

Antibody cross-linking of human CD9 and the high-affinity immunoglobulin E receptor stimulates secretion from transfected rat basophilic leukaemia cells

A. HIGGINBOTTOM,* I. WILKINSON,* B. MCCULLOUGH,* F. LANZA,† D. O. AZORSA,‡ L. J. PARTRIDGE* & P. N. MONK*
*Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield, UK, †INSERM U.311, Etablissement de Transfusion, Strasbourg, France, ‡Cancer Genetics Branch, National Human Genome Research Institute/NIH, Bethesda, MD, USA

SUMMARY

Previous studies have shown that antibody cross-linking of the tetraspanin protein CD9 stimulates the degranulation of platelets and eosinophils, although the mechanism of activation is unclear. In this work we transfected human CD9 into the rat basophilic leukaemia (RBL-2H3) cell line and studied the stimulation of secretion from these cells in response to a panel of anti-CD9 antibodies. Intact immunoglobulin G1 (IgG1) antibodies activated transfected cells whereas F(ab')₂ fragments of antibody and an intact IgG2a did not. Stimulation of secretion was inhibited by co-incubation with monomer murine immunoglobulin E (IgE) but not with an IgG1 isotype control, indicating that the response involves the endogenous high-affinity IgE receptor (FcεRI). The anti-CD9 antibody activation curve was biphasic, and supraoptimal antibody concentrations stimulated little or no degranulation, indicating that multivalent binding of human CD9 molecules is necessary for the formation of an active complex with rat FcεRI. Immunoprecipitation of FcεRI under mild detergent conditions co-precipitated CD9, suggesting the presence of pre-existing complexes of CD9 and FcεRI that could be activated by antibody cross-linking. These data are further evidence that tetraspanins are involved in FcεRI signalling and may reflect the participation of tetraspanins in the formation of complexes with other membrane proteins that use components of Fc receptors for signal transduction.

INTRODUCTION

At least 17 mammalian tetraspanin proteins have been identified, each containing four highly homologous transmembrane domains and short intracellular N- and C-termini. The proteins are characterized by the presence of a large extracellular domain, which shows great amino acid diversity except for four highly conserved cysteine residues that are predicted to be involved in disulphide linkages. The conserved nature of the transmembrane and intracellular regions indicates that tetraspanin proteins interact with a limited range of intracellular components. In contrast, the divergent extracellular domain sequences suggest that extracellular interac-

tions will probably be more complex, with different ligands for each tetraspanin protein.¹ Tetraspanin involvement in cell function is diverse and includes adhesion, cell-cell contact and motility, Fc receptor signalling, and viral susceptibility. Immunoprecipitation experiments have shown that several types of protein appear to associate with tetraspanins, leading to suggestions of a membrane network in which tetraspanin proteins perform a largely structural function and maintain correct spatial relationships between proteins.² Other data suggest that tetraspanins may have a role in signal transduction, interacting with protein and lipid kinases and phosphatases [reviewed in references 1, 3 and 4]. The most intensively studied tetraspanin, CD9, is expressed on a wide variety of cells (including B lymphoblasts, T cells, colon carcinoma and endothelial cells) and functional data is available from many of these cell types.^{5–11} Several monoclonal antibodies (mAb) to CD9 induce platelet aggregation and granule release, which is dependent on FcγRIIa, although the signal generated is distinct from FcγRIIa activation alone.¹² In eosinophils, a panel of soluble anti-CD9 mAbs can prolong cellular survival by increasing the production of granulocyte-macrophage colony-stimulating factor (GM-CSF). This stimulatory activity is inhibited by an anti-β₂-integrin mAb.¹³

Received 17 November 1999; accepted 1 December 1999.

Abbreviations: BSS, balanced salts solution; FcεRI, high-affinity IgE Fc receptor; FcγR, IgG Fc receptor; GM-CSF, granulocyte-macrophage colony-stimulating factor; 5-HT, 5-hydroxytryptamine; RBL, rat basophilic leukaemia.

Correspondence: Peter N. Monk, Department of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Western Bank, Sheffield S10 2UH, UK.

The rat basophilic leukaemia (RBL-2H3) cell line is a widely used model cell line for signalling via the high-affinity receptor for immunoglobulin E (IgE) (FcεRI). Previous work has shown that anti-rat CD63 and CD81 mAbs are capable of inhibiting IgE-mediated secretion^{14,15} in RBL cells. In contrast, we have shown previously that human CD63 is capable of mediating mAb-dependent secretion when transfected into RBL cells.¹⁶ In this report, we have used the rat CD9-negative RBL cell line to study the participation of human CD9 in the secretory response. Immunoglobulin G1 (IgG1) isotype anti-human CD9 antibodies were found to activate transfected RBL cells, probably through FcεRI. We showed, for the first time, that a tetraspanin can co-precipitate with FcεRI and discussed possible mechanisms of action for antitetraspanin mAbs on RBL cells.

MATERIALS AND METHODS

Antibodies

Fluorescein isothiocyanate (FITC)-labelled anti-CD9 antibody and ALB6 anti-CD9 IgG1 were purchased from Coulter (München, Germany). Anti-CD63 mAb H5C6 was obtained from the American Type Culture Collection (ATCC) (Rockville, MD). The ALMA anti-CD9 mAbs were generated from mice immunized with human platelets, as described previously.¹⁷ The anti-CD9 antibodies recognized adjacent or identical epitopes on the large extracellular domain of CD9.¹⁷

Tissue culture

Rat basophilic leukaemia (RBL-2H3) cells were routinely cultured in Dulbecco's modified Eagle's medium, containing 10% (v/v) fetal calf serum (FCS), at 37° in an atmosphere of 10% CO₂. Media for transfected cells was supplemented with 400 mg/l of G-418 for transfectants. Transfected and untransfected RBL cells were harvested with non-enzymatic cell dissociation solution.

Expression of human CD9 in RBL cells

RBL-2H3 cells were transfected by electroporation, as previously described,¹⁶ using the pEE6hCMV.neo vector containing the human CD9 cDNA insert. Cells were sorted using FITC-labelled anti-CD9 on a Becton-Dickinson Vantage FACSort (Becton Dickinson, Franklin, NJ) selecting the top 10% on two occasions. Following the second round of sorting, receptor number was quantified by using the Quantum 'Simply Cellular' kit (Sigma, Poole, Dorset, UK).

F(ab')₂ generation

Single peaks of IgG antibody were isolated from ascitic fluid or culture supernatant using protein G-Sepharose. Pepsin digestion was then performed at 37° for 10 hr, with a final enzyme concentration of 25 mg/l. Completion of digestion was checked by silver staining sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) gels. Reactions were then quenched with 3 M Tris (pH 8.6) and centrifuged at 10 000 g. Intact antibody was removed by incubation overnight with modified protein G-Sepharose.

Measurement of cell activation

Using soluble anti-CD9 mAb. This was determined as the

release of [³H]5-hydroxytryptamine (5-HT) from intracellular granules in response to incubation with mAbs for 30 min, as described previously.¹⁶ In some cases, cells were co-incubated with either IgE or IgG1 and anti-CD9 mAb. mAbs were always centrifuged at 100 000 g for 1 hr prior to use to remove aggregations. All values of release are quoted as a percentage of total cell-associated radioactivity adjusted for background (spontaneous) release or as a percentage of maximal antibody-stimulated release. Anti-CD9 mAb-stimulated secretion was typically 50–75% of the maximal secretory response to cross-linkage of surface-bound mIgE by optimal concentrations of antigen.

Using anti-CD9 mAb-coated fixed cells. In some experiments, the capacity of anti-CD9 mAb-coated fixed cells to stimulate secretion from unfixed cells was analysed. After harvesting, cells were fixed by incubation with 1% (w/v) paraformaldehyde in balanced salts solution (BSS) for 30 min at 4°. After two washes in BSS+0.2% (w/v) bovine serum albumin (BSA) solution, fixed cells were resuspended in BSS/BSA alone with either ALMA 1 or isotype control Ab at 1 µg/2 × 10⁵ cells (the ratio giving maximal release in the antibody dose-response assay). The cells were incubated for 1 hr at 4° then washed three times with BSS to remove excess mAb. The mAb-coated fixed cells were added to monolayers of live RBL cells prelabelled with [³H]5HT and incubated for 30 min at 37°. The degranulatory response of the live cells to the mAb-coated fixed cells was determined as the release of [³H]5HT into the supernatant, as described above. Aliquots of fixed cells were also analysed, by using flow cytometry, to determine mAb binding.

Immunoprecipitation of Fc receptors and CD9

Human CD9 and mock-transfected RBL cells were incubated overnight with 1 µg/ml of dinitrophenol (DNP)-specific mouse IgE and then washed with release buffer prior to incubation at 37° for 30 min, with or without 100 ng/ml of DNP-human serum albumin (HSA). After two washes with release buffer and one with lysis buffer without detergent (50 mM Tris-HCl, pH 7.6, 100 mM NaCl, 5 mM EDTA, 5 mM NaF, 1 mM phenylmethylsulphonyl fluoride [PMSF], 1 mM Na₃VO₄ and 2 µg/ml of aprotinin, pepstatin and leupeptin), cells were lysed with 1% (v/v) (3-[(3-cholamidopropyl)-dimethylammonio]-l-propane sulphanate (CHAPS) in lysis buffer at 4° for 30 min. After centrifugation at 15 000 g (for 5 min at 4°), lysates were cleared three times with protein A-agarose at 4° for 1 hr and then incubated with varying concentrations of rabbit anti-mouse antibody at 4° for 2 hr. Immune complexes were isolated by incubation with protein A-agarose beads at 4° for 1 hr, then washed three times with lysis buffer, eluted by boiling and resolved by 15% SDS-PAGE under non-reducing conditions and subsequently transferred to nitrocellulose membranes. Membranes were probed using anti-CD9 antibodies and then visualized using enhanced chemiluminescence.

RESULTS

Generation of a CD9-expressing RBL cell line

RBL cells transfected with CD9 cDNA were selected by fluorescence-activated cell sorter (FACS) analysis using an FITC-labelled anti-CD9 antibody. Two rounds of selection

were performed, isolating the top 10% of fluorescent cells. A mock transfectant cell line was established by transfection of RBL cells with vector alone (pEE6) and grown continually in selection media (containing G418). The antibody-binding capacity of the CD9-RBL cells was found to be 1.6×10^5 /cell, compared with an IgE-binding capacity of 1.3×10^5 /cell. The cellular distribution of CD9 in RBL cells was analysed by fluorescence microscopy and found to be predominantly at the plasma membrane (data not shown). Mock-transfected RBL cells did not bind detectable levels of anti-CD9 in either direct or indirect immunofluorescence assays (results not shown).

Stimulation of degranulation in RBL by anti-CD9 mAbs

The anti-CD9 mAbs, ALMA 1, ALMA 11 and ALB6 (IgG1), and ALMA 3 (IgG2a), bound specifically to CD9-transfected RBL cells, with half-maximal binding, at 1–10 nM (Fig. 1a, 1b, 1c, 1d). These mAb were tested for their ability to stimulate degranulation from transfected RBL cells. ALMA 1, 11 and ALB 6 all stimulate secretion according to a biphasic dose-response curve, whereas ALMA 3 had no effect at any of the concentrations used. Stimulation was maximal at 10–100 nM but decreased strongly again at 100–1000 nM (Fig. 1a, 1b, 1c, 1d). At concentrations where stimulation of degranulation was half maximal, binding was $\approx 80\%$ of the

maximum. At peak stimulatory levels of mAb, antibody binding was 100%, with stimulation decreasing as binding remained at saturation levels. The secretory response to anti-CD9 IgG1 mAb was specific; an IgG1 mAb that recognizes the human receptor for C5a (hC5aR) was tested on RBL cells transfected with this receptor.¹⁸ Expression levels of hC5aR, a G-protein-coupled receptor, were comparable to CD9 (1.7×10^5 /cell and 1.6×10^5 /cell, respectively) but mAb at concentrations up to 67 nM failed to stimulate any 5-HT secretion. Mock-transfected RBL cells did not secrete [³H]5-HT in response to any of the mAbs tested (results not shown). F(ab')₂ fragments were generated from intact ALMA 1 by pepsin digestion followed by purification using protein G. Despite binding to transfected RBL cells with an avidity similar to intact mAb (Fig. 1a), these fragments failed to stimulate degranulation at concentrations up to 1 μ M, 10–100-fold higher than the amounts of intact mAb required to stimulate maximal degranulation. F(ab')₂ fragments of ALMA 11 also failed to stimulate detectable levels of degranulation at these concentrations (data not shown).

Effects of co-incubation with monomer IgE (mIgE) and mIgG1 on anti-CD9 mAb-stimulated degranulation in RBL cells.

The possibility that an Fc receptor interaction with anti-

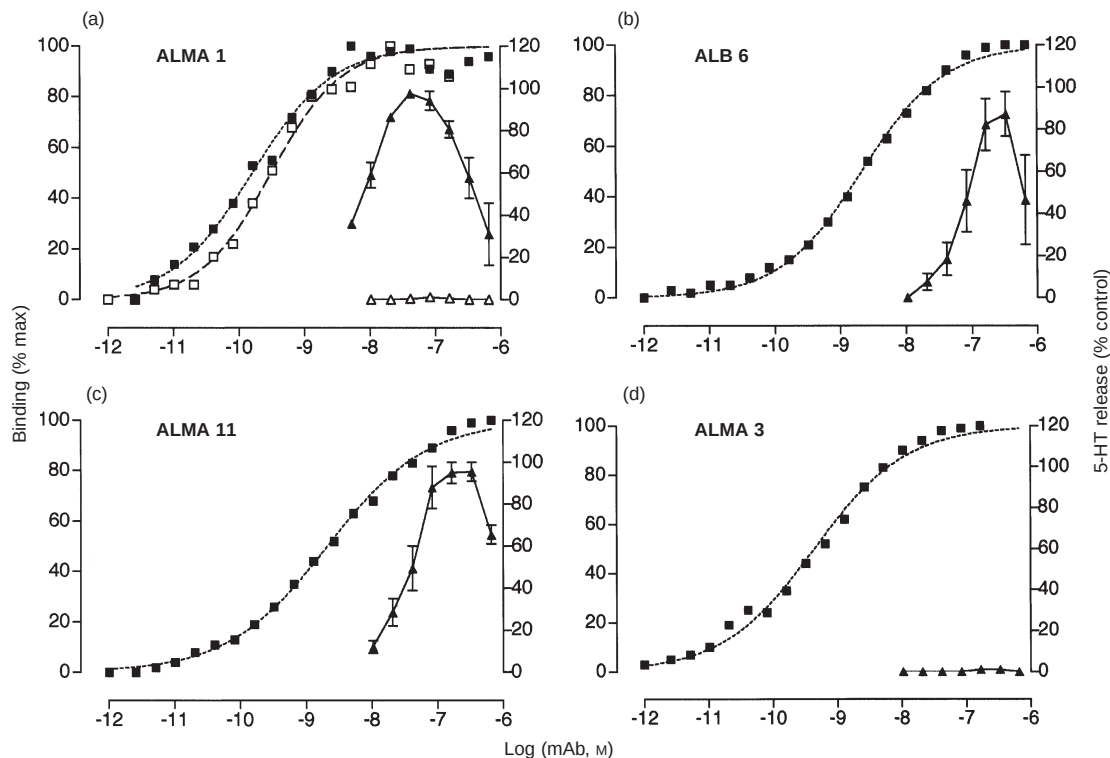


Figure 1. Antibody binding and activation of rat basophilic leukaemia (RBL-2H3) cells transfected with human CD9. RBL-2H3 cells transfected with human CD9 were incubated with anti-CD9 antibodies at 4° for measurement of binding by indirect immunofluorescence (squares, left axes). RBL-2H3 cells labelled with [³H]5-hydroxytryptamine (5-HT) were incubated with antibodies at 37° for the measurement of cell activation (triangles, right axes), as described in the Materials and methods. The binding data is the mean of one experiment performed in duplicate, typical of two similar experiments. Cell activation data is expressed as the mean $\pm$ SEM of three experiments performed in duplicate. The open symbols on (a) show the binding and cell activation measured using F(ab')₂ fragments of ALMA 1.

CD9 mAb must occur in order to stimulate degranulation from transfected RBL cells was investigated by attempting to inhibit the response by co-incubation with either mIgG1 or mIgE. The data showed that addition of mIgE inhibited the response to ALMA 1 with an IC_{50} of ≈ 1 – 10 nM, whereas mIgG1 did not inhibit at concentrations of up to $1 \mu\text{M}$ (Fig. 2). The binding of ALMA 1 to transfected RBL cells was not inhibited by co-incubation with mIgE (results not shown). This strongly suggests that co-ligation of CD9 and the high-affinity IgE receptor (Fc ϵ RI) occurs during incubation with the IgG1 mAbs.

Effects of anti-CD9 mAb presented on fixed cells

An alternative explanation of our data is that anti-CD9 mAb bound to the surface of RBL cells is capable of ligating and activating Fc receptors on adjacent cells (*trans*-activation) rather than co-ligating CD9 and Fc ϵ RI on the same cell (*cis*-activation). RBL cells transfected with CD9 were fixed using paraformaldehyde and saturated with an anti-CD9 mAb (ALMA 1). This fixation method did not affect the amount of mAb bound to the cell surface, as measured by using flow cytometry (data not shown). These loaded cells were then washed and added to unfixed target cells, labelled with [^3H]5-HT. Using live CD9 $^+$ RBL cells, some release of radiolabel was observed at ratios of 5:1–20:1 fixed:target cells (at 20:1, $31 \pm 12\%$ of response to direct addition of ALMA 1, mean $\pm$ SEM, $n=3$). The 5-HT release observed was presumed to be caused by leakage of anti-CD9 mAb from the fixed cells because live untransfected cells used in the same assay produced no release at these ratios. This suggests that cellular activation by anti-CD9 mAb is not a result of *trans*-activation but is a consequence of the co-ligation of Fc ϵ RI and CD9 on the surface of the same cell.

Immunoprecipitation of Fc ϵ RI and CD9

To determine if CD9 and Fc ϵ RI can form stable complexes prior to the addition of anti-CD9 mAb, RBL cells were loaded with anti-DNP mIgE and then the plasma membranes were

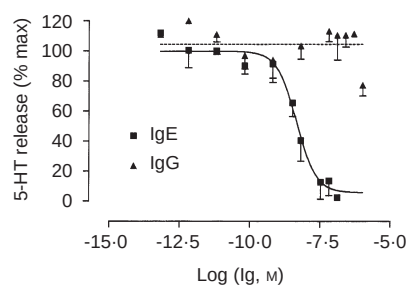


Figure 2. Cell activation by anti-CD9 antibodies is inhibited by murine immunoglobulin E (IgE) but not by murine immunoglobulin G1 (IgG1). Rat basophilic leukaemia (RBL-2H3) cells labelled with [^3H]5-hydroxytryptamine (5-HT) were co-incubated with different concentrations of either murine IgE or IgG1 and 67 nM anti-CD9 antibody (ALMA 1), and cell activation was measured as described in the Materials and methods. The results show 5-HT release relative to the effect of ALMA 1 alone (=100) and are expressed as the mean $\pm$ SEM of three experiments performed in duplicate.

solubilized using the detergent, CHAPS. IgE bound to Fc ϵ RI was immunoprecipitated by anti-mouse immunoglobulin antibodies. Development of Western blots with anti-CD9 mAb showed clearly that CD9 (MW 24 000) was co-precipitated with Fc ϵ RI in unactivated cells (Fig. 3a). A similar result was obtained by direct immunoprecipitation with an antibody against the α subunit of Fc ϵ RI (results not shown). This suggests that CD9 forms complexes with Fc ϵ RI in the absence of anti-CD9 mAbs. Activation of RBL cells by the polyvalent antigen, DNP-HSA, increased the amounts of CD9 associated with Fc ϵ RI (Fig. 3b). In total detergent lysates, a lower molecular weight form of CD9 was also present (≈ 21 000), which may be a degradation product of CD9 or the aglycosylated form; lysates from mock transfected cells did not contain either form of CD9 (results not shown). The 21 000-MW form of CD9 did not appear to associate with Fc ϵ RI.

DISCUSSION

CD9 has been implicated in the activation of eosinophils and platelets through integrins and Fc γ RIIa, respectively. In this work we investigated the potential of transfected human CD9 to support activation in a model system, the antibody-mediated degranulation of RBL cells.

The comparison of binding to RBL cells and the dose–

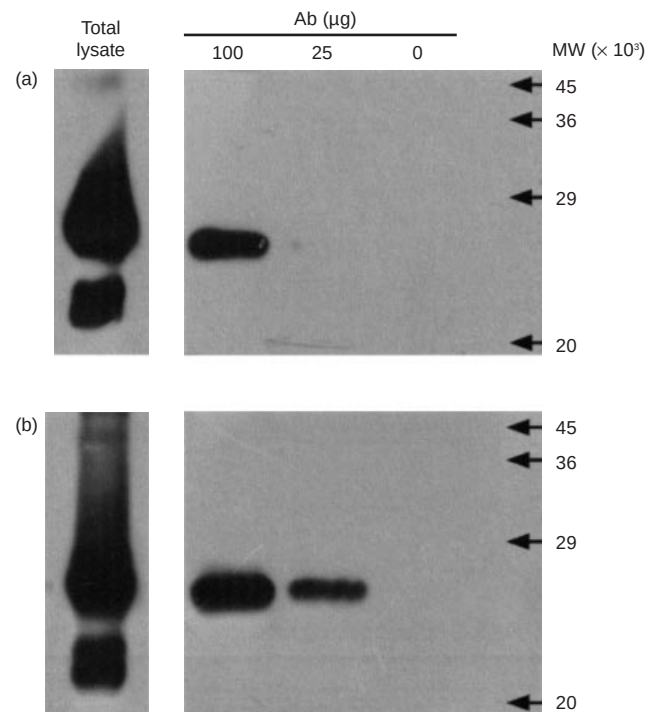


Figure 3. Co-precipitation of CD9 and Fc ϵ RI from solubilized cell membranes. Immunoglobulin E (IgE)/Fc ϵ RI complexes were immunoprecipitated by anti- κ light-chain antibodies (Ab; 0–100 μg) immobilized on protein A–agarose beads from either resting rat basophilic leukaemia (RBL) cells (a) or from cells activated with polyvalent antigen (b) prior to solubilization in 1% CHAPS. Western blots were developed using ALMA 1 anti-CD9 monoclonal antibodies (mAb) and visualized by enhanced chemiluminescence. The results shown are representative of four similar experiments.

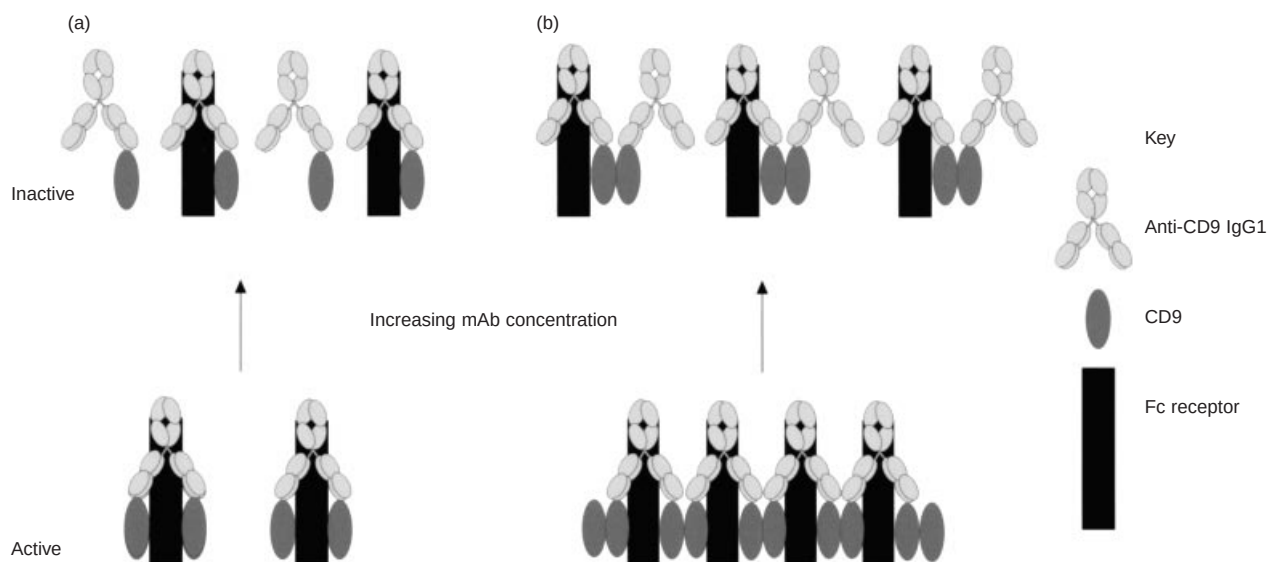


Figure 4. Models of anti-CD9-mediated Fc receptor activation. Supraoptimal concentrations of anti-CD9 immunoglobulin G1 (IgG1) inhibit degranulation of transfected rat basophilic leukaemia (RBL) cells. In (a), increasing competition for limiting CD9 epitopes decreases the proportion of complexes between CD9 and Fc ϵ RI that are capable of stimulating degranulation. In (b), the active complex is a large network of Fc receptors linked by CD9 dimers. The size of these networks is diminished at high antibody concentrations, leading to inhibition of the antibody response.

response for activation suggests that the primary event in cell stimulation is not the binding of mAb to CD9, but a lower affinity event that occurs only at high surface concentrations of mAb. F(ab')₂ fragments of two mAb, ALMA 1 and ALMA 11, failed to stimulate degranulation although they retained the ability to bind to cells transfected with CD9. RBL cells did, however, degranulate in response to cross-linkage of the anti-CD9 F(ab')₂ fragments by intact anti-F(ab')₂ antibodies (data not shown). Taken together, this evidence indicates that an Fc receptor is involved in cell activation and that simple cross-linking of CD9 molecules is insufficient to stimulate degranulation. The identity of this Fc receptor was revealed when it was found that mIgE was far more potent than mIgG1 for inhibition of the response to anti-CD9 mAb. It has been shown previously that Fc ϵ RI has a higher affinity for mIgG1 than mIgG2a, explaining the inactivity of mAb ALMA 3 in the RBL cell degranulation assay.^{19,20} RBL cells have been shown to possess low-affinity IgG Fc receptors (Fc γ RII and Fc γ RIII).^{21,22} The cross-linking of these receptors by saturating concentrations of aggregated IgG does not elicit degranulation²¹ and they may, in fact, be negative regulators of secretion.²² Previous work has also shown that mouse IgE can only partly inhibit IgG binding to RBL-cell Fc γ receptors, whereas we found that low concentrations of monomeric mouse IgE could completely inhibit the anti-CD9 mAb response.¹⁹ For these reasons, Fc γ receptors are unlikely to be involved in the secretory response to anti-CD9 antibodies.

The interaction between monomeric IgG1 Fc domains and the rat Fc ϵ RI receptor is a low-affinity event that is undetectable in indirect immunofluorescence assays¹⁹ and therefore no binding of anti-CD9 mAb to untransfected RBL cells is observed. Additionally, we found that prior incubation of RBL cells with IgE did not inhibit anti-CD9 mAb binding. It appears probable from our immuno-

precipitation data that the binding of mIgG1 to the Fc receptor is driven by a pre-existing association between CD9 and Fc ϵ RI which specifically increases the concentration of antibody in the immediate vicinity of the Fc receptor. The biphasic nature of the IgG1 activation curve suggests that multimers, possibly dimers, of CD9 molecules may interact with Fc ϵ RI molecules to produce cell stimulation. At high mAb concentrations, competition between mAb molecules may result in monovalent CD9 binding and prevent formation of the putative activation complex. A similar biphasic response curve is also observed when an anti-IgE antibody is used to stimulate RBL cells,²³ although this phenomenon has been ascribed to the formation of inhibitory signalling complexes at high antibody concentrations. We can disregard the possibility that the Fc regions of anti-CD9 mAb bound to one cell may interact with the Fc receptors of neighbouring cells. Fixed cells saturated with an activating anti-CD9 mAb (ALMA 1) had no effect on secretion from live untransfected cells and only a small effect on CD9-transfected RBL cells. The failure of anti-hC5aR IgG1 to stimulate secretion also argues against this and so the simple display of mIgG1 on the cell surface is insufficient to cause cell activation.

There is a considerable body of evidence showing that stimulation of RBL cells by IgE requires the formation of dimers and higher multimers of Fc ϵ RI,²⁴ although this picture is complicated by experiments using rigid cross-linkers which appear to show that physical contact between receptors is not essential for activation.²⁵ Our immunoprecipitation data shows that CD9 and Fc ϵ RI are associated on the surface of RBL cells. However, the failure of anti-CD9 F(ab')₂ fragments to trigger degranulation suggests that simple cross-linkage of the putative CD9/Fc ϵ RI complexes is insufficient to cause activation; an interaction between the Fc region of the antibody and Fc ϵ RI is required. The importance of this interaction in IgE-mediated

signalling has recently been highlighted by work using the SH2-domain-containing inositol polyphosphate 5-phosphatase (SHIP) knockout mouse.²⁶ Mast cells isolated from SHIP^{-/-} mice are extremely sensitive to occupancy of the FcεRI by purified IgE monomers and complete degranulation can apparently occur in the absence of the cross-linking of Fc receptors. The signal generated by the binding of monomeric Fc domains to FcεRI is undetectable in mast cells from normal mice, presumably owing to down-regulation of signalling by SHIP. In our system, it is possible that the presence of CD9 in complexes with FcεRI can override this negative regulatory signal. However, mIgE monomers were not observed to stimulate CD9⁺ RBL cells, and mIgE inhibited degranulation by anti-CD9 mAb. This suggests that activation requires conformational changes in the CD9/FcεRI complex, induced specifically by the anti-CD9 IgG1 Fc domain.

Two models of the activation complex that fit these observations are shown in Fig. 4. In the first model (Fig. 4a), two CD9 molecules are brought into a complex with an FcεRI molecule, which results in Fc receptor activation. This model does not require the dimerization of CD9 prior to anti-CD9 mAb addition but does suggest a direct association between CD9 and FcεRI. In the second model (Fig. 4b), pre-existing multimers of CD9 are cross-linked by IgG1 to form a network in which FcεRI molecules become enmeshed as a result of interactions with antibody Fc domains. Degranulation is a consequence of this immobilization of Fc receptors, which is similar to the direct formation of FcεRI multimers by antigen cross-linking of bound IgE. Direct association between CD9 and FcεRI is not essential in this model but these molecules must be in close proximity to drive the IgG1/Fc receptor interaction. Although CD9 multimers did not co-precipitate with FcεRI, dimers were seen in SDS-PAGE of plasma membrane preparations, suggesting that CD9 can form stable multimers, even in the presence of SDS. The formation of a network involving CD9 and Fc receptors is in agreement with the 'tetraspanin web' theory recently proposed by Rubinstein *et al.*,² in which membrane proteins are organized into functional domains or clusters.

The significance of the interaction between human CD9 and endogenous FcεRI in RBL cells is unclear. However, the documented examples of interactions among CD9, FcγRIIa, integrins and the tyrosine kinase p72syk in platelets suggest a role for CD9 in signalling clusters or networks of membrane proteins. CD9 is known to interact directly with platelet integrins (e.g. αIIb/β3) and, following anti-CD9 stimulation, with FcγRIIa and gpIIb/IIIa.^{12,27,28} The gpIb-IX-V complex (which mediates platelet activation by von Willebrand factor and thrombin) also interacts with FcγRIIa in platelets.²⁹ It is interesting to note that platelet activation by collagen requires an interaction between the Fc receptor γ chain and gpVI, in addition to engagement of the integrin gpIa-Iia.³⁰ The Fc receptor γ chain is normally associated with FcγRI and FcεRI (neither of which are present on platelets) and is the primary signalling protein for these Fc receptors. The γ chain has also been implicated in the induction of mouse mast cell adhesion to fibronectin following aggregation of the low-affinity IgG receptor, FcγRIII.³¹ In RBL cells, transfected human CD9 may be mimicking the role of endogenous tetraspanins by interacting with FcεRI. FcεRI/tetraspanin complexes may mediate interactions with other cluster components, giving

access to the FcεRI γ chains that could act as signal transducers for a range of membrane proteins. A common component of the platelet signalling pathways activated through CD9/FcγRIIa and gpVI/FcR γ chains is the protein tyrosine kinase p72syk, which is also involved in gpIIb/IIIa signalling.^{32,32,33} The role of CD9 in these signalling clusters, which can include β1 and β3-integrins and FcγRII, may involve the incorporation and organization of specific components in clusters, linking diverse cell-surface proteins with common downstream signal transducers such as p72syk. In RBL cells, IgE-mediated degranulation is potentiated by cell adhesion and spreading on physiological substrates, such as fibronectin, or non-physiological substrates, such as poly-L-lysine and anti-major histocompatibility complex (MHC) class I antibodies.^{34,35} The mechanism for this may involve the presence of signalling clusters similar to those occurring in platelets, containing integrins, MHC I, FcεRI and other proteins, which functionally interact and modulate signal transduction. Such clusters will probably involve tetraspanins (such as CD81 and CD63) that are known to associate with these proteins in other systems. Antibodies against tetraspanins are thus able to modulate cluster function, either inhibiting FcεRI signalling (in the case of endogenous CD63 and CD81^{14,15}) or stimulating it [in the case of transfected human CD9 and CD63 (this work and reference 16)]. Other potential components of these clusters have been identified by mAbs that interfere with IgE-mediated secretion from RBL cells: antibodies B17 and AA4 recognize a ganglioside, G_{D1b}.^{36,37} G63 recognizes the mast cell function-associated antigen, a C-type lectin,³⁸ and BD6 recognizes a 40 000-MW protein that associates with FcεRI.³⁹ BD6 treatment of RBL cells both inhibits IgE binding and causes morphological changes and detachment from some substrates, suggesting that integrin activity can be selectively modulated by this mAb. It is interesting to note that mast cell degranulation can also be stimulated by antibodies against MHC I,⁴⁰ a dimeric protein that has previously been shown to associate with a number of tetraspanins, including CD9.¹

In summary, we have shown that RBL cells transfected with human CD9 degranulate, in response to anti-CD9 IgG1 mAbs, by co-ligation with FcεRI. The activation mechanism probably involves the association of two CD9 molecules with one Fc receptor and may bypass the normal requirement for the direct multimerization of Fc receptors by IgE/antigen complexes.

ACKNOWLEDGMENTS

P. N. M. is a fellow of the Arthritis and Rheumatism Campaign (M0543). This work was supported by a grant from the British Heart Foundation (PG/95058) to P. N. M. and L. J. P. FACS facilities were provided by the Yorkshire Cancer Research Campaign.

REFERENCES

1. WRIGHT M.D. & TOMLINSON M.G. (1994) The ins and outs of the transmembrane-4 superfamily. *Immunol Today* **15**, 588.
2. RUBEISTEIN E., LENAOUR F., LAGAUDRIERE-GESBERT C., BILLARD M., CONJEAUD H. & 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.
3. HEMLER M.E., MANNION B.A. & BERDITCHEVSKI F. (1996) Association of TM4SF proteins with integrins – relevance to cancer. *Biochim Biophys Acta* **1287**, 67.

4. MAECKER H.T., TODD S.C. & LEVY S. (1997) The tetraspanin superfamily: molecular facilitators. *FASEB J* **11**, 428.
5. JONES P.H., BISHOP L.A. & WATT F.M. (1996) Functional significance of CD9 association with beta (1) integrins in human epidermal keratinocytes. *Cell Adhesion Commun* **4**, 297.
6. MASSELLI-SMITH A. & SHAW A.R.E. (1994) CD9-regulated adhesion – anti-CD9 monoclonal-antibody induce pre-B-cell adhesion to bone-marrow fibroblasts through de-novo recognition of fibronectin. *J Immunol* **152**, 2768.
7. TAI X.J., TOYOOKA K., YASHIRO Y. *et al.* (1997) CD9-mediated costimulation of TCR-triggered naive T cells leads to activation followed by apoptosis. *J Immunol* **159**, 3799.
8. LI W.Z. & TAIT J.F. (1998) Regulatory effect of CD9 on calcium-stimulated phosphatidylserine exposure in Jurkat T lymphocytes. *Arch Biochem Biophys* **351**, 89.
9. CAJOT J.F., SORDAT I., SILVESTRE T. & SORDAT B. (1997) Differential display cloning identifies motility-related protein (MRP1/CD9) as highly expressed in primary compared to metastatic human colon carcinoma cells. *Cancer Res* **57**, 2593.
10. IKEYAMA S., KOYAMA M., YAMAOKA M., SASADA R. & MIYAKE M. (1993) Suppression of cell motility and metastasis by transfection with human motility-related protein (MRP-1/CD9) DNA. *J Exp Med* **177**, 1231.
11. FORSYTH K.D. (1991) Anti-CD9 antibodies augment neutrophil adherence to endothelium. *Immunology* **72**, 292.
12. QI R.M., OZAKI Y., KURODA K. *et al.* (1996) Differential activation of human platelets induced by Fc γ RII cross-linking and by anti-CD9 monoclonal-antibody. *J Immunol* **157**, 5638.
13. KIM J.T., GLEICH G.J. & KITA H. (1997) Roles of CD9 molecules in survival and activation of human eosinophils. *J Immunol* **159**, 926.
14. KITANI S., BERENSTEIN E., MERGENHAGEN S., TEMPST P. & SIRAGANIAN R.P. (1991) A cell-surface glycoprotein of rat basophilic leukemia cells close to the high-affinity IgE receptor (Fc ϵ RI) – similarity to human-melanoma differentiation antigen ME491. *J Biol Chem* **266**, 1903.
15. FLEMING T.J., DONNADIEU E., SONG C.H., VAN LAETHAM F., GALLI S.J. & KINET J.P. (1997) Negative regulation of Fc epsilon RI-mediated degranulation by CD81. *J Exp Med* **186**, 1307.
16. SMITH D.A., MONK P.N. & PARTRIDGE L.J. (1995) Antibodies against human CD63 activate transfected rat basophilic leukemia (RBL-2H3) cells. *Mol Immunol* **32**, 1339.
17. AZORSA D.O., MOOG S., CAZENAVE J.-P. & LANZA F. (1999) A general method for the development of monoclonal antibodies to members of the tetraspanin superfamily with the use of recombinant GST-proteins. *J Immunol Methods* **29**, 35.
18. MONK P.N., PEASE J.E., MARLAND G. & BARKER M.D. (1994) Mutation of aspartate-82 of the human C5a receptor abolishes the secretory response to human C5a in transfected rat basophilic leukemia-cells. *Eur J Immunol* **24**, 2922.
19. HALL T.J. & RITTENBURG M.B. (1986) The interaction of monomeric and complexed mouse monoclonal IgG with rat basophilic leukemia cells – a subset of IgG molecules can bind to the RBL cell Fc receptors for IgG. *J Immunol* **137**, 2331.
20. KEPRON M.R., BAZIN H. & FROESE A. (1988) The interaction of IgG subclasses with solubilized Fc receptors of rat basophilic leukemia cells. *Mol Immunol* **25**, 597.
21. BOCEK P., DRÁBEROVÁ L., DRÁBER P. & PECHT I. (1995) Characterization of Fc gamma receptors on rat mucosal mast cells using a mutant Fc ϵ RI deficient rat basophilic leukemia line. *Eur J Immunol* **25**, 2948.
22. SEGAL D.M., TAIT S.O., JONES J.F. & SIRAGANIAN R.P. (1981) Fc (IgG) receptors on rat basophilic leukemia cells. *J Immunol* **126**, 138.
23. SEAGRAVE J., PFEIFFER J.R., WOFSY C. & OLIVER J.M. (1991) Relationship of IgE receptor topography to secretion in RBL-2H3 mast cells. *J Cell Physiol* **148**, 139.
24. METZGER H. (1991) The high affinity receptor for IgE on mast cells. *Clin Exp Immunol* **21**, 269.
25. KANE P.M., HOLOWKA D. & BAIRD B. (1988) Cross linking of IgE-receptor complexes by rigid bivalent antigens greater than 200Å in length triggers cellular degranulation. *J Cell Biol* **107**, 969.
26. HUBER M., HELAGASON C.D., DAMEN J.E., LIU L., HUMPHRIES R.K. & KRYSAL G. (1998) The src homology 2-containing inositol phosphatase (SHIP) is the gatekeeper of mast cell degranulation. *Proc Natl Acad Sci USA* **95**, 11330.
27. INDIG F.E., DIAZ-GONZALEZ F. & GINSBERG M.H. (1997) Analysis of the tetraspanin CD9-integrin alpha (IIb) beta (3) (GPIIb-IIIa) complex in platelet membranes and transfected cells. *Biochem J* **327**, 291.
28. SLUPSKY J.R., SEEHAFFER J.G., TANG S.C., MASSELLI-SMITH A. & SHAW A.R.E. (1989) Evidence that monoclonal-antibodies against CD9 antigen induce specific association between CD9 and the platelet glycoprotein-IIb-glycoprotein-IIIa complex. *J Biol Chem* **264**, 12289.
29. SULLAM P.M., HYUN W.C., SZOLLOSI J., DONG J.F., FOSS W.M. & LOPEZ J.A. (1998) Physical proximity and functional interplay of the glycoprotein Ib-IX-V complex and the Fc receptor Fc gamma RIIa on the platelet plasma membrane. *J Biol Chem* **273**, 5331.
30. TSUIJI M., EZUMI Y., ARAI M. & TAKAYAMA H. (1997) A novel association of Fc receptor gamma-chain with glycoprotein VI and their co-expression as a collagen receptor in human platelets. *J Biol Chem* **272**, 23528.
31. DASTYCH J., HARDISON M.C. & METCALFE D.D. (1997) Aggregation of low affinity IgG receptors induces mast cell adherence to fibronectin – requirement for the common FcR gamma-chain. *J Immunol* **158**, 1803.
32. OZAKI Y., SATOH K., KURODA K. *et al.* (1995) Anti-CD9 monoclonal-antibody activates p72 (syk) in human platelets. *J Biol Chem* **270**, 15119.
33. GAO J., ZOLLER K.E., GINSBERG M.H., BRUGGE J.S. & SHATTIL S.J. (1997) Regulation of the pp72 (syk) protein tyrosine kinase by platelet integrin alpha (IIb) beta (3). *EMBO J* **16**, 6414.
34. HAMAWY M.M., OLIVER C., MERGENHAGEN S.E. & SIRAGANIAN R.P. (1992) Adherence of rat basophilic leukemia (RBL-2H3) cells to fibronectin-coated surfaces enhances secretion. *J Immunol* **149**, 615.
35. ARGAR J.R. (1997) Increased degranulation and phospholipase A₂, C, and D activity in RBL cells stimulated through Fc ϵ RI is due to spreading and not simply adhesion. *J Cell Sci* **110**, 771.
36. ORTEGA E., LICHT A., BIENER Y. & PECHT I. (1990) A glycolipid-specific monoclonal antibody modulates Fc-epsilon receptor stimulation of mast cells. *Mol Immunol* **27**, 1269.
37. STEPHAN V., GUO N.H., GINSBURG V. & SIRAGANIAN R.P. (1991) Immunoprecipitation of membrane proteins from rat basophilic leukemia cells by the antiganglioside monoclonal antibody AA4. *J Immunol* **146**, 4271.
38. GUTHMANN M.D., TAL M. & PECHT I. (1995) A new member of the C-type lectin family is a modulator of the mast cell secretory response. *Int Arch Allergy Immunol* **107**, 82.
39. HAMAWY M.M., OLIVER C. & SIRAGANIAN R.P. (1992) Inhibition of IgE binding to RBL-2H3 cells by a monoclonal antibody (BD6) to a surface protein other than the high affinity IgE receptor. *J Immunol* **148**, 524.
40. DAERON M., DUC H.T., KANELLOPOULOS J., LE BOUTELLIER P., KINSKY R. & VOISIN G.A. (1975) Allogeneic mast cell degranulation induced by histocompatibility antibodies: an *in vitro* model of transplantation anaphylaxis. *Cell Immunol* **20**, 133.