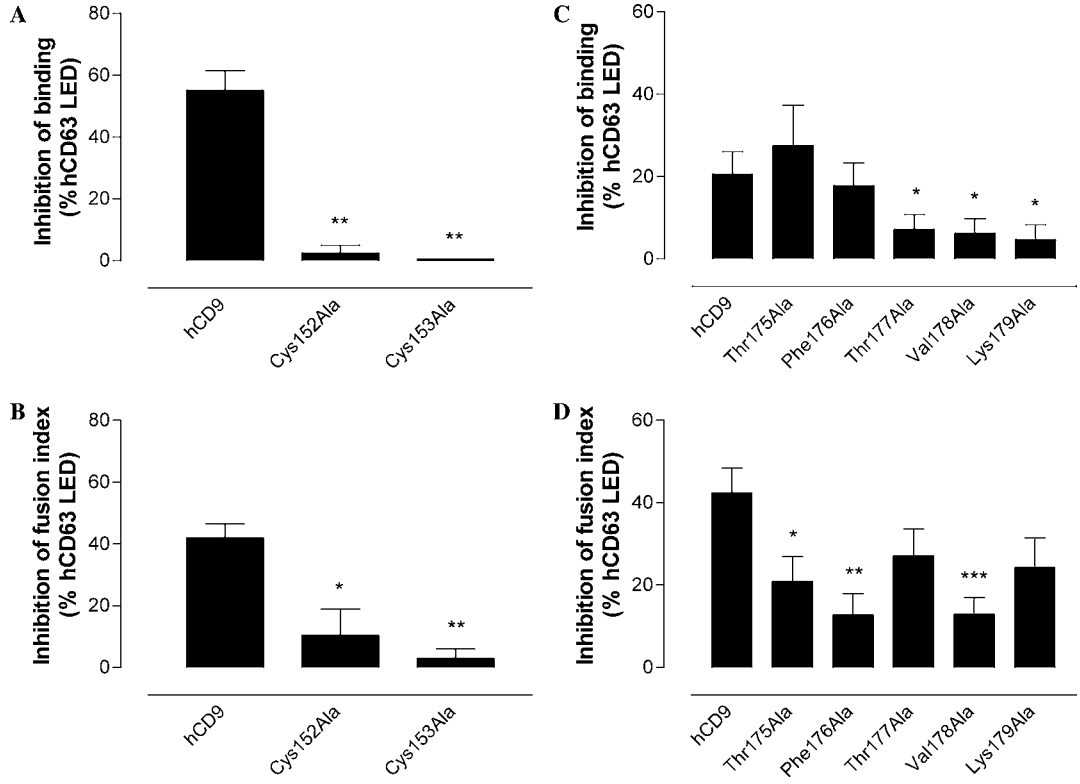

Fig. 4. Effects of mutant human CD9 extracellular domain proteins on sperm/oocyte binding and fusion. Zona free mouse oocytes (10–15 per sample) prelabelled with Hoechst dye were treated with the indicated amounts of wild type (hCD9) or mutant (Cys^{152}Ala, Cys^{153}Ala, Thr^{175}Ala, Phe^{176}Ala, Thr^{177}Ala, Val^{178}Ala, and Lys^{179}Ala) human CD9 large extracellular domain-GST fusion proteins (LED) prior to the addition of mouse sperm. By fluorescence microscopy, the mean number of sperm bound per oocyte (A,C) and the percentage of oocytes with fused sperm (B,D) were counted. Inhibition was calculated as a percentage of control (GST-human CD63 large extracellular domain) values, mean ± SEM of: (A,B) (two (Cys mutants) or five (wild type CD9)); (C,D) (8–17 (mutants) or 19 (wild type CD9)) separate determinations performed in duplicate. Significantly different from CD63 peptide values: $^{**}P < 0.005$; unpaired t test.

which is conserved in mammalian species and predicted to have an exposed side-chain in the vicinity of Phe^{176} [17], has a major role in fusion. Mutation of Val^{178}, outside of the Ser–Phe–Gln triplet observed in mCD9 LED, has the largest effect on the inhibition of fusion, suggesting that it forms a significant part of the critical site. This is supported by the degree of conservation of this residue between species; only Ile, Thr, and Leu substitute for Val^{178} in CD9 from other species.

The mechanism of action of soluble CD9 LED remains unclear. CD9 LED does not bind to sperm and so is unlikely to be directly inhibiting an interaction with a sperm protein such as an ADAM, previously implicated in interactions leading to fusion. This view is supported by our unpublished data (Y. Takahashi, B. Tomczuk, and J. White) showing that somatic cell binding to immobilised ADAM 2 and 3 disintegrin domains is not inhibited by anti-CD9 mAb and that CD9 nullizygous oocytes can still bind these ADAM domains. It is more likely that CD9 acts indirectly, modifying the function of additional oocyte proteins, which themselves act as the sperm binding and fusion sites. We have also shown that sperm fusion with CD9 nullizygous oocytes is not restored by soluble CD9 LED (Y. Takahashi and J. White, unpublished data), indicating that the remainder of the CD9 molecule is important, perhaps to provide binding sites for additional membrane or cytosolic proteins. Soluble CD9 LED may act to disrupt key interactions in this protein complex, with a small effect on sperm binding (a relatively simple event or a function that is duplicated by one or more additional tetraspanins) and a substantially stronger effect on fusion (an event requiring a higher degree of order in the membrane complex). It appears likely that sperm binding and fusion are controlled at separate but overlapping sites and this may indicate that different oocyte proteins can interact with CD9 LED at this binding surface, perhaps bringing together into close proximity proteins involved in sperm binding and fusion. A similar organising role has been proposed for tetraspanins in myoblast fusion [19] and virus-induced cell–cell fusion [20].

Acknowledgments

This work was supported by Arthritis Research Campaign and British Heart Foundation project Grants M0648 and PG/98163 to P.N.M. and NIH Grant, GM48739 to J.M.W.

References

[1] H.T. Maecker, S. Levy, Normal lymphocyte development but delayed humoral immune response in CD81-null mice, J. Exp. Med. 185 (1997) 1505–1510.

[2] M. Gourgues, P.H. Clergeot, C. Veneault, J. Cots, S. Sibuet, A. Brunet-Simon, C. Levis, T. Langin, M.H. Lebrun, A new class of tetraspanins in fungi, Biochem. Biophys. Res. Commun. 297 (2002) 1197–1204.

[3] M.E. Hemler, Specific tetraspanin functions, J. Cell Biol. 155 (2001) 1103–1107.

[4] E. Todres, J.B. Nardi, H.M. Robertson, The tetraspanin superfamily in insects, Insect. Mol. Biol. 9 (2000) 581–590.

[5] L.G. Fradkin, J.T. Kamphorst, A. DiAntonio, C.S. Goodman, J.N. Noordermeer, Genomewide analysis of the *Drosophila* tetraspanins reveals a subset with similar function in the formation of the embryonic synapse, Proc. Natl. Acad. Sci. USA 99 (2002) 13663–13668.

[6] C.S. Stipp, T.V. Kolesnikova, M.E. Hemler, Functional domains in tetraspanin proteins, Trends Biochem. Sci. 28 (2003) 106–112.

[7] M.S. Chen, K.S. Tung, S.A. Coonrod, Y. Takahashi, D. Bigler, A. Chang, Y. Yamashita, P.W. Kincade, J.C. Herr, J.M. White, Role of the integrin-associated protein CD9 in binding between sperm ADAM2 and the egg integrin alpha6beta1: implications for murine fertilization, Proc. Natl. Acad. Sci. USA 96 (1999) 11830–11835.

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

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

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

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

[12] G.Z. Zhu, B.J. Miller, C. Boucheix, E. Rubinstein, C.C. Liu, R.O. Hynes, D.G. Myles, P. Primakoff, Residues SFQ (173–175) in the large extracellular loop of CD9 are required for gamete fusion, Development 129 (2002) 1995–2002.

[13] K. Kaji, S. Oda, S. Miyazaki, A. Kudo, Infertility of CD9-deficient mouse eggs is reversed by mouse CD9, human CD9, or mouse CD81; polyadenylated mRNA injection developed for molecular analysis of sperm–egg fusion, Dev. Biol. 247 (2002) 327–334.

[14] D.A. Smith, P.N. Monk, L.J. Partridge, Antibodies against human CD63 activate transfected rat basophilic leukemia (RBL-2H3) cells, Mol. Immunol. 32 (1995) 1339–1344.

[15] M.S. Chen, K.S. Tung, S.A. Coonrod, Y. Takahashi, D. Bigler, A. Chang, Y. Yamashita, P.W. Kincade, J.C. Herr, J.M. White, 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 (1999) 11830–11835.

[16] A. Higginbottom, I. Wilkinson, B. McCullough, F. Lanza, D.O. Azorsa, L.J. Partridge, P.N. Monk, Antibody cross-linking of human CD9 and the high-affinity immunoglobulin E receptor stimulates secretion from transfected rat basophilic leukaemia cells, Immunology 99 (2000) 546–552.

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

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

[19] I. Tachibana, M.E. Hemler, Role of transmembrane 4 superfamily (TM4SF) proteins CD9 and CD81 in muscle cell fusion and myotube maintenance, J. Cell Biol. 146 (1999) 893–904.

[20] S. Loffler, F. Lottspeich, F. Lanza, D.O. Azorsa, V. ter Meulen, J. Schneider-Schaulies, CD9, a tetraspan transmembrane protein, renders cells susceptible to canine distemper virus, J. Virol. 71 (1997) 42–49.