Transmembrane 4 superfamily protein CD151 (PETA-3) associates with β_1 and $\alpha_{IIb}\beta_3$ integrins in haemopoietic cell lines and modulates cell–cell adhesion

Stephen FITTER*, Paul M. SINCOCK*†, Corina N. JOLLIFFE* and Leonie K. ASHMAN*†¹

*Division of Haematology, Hanson Centre for Cancer Research, Institute of Medical and Veterinary Science, PO Box 14 Rundle Mall, Adelaide, SA 5000, Australia, and

†Department of Medicine, University of Adelaide, Adelaide, SA 5005, Australia

CD151 (PETA-3/SFA-1) is a member of the transmembrane 4 superfamily (TM4SF) of cell-surface proteins and is expressed abundantly both on the cell surface and in intracellular membranes by the haemopoietic cell lines M07e, HEL and K562. In the presence of mild detergent (CHAPS), CD151 co-immunoprecipitated with integrin $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$ and $\alpha_{IIb}\beta_3$. The association of CD151 with $\alpha_4\beta_1$ and $\alpha_5\beta_1$ seemed to be constitutive, as it was not modified by treatment of M07e cells with cytokines that regulate integrin function by ‘inside-out’ signalling. CD151 also associated with other tetraspans in an apparently cell-type-specific fashion, as defined by its co-precipitation with CD9, CD63 and CD81 from M07e cells, but not from K562 cells, which express similar levels of these proteins. F(ab')₂ fragments of monoclonal antibodies (mAbs) against CD151 caused homotypic adhesion of HEL and K562 cells that was dependent on

energy and cytoskeletal integrity and was augmented in the presence of RGDS peptides. The adhesion was not blocked by function-inhibiting mAbs against β_1 or β_3 integrins, suggesting that cell–cell adhesion was not mediated by the binding of integrin to a cell-associated ligand. Furthermore, mAb CD151 did not affect adhesion of the cells to fibronectin, laminin, collagen or fibrinogen, which are ligands for $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$ and $\alpha_{IIb}\beta_3$ integrins. Taken together, these results indicate that the ligation of CD151 does not induce the up-regulation of integrin avidity, but might act as a component of integrin signalling complexes.

Key words: cell-surface proteins, cell adhesion, integrin–signalling complexes.

INTRODUCTION

CD151 (PETA-3/SFA-1) [1–3] is a member of the transmembrane 4 superfamily (TM4SF) and is expressed on platelets, megakaryocytes and activated T lymphocytes [2], and on endothelial, epithelial, Schwann and muscle cells [4,5]. Analogously to monoclonal antibodies (mAbs) against another TM4SF protein, CD9 [6,7], the anti-CD151 mAb 14A2.H1 induces Fc-dependent platelet aggregation and mediator release [4], and synergizes with subthreshold doses of other agonists, ADP, adrenaline, collagen and serotonin, in mediating platelet activation [8]. Another mAb (11B1.G4) [5] against this protein acts as a platelet agonist independent of the Fc domain (P. M. Sincock and L. K. Ashman, unpublished work), indicating that the ligation of CD151 can itself initiate transmembrane signalling.

The TM4SF consists of over 20 members, characterized by the presence of four highly conserved hydrophobic membrane-spanning domains and two divergent extracellular domains. These proteins are expressed on a wide variety of cell and tissue types and in evolutionarily diverse species such as mammals, schistosomes, *Drosophila* and *Caenorhabditis elegans*, suggesting a key role in cellular function [9,10]. The involvement of TM4SF proteins in signal transduction, cell activation, proliferation, motility and adhesion has been reported (reviewed in [10]). The diverse range of cellular responses attributed to TM4SF molecules is thought to be due in part to the formation of multiprotein complexes. For example, CD81 forms part of a signalling complex

on B cells with CD19, Leu13 and CD21 [11] and, together with another TM4SF protein CD82, complexes with CD4 or CD8 on T cells [12]. CD53 is associated with CD2 on rat T cells and NK cells [13] and with CD37, CD81, CD82 and MHC class I and II molecules in B cells [14]. Similarly, several TM4SF members have been shown to associate with a subset of β_1 integrins: CD9, CD63, CD81 and CD82 have been shown to associate specifically with integrins $\alpha_3\beta_1$ [15–18], $\alpha_4\beta_1$ [19,20] and $\alpha_6\beta_1$ [15,17,18]. Studies with mAbs against TM4SF proteins suggest that these complexes are involved in cell adhesion and migration. For example, perturbation of CD9 with mAbs induced the enhanced adhesion of pre-B cells to bone-marrow fibroblasts [21], augmented the adherence of neutrophils to endothelial cells [22] and induced the homotypic adhesion of pre-B cells [23]. Similarly, anti-CD81 mAb induced B-cell adhesion to tonsil interfollicular stroma [24] and homotypic adhesion of thymocytes [25]. Furthermore, the ectopic expression of CD9 in poorly motile Raji cells enhanced their motility on fibronectin-coated and laminin-coated filters [26]. Recently, we and others [27] have demonstrated the association of CD151 with $\alpha_3\beta_1$ in endothelial cells (P. M. Sincock, S. Fitter and L. K. Ashman, unpublished work). CD151 mAbs inhibit endothelial cell migration, and capillary formation *in vitro*, suggesting a role for these complexes in modulating endothelial cell function.

Here we report the association of CD151 with members of the β_1 and β_3 integrin family and with other TM4SF proteins in haemopoietic cell lines M07e, HEL and K562, which display

Abbreviations used: ECL, enhanced chemiluminescence; ECM, extracellular matrix; GM-CSF, granulocyte/macrophage colony-stimulating factor; HRP, horseradish peroxidase; mAb, monoclonal antibody; NRS, normal rabbit serum; SCF, stem-cell factor; TM4SF, transmembrane 4 superfamily.

¹ To whom correspondence should be addressed (e-mail leonie.ashman@imvs.sa.gov.au).

megakaryocytic characteristics. CD151 co-immunoprecipitated with $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$ and $\alpha_{IIb}\beta_3$ integrins from these cell types, although with differing detergent sensitivity. We have also investigated the possible functional consequences of CD151 association with integrins. Agonistic mAb against CD151 did not affect integrin avidity for extracellular matrix (ECM), but induced cell-cell adhesion by an energy- and cytoskeleton-dependent process.

EXPERIMENTAL

Antibodies

The anti-TM4SF mAbs used were as follows: anti-PETA-3, 11B1.G4, and anti-CD9, 1AA2.H9 [5]; anti-CD63, 12F12.1G2 [28]; and anti-CD81, JS41 (a gift from Dr. T. F. Tedder, Duke University Medical Centre, NC, U.S.A.). The anti-integrin mAbs used were as follows: anti- β_1 , 61-2C4 [5] (a gift from Dr. J. Gamble, Hanson Centre, Adelaide, Australia), QE.2E5 [29] (gift from Dr. R. Faull, Royal Adelaide Hospital, Adelaide, Australia) and 8A2 [30] (kindly provided by Dr. N. Kovak, University of Washington, Seattle, WA, U.S.A.); anti- α_2 , 16B4 (Serotec, Oxford, U.K.); anti- α_3 , P1B5 (Gibco BRL, Gaithersburg, MD, U.S.A.); anti- α_4 , HP2/1 (Gibco BRL); anti- α_5 , PHM2 [31]; anti- α_6 , 4F10 (Serotec); anti- β_2 , HB203 (American Type Culture Collection, Rockville, MD, U.S.A.); anti- $\alpha_{IIb}\beta_3$, m10E5 (gift from Dr. B. Collier, Mt. Sinai Hospital, New York, NY, U.S.A.). The anti-CD31 mAb B2B1 and the IgG_{2a} isotype negative control mAb 1D4.5 have been described elsewhere [32]. F(ab')₂ fragments of 11B1.G4 and 1D4.5 were prepared by digestion with ficin and purification with Protein A as described previously [8]. Briefly, Ig at 1 mg/ml was digested with 100 μ g/ml ficin for 7 h at 37 °C. Undigested Ig was separated from F(ab')₂ fragments by passage through Protein A-Sepharose (Pharmacia, Uppsala, Sweden). Eluted F(ab')₂ fragments were dialysed against Tyrode's buffer and their purity was confirmed by analysis on SDS/PAGE gels.

Cell culture, immunofluorescence and flow cytometry

The human erythroleukaemia cell lines HEL and K562 were cultured in RPMI-1640 medium supplemented with 10% (v/v) foetal bovine serum. The factor-dependent human megakaryoblastic cell line M07e was cultured in Dulbecco's modified Eagle's medium with 10% (v/v) foetal-bovine serum, supplemented with interleukin 3 (10 ng/ml). The expression of surface molecules was measured by indirect immunofluorescence and flow cytometry. In brief, cells were washed twice in cold PBA [PBS (pH 7.4)/0.1% BSA/0.1% (w/v) sodium azide], and resuspended at 10⁷/ml in 10% (v/v) heat-inactivated normal rabbit serum (NRS) in PBA, before incubation with primary mAb for 1 h at 4 °C. After being washed twice in cold PBA, bound mAb was detected by incubation with r-phycoerythrin-conjugated goat anti-(mouse Ig) (Caltag Laboratories, Burlingame, CA, U.S.A.) diluted 1:50 in PBA containing 10% (v/v) NRS for 1 h at 4 °C. Cells were washed twice in cold PBA before fixation in 0.5 ml of cold 1% (w/v) paraformaldehyde. Binding of mAb was quantified with an EPICS XL2 flow cytometer (Coulter, Miami, FL, U.S.A.). Antigen copy number was determined by using Qifikit microspheres (Biotex, Marseille, France) as described previously [33]. Detection of intracellular antigens was performed by using saponin permeabilization as described previously [34]. In brief, cells were fixed with 2% (v/v) formaldehyde for 10 min at room temperature, washed in PBA, then resuspended in PBA containing 0.1% saponin (Sigma, St. Louis, MO, U.S.A.). Saponin-treated cells were incubated at

4 °C for 1 h with primary mAb supplemented with 0.1% saponin/10% (v/v) NRS. Cells were washed in cold PBA containing 0.1% saponin, then bound mAb was detected by incubation for 1 h at 4 °C with r-phycoerythrin-conjugated goat anti-(mouse Ig) (Caltag) supplemented with 0.1% saponin. Non-permeabilized cells were treated similarly, except that saponin was omitted. Cells were fixed in cold 1% (w/v) paraformaldehyde and analysed by flow cytometry as described above. For confocal microscopy, cytocentrifuge smears were fixed and permeabilized with acetone/methanol/formaldehyde (19:19:2, by vol.) for 30 s at 4 °C. Smears were washed in PBS, then incubated with primary mAb diluted in PBA containing 10% (v/v) NRS for 45 min at 4 °C. Bound antibody was detected with a 1:30 dilution of FITC-labelled sheep antibody against mouse Ig (Silenus, Hawthorn, Australia). Specimens were fixed with 1% (w/v) paraformaldehyde, mounted in 86% (v/v) glycerol containing 1% (w/v) propyl gallate (Sigma) and examined with an MRC 600 confocal microscope (Bio-Rad, Hercules, CA, U.S.A.).

Immunoprecipitation and Western blotting

To label cell surface molecules, cells were washed with PBS, pH 7.4, then incubated with 0.5 mg per 2 $\times$ 10⁷ cells/ml of sulpho-(*N*-hydroxysulphosuccinimide)-biotin (Pierce, Rockford, IL, U.S.A.) for 30 min at room temperature. Unreacted biotin was washed from the cells; lysates were then prepared with CHAPS lysis buffer [1% (w/v) CHAPS/10 mM Tris/HCl (pH 7.4)/140 mM NaCl/0.5 mM CaCl₂/0.5 mM MgCl₂/40 μ g/ml PMSF/40 μ g/ml tosylphenylalanylchloromethane (TPCK)/40 μ g/ml tosyl-lysylchloromethane (TLCK)/40 μ g/ml *p*-nitrophenyl-*p*'-guanidinobenzoate hydrochloride (all from Sigma)] for 30 min on ice. Insoluble material was pelleted by centrifugation at 150 000 *g* for 30 min at 4 °C, then cellular lysate was precleared with goat anti-(mouse Ig) coupled Sepharose for 1 h at 4 °C. Specific molecules were immunoprecipitated by the incubation of lysate from 10⁷ cells with 5 μ g of purified antibody for 1 h at 4 °C followed by incubation with goat anti-(mouse Ig)-Sepharose for 1 h at 4 °C. Immunoprecipitates were washed four times in 1% (w/v) CHAPS lysis buffer then twice in 0.5% (v/v) CHAPS lysis buffer, eluted with reducing SDS/PAGE loading buffer at 100 °C for 3 min, then separated by 11% (w/v) PAGE. Proteins were transferred to PVDF membranes (Micron Separations, Westborough, MA, U.S.A.) and labelled proteins were detected with streptavidin-biotinylated horseradish peroxidase (HRP) (Amersham, Little Chalfont, Bucks., U.K.) and detected with the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham). For reprecipitation experiments, immunoprecipitates were washed as above, then immunoprecipitated proteins were eluted for 2 h at 4 °C in NP40 buffer [1% (v/v) NP40/50 mM Tris/HCl, pH 7.4/150 mM NaCl/1 mM EDTA] supplemented with 0.2% SDS. Eluted proteins were diluted 1:2.5 with NP40 buffer and precleared, then reprecipitated as described above. Pellets containing reprecipitated proteins were washed twice in NP40 buffer, then analysed as described above. For immunoprecipitation and Western blotting, unlabelled lysate was prepared with 1% (w/v) CHAPS lysis solution, and immunoprecipitations and electrophoresis were performed as described above, except that non-reducing conditions were used. After transfer to PVDF membranes, blots were blocked with TBS-T [20 mM Tris/HCl (pH 7.5)/150 mM NaCl/0.1% (v/v) Tween 20] then probed with primary mAbs for 2 h at room temperature. Bound biotinylated primary mAbs (anti-CD151 11B1.G4 and anti- β_1 QE.2E5) were then detected with streptavidin-biotinylated HRP (Amersham) and proteins were detected as described above. Bound unlabelled primary mAbs (anti-CD9

1AA2.H9, anti-CD63 12F12.1G2 and anti-CD81 JS81) were detected with biotinylated sheep antibody against mouse immunoglobulins (Amersham) followed by streptavidin-biotinylated HRP then detected as described above.

Constitutive association experiments

M07e cells were cultured for 2 h in serum- and factor-free conditions (10^6 /ml), resuspended at 10^7 /ml then stimulated at 37 °C with stem-cell factor (SCF) (50 ng/ml) or granulocyte-macrophage colony-stimulating factor (GM-CSF) (2 ng/ml) for various durations. Cell lysate was then prepared and immunoprecipitations were performed, all as described above. Immunoprecipitated proteins were separated under non-reducing conditions 11% (w/v) PAGE and transferred to PVDF membranes, then Western blots were performed with saturating amounts of biotinylated anti- β_1 mAb (QE.2E5) and anti-CD151 mAb (11B1.G4). Bound mAb was then detected by using 0.25 μ Ci/ml ^{125} I-streptavidin (33 μ Ci/ μ g) (Amersham). Immunoblotted molecules were then detected with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Cell aggregation

Aliquots of HEL cells (2×10^4) in 200 μ l of complete medium were placed in 96-well flat-bottomed microtitre plates. Purified 11B1.G4 F(ab')₂ fragments were added at 1 μ g/ml and plates were incubated at 37 °C under air/CO₂ (19:1) for up to 24 h. For specific treatments, HEL cells were preincubated for 15 min with 0.5 μ g/ml cytochalasin D (Sigma), 1.5–500 μ M GRGDSP (one-letter code) or GRGESP peptides (Gibco), or 5 mM 2-deoxyglucose (Sigma)/0.1% sodium azide before the addition of 11B1.G4 F(ab')₂ fragments.

Cell adhesion assays

Adhesion assays were performed essentially as described previously [35]. In brief, wells of a 96-well plate were coated with 0.1–10 μ g of fibronectin per well (Boehringer Mannheim, Mannheim, Germany), collagen I or collagen IV (Collaborative Biomedical, Bedford, MA, U.S.A.), laminin (Boehringer Mannheim) or fibrinogen (Calbiochem, San Diego, CA, U.S.A.) at 37 °C for 1 h. Non-specific binding was blocked by incubation with 2% (w/v) BSA (CSL, Victoria, Australia) in serum-free culture medium for 2 h at 37 °C. Exponential-phase cells (4×10^6) were labelled with 100 μ Ci of ^{51} Cr (NEN, Boston, MA, U.S.A.) for 1 h at 37 °C in a volume of 500 μ l. Cells were washed three times in binding buffer (serum-free culture medium containing 0.2% BSA), resuspended at 4×10^5 /ml and stimulated with antibodies against CD151 (11B1.G4) and β_1 integrin (8A2) for 15 min on ice. Aliquots (100 μ l) of cells were then added to the coated wells, warmed rapidly to 37 °C, then incubated for 30 min. Non-adherent cells were flicked from the wells and each well was washed three times with binding buffer. Adherent cells were lysed in 150 μ l of 0.5% SDS/0.1% NaOH for 10 min at room temperature and radioactivity was quantified with a γ -counter (Packard, Meriden, CT, U.S.A.). The percentage adhesion was determined from the average of quadruplicate wells and the total counts loaded in each well.

RESULTS

Integrin and TM4SF surface expression on haemopoietic cell lines

As a prelude to analysing CD151 associations, the surface expression of integrin and TM4SF was determined on the cell lines by indirect immunofluorescence and flow cytometry (Figure

Figure 1 Surface expression levels of TM4SF molecules and integrins on haemopoietic cell lines

Expression of the indicated molecules on M07e, HEL and K562 cells was measured by indirect immunofluorescence and flow cytometry. Antigen copy number was determined by using Qifit kit microspheres.

1). Copy number was determined relative to a standard curve constructed by using a series of Qifit beads coated with known numbers of mouse Ig molecules. CD151 was abundant on M07e cells, whereas its expression on HEL and K562 cells was approximately half that on M07e. CD9 and CD63 levels were comparable between the cell lines, and CD81 expression was strong on M07e and K562 cells. M07e cells also expressed abundant β_1 integrin, whereas HEL and K562 had only 50% and 20% respectively of that on M07e. These differences in β_1 levels were reflected in the expression of integrin α chains on these cell lines. The predominant integrin α chains present on M07e cells were found to be α_4 , α_5 and α_6 . HEL cells express α_2 , α_4 , α_5 and $\alpha_{IIb}\beta_3$; K562 expresses the α_5 subunit only. No α_1 or α_3 chains were detected, and β_2 integrin expression was very low (results not shown).

CD151 associates with β_1 and $\alpha_{IIb}\beta_3$ integrins on the cell surface

On the basis of this expression pattern, immunoprecipitations were performed from cell lysates prepared with the zwitterionic detergent CHAPS. The requirement of mild lysis conditions to maintain TM4SF–integrin associations has been demonstrated for other cell types [15,17]. From the three cell lines tested, antibody against CD151 consistently immunoprecipitated molecules in the range 110–150 kDa (Figure 2A). These were similar to bands precipitated with antibodies against integrin α and β chains and the Ig superfamily member CD31, which was used as a control for the specificity of association. A band corresponding in size to CD151 (28–30 kDa) was also detected in CD151, β_1 and α_6 immunoprecipitations from M07e cells. However, this band was not evident when samples were run under non-reducing conditions, suggesting that it was generated from cleavage of the α_6 chain (results not shown). Similarly, a band of approx. 20 kDa was also present in anti-CD151, β_1 and α_5 immunoprecipitates from M07e, HEL and K562 cells; these could also be cleavage products of the α chains. The failure to detect CD151 suggests that insufficient labelled lysine residues were present to enable subsequent detection by ECL, because immunoprecipitated protein could be readily detected by Western blotting (see below).

Figure 2 Reprecipitation of integrins from anti-CD151 immunoprecipitates

(A) Cells were surface-labelled with biotin then lysed with the mild zwitterionic detergent CHAPS. Immunoprecipitations were then performed with mAb against the indicated molecules. Immunoprecipitated molecules were separated by 11% (w/v) PAGE under reducing conditions and transferred to PVDF membranes; labelled proteins were then detected with streptavidin-biotinylated HRP and detected with the ECL Western blotting detection reagents. (B) Molecules immunoprecipitated with anti-CD151 mAb 11B1.G4 were stripped from the Sepharose with 1% (v/v) NP40/0.2% SDS then reprecipitated with mAb against the indicated molecules. Reprecipitated proteins were separated by 11% (w/v) PAGE under reducing conditions and transferred to PVDF membranes; labelled proteins were then detected as described above.

Figure 3 Reciprocal precipitation of CD151 with β_1 and $\alpha_{11b}\beta_3$ integrins

Cell lysates were prepared by using 1% (w/v) CHAPS, and immunoprecipitations were performed with antibodies against the indicated molecules. Immunoprecipitated proteins were separated by 11% (w/v) PAGE under non-reducing conditions and transferred to PVDF membrane; Western blots were then performed with biotin-labelled anti- β_1 mAb QE.2E5 and biotin-labelled anti-CD151 mAb 11B1.G4. Bound mAb was detected with streptavidin-HRP and detected by using ECL.

To determine the specificity of the CD151 associations, proteins precipitated with CD151 were disassociated from the complex with 1% (v/v) NP40/0.2% SDS, then reprecipitated with anti-integrin and anti-CD31 mAbs. All mAbs were shown to bind their respective antigens in the SDS-supplemented buffer (results not shown). Reprecipitated material was subjected to SDS/PAGE under reducing conditions. For M07e cells, β_1 , α_4 (evident as a weak 150 kDa band), α_5 and α_6 chains were reprecipitated from anti-CD151 primary immunoprecipitates (Figure 2B). Similarly, β_1 and α_5 , but not α_4 , chains were reprecipitated from HEL cells, and β_1 and α_5 were also reprecipitated from K562. Furthermore the integrin $\alpha_{11b}\beta_3$, present as 125 and 105 kDa bands, was also reprecipitated from anti-CD151 primary immunoprecipitates from HEL cells. No reprecipitated material was detected with anti- α_2 or anti-CD31 mAbs.

Figure 4 Influence of solubilizing agents on CD151-integrin complexes

M07e cell lysates were prepared by using 1% (w/v) or 1% (v/v) solubilizing reagents; immunoprecipitations were performed with antibodies to the indicated molecules. Immunoprecipitated proteins were separated by 11% (w/v) PAGE under non-reducing conditions and transferred to PVDF membrane; Western blots were then performed with biotin-labelled anti- β_1 mAb QE.2E5 and biotin-labelled anti-CD151 mAb 11B1.G4. Bound mAb was detected with 0.25 μ Ci/ml ^{125} I-streptavidin (33 μ Ci/ μ g), then detected with a PhosphorImager.

Reciprocal association of CD151 and integrins

Because CD151 failed to be biotinylated under the described labelling conditions, Western blots were performed to determine

Figure 5 Comparison of cell-surface and total cellular staining of TM4SF molecules and β_1 integrins on M07e cells

(A) Expression of the indicated molecules on unpermeabilized (open bars) and saponin-permeabilized (filled bars) M07e cells was measured by indirect immunofluorescence and flow cytometry. Mean fluorescence intensity (MFI) was determined on triplicate samples; background fluorescence, determined with isotype-matched control mAbs ($MFI \leq 1$) was subtracted. Error bars show the S.E.M. for each triplicate. The difference between the filled and open bars indicates the amount of cytoplasmic staining. (B) Confocal microscopy analysis of the distribution of CD151, β_1 , α_4 , α_5 , α_6 and CD63 in M07e cells.

whether the associations were reciprocal. CD151 appeared as a doublet on Western blots owing to the presence of both glycosylated (upper band) and non-glycosylated forms. As expected from the reprecipitation data, CD151 was detected in β_1 , α_4 , α_5 and α_6 immunoprecipitates from M07e cells (Figure 3). Similarly, CD151 was detected in β_1 , α_4 , α_5 and $\alpha_{IIb}\beta_3$ immunoprecipitates from HEL cells and in β_1 and α_5 immunoprecipitates from K562 cells. No CD151 was detected in α_2 or CD31 immunoprecipitates, suggesting that CD151 associates specifically with a range of integrins on these cells.

CD9, but not other TM4SF members (CD63, CD81, CD82), has recently been shown to associate with the pre- β_1 chain in a number of cell types [36]. To determine whether this phenomenon was restricted to CD9, we analysed the level of mature β_1 and pre- β_1 associated with CD151 on these cells. The mature β_1 chain was clearly visible in all CD151 and β_1 integrin immunoprecipitates (Figure 3). In contrast, a distinct glycosylation variant indicative of the pre- β_1 form [37] was detected only in β_1 and α_4 immunoprecipitates from HEL cells and in β_1 immuno-

precipitates from K562 cells. For CD151, a weak band corresponding to the pre- β_1 chain was detected in immunoprecipitates from K562 cells (Figure 3).

Detergent sensitivity of CD151–integrin complexes

Of the TM4SF–integrin associations cited in the literature, only one example of a TM4SF– $\alpha_5\beta_1$ association has been reported [19]. More recent evidence has suggested that specific TM4SF–integrin associations are detergent-dependent [20,38]. We therefore looked at the sensitivity of CD151–integrin complexes in M07e cells to a range of detergents used previously to analyse TM4SF–integrin complexes, i.e. CHAPS, Brij 58 and Brij 97. Western blots were performed, and the β_1 chain and CD151 were detected. Consistently with our previous observations, CD151 was co-precipitated with $\alpha_4\beta_1$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$ in CHAPS lysates (Figure 4). This pattern of co-precipitation was also observed in Brij 58 lysates. However, the association of CD151 with $\alpha_4\beta_1$ and $\alpha_5\beta_1$ was not observed in Brij 97 lysates. Surprisingly, appreciable

Figure 6 CD151 associates with other TM4SF members CD9, CD63 and CD81

(A) Cell lysates were prepared by using 1% (w/v) CHAPS; immunoprecipitations (IP) were then performed with mAb against the indicated TM4SF molecules. Immunoprecipitated proteins were separated by 11% (w/v) PAGE under non-reducing conditions and transferred to PVDF membranes; Western blots were then performed with 1AA2.H9 (α -CD9), 12F12.1G2 (α -CD63), JS81 (α -CD81) or 11B1.G4 (α -CD151). Bound mAb was detected with biotinylated sheep antibody against mouse immunoglobulins followed by streptavidin-HRP, then detected with ECL. (B) Stability of CD151-TM4SF complexes. M07e cell lysates were prepared by using 1% (w/v) or 1% (v/v) solubilizing reagents and immunoprecipitations, then Western blots were performed as described above. Bound mAb was detected with 0.25 μ Ci/ml ^{125} I-streptavidin (33 μ Ci/ μ g), then detected with a PhosphorImager.

amounts of CD151 was detected in $\alpha_6\beta_1$ immunoprecipitates from lysates prepared with the stronger non-ionic detergent NP40.

M07e cells contain substantial cytoplasmic pools of CD151

To determine the cellular distribution of CD151, we used immunofluorescence and flow cytometry to quantify the expression of TM4SF protein and integrin on permeabilized and non-permeabilized M07e cells. Fluorescence signals generated from saponin-permeabilized M07e cells represent the total cellular pool (i.e. surface and intracellular) of antigen, whereas signals from non-permeabilized cells represent surface-expressed molecules only. The difference between the fluorescence signals therefore represents the amount of antigen expressed in the cytoplasm. CD151 was found to have a considerable cytoplasmic pool, constituting approx. 85% of the total CD151 in these cells (Figure 5A). Similarly, 94% of CD63 was present inside the cell, whereas 62% of CD81 and 30% of CD9 was intracellular. Of the β_1 integrins, 50% of α_4 and 30% of β_1 was cytoplasmic, whereas α_5 and α_6 had no apparent intracellular pool. These results were confirmed by confocal microscopy (Figure 5B). In HEL and K562 cells, 60% and 70% respectively of CD151 was intracellular (results not shown).

CD151 is constitutively associated with β_1 integrins during the modulation of integrin avidity

So far, little is known of the dynamics of the TM4SF-integrin associations. Early experiments indicated that the association of CD9 with integrin $\alpha_{IIb}\beta_3$ on platelets required stimulation with agonistic CD9 mAb [39]. It was possible that the association of TM4SF proteins with integrins could be involved in the process

of up-regulation of integrin avidity. To address this question for CD151, the amount of this protein associated with β_1 integrins was analysed under conditions in which integrin avidity was modulated by an 'inside-out' signalling mechanism [40]. M07e cells were factor-deprived for 2 h, then stimulated with either SCF or GM-CSF, which transiently up-regulated both $\alpha_4\beta_1$ and $\alpha_5\beta_1$ avidity for fibronectin [35]. The effectiveness of the treatment was shown in a standard adhesion assay on fibronectin-coated plates. The amount of CD151 associated with α_4 , α_5 and α_6 integrins was then compared between steady-state and cytokine-stimulated cells by immunoprecipitation and quantitative Western blotting. No appreciable differences in the amounts of CD151 associated with these integrins were observed in cells isolated from factor-deprived as compared with factor-stimulated cells (results not shown).

CD151 associates with other TM4SF proteins

In addition to forming complexes with other cell-surface molecules including β_1 integrins and HLA antigens, CD9, CD63 and CD81 have been demonstrated to associate with each other in these same complexes [17,41,42]. This organization of cell surface molecules has been termed the tetraspan network [41] and might function to connect and arrange several cell-surface proteins. Because CD151 is often co-expressed with CD9, CD63 and CD81, we determined whether CD151 could complex with these TM4SF members. Using a combination of immunoprecipitation and Western blotting, we detected CD151 in CD9, CD63 and CD81 immunoprecipitates from M07e cells (Figure 6A). However, CD151 was detected only in CD63 immunoprecipitates from HEL cells. No co-precipitating TM4SF molecules were detected for K562 cells, despite the detection of a similar level of

Figure 7 mAb against CD151 induces homotypic cell adhesion

HEL cells (5×10^4) were incubated with $1 \mu\text{g/ml}$ F(ab')_2 fragments of anti-CD151 mAb 11B1.G4, then incubated at 37°C under air/ CO_2 (19:1) for up to 24 h. Cell aggregates were photographed immediately after the addition of mAb (**A**) or at 1 h (**B**), 4 h (**C**), 12 h (**D**) or 24 h (**E**) afterwards. (**F–H**) Cells incubated with $1 \mu\text{g/ml}$ 11B1.G4 F(ab')_2 fragments and $50 \mu\text{M}$ GRGDSP peptides at 1 h (**F**); cells incubated with $1 \mu\text{g/ml}$ IgG_{2a} control mAb 1D4.5 F(ab')_2 fragments at 24 h (**G**); cells incubated with 11B1.G4 F(ab')_2 fragments and $0.5 \mu\text{g/ml}$ cytochalasin D at 24 h (**H**).

immunoprecipitated proteins on control Western blots (Figure 6A).

The stability of CD151–CD9 and CD151–CD63 complexes in M07e cells was then tested by using the same detergents as in the experiments shown in Figure 4. CD151 was detected in CD9 and CD63 immunoprecipitations from cell lysates prepared with 1 % CHAPS, Brij 58 and Brij 97, but not in those prepared with NP40 (Figure 6B).

Anti-CD151 mAbs induce homotypic cell adhesion

Having established that CD151 associates with integrins, we

investigated whether it is involved in adhesive processes in these cells. The incubation of HEL cells with F(ab')_2 fragments of the anti-CD151 mAb 11B1.G4 resulted in the appearance of homotypic aggregates within 3 h of stimulation; this was maximal by 24 h (Figures 7A–7E). No aggregation was seen in control experiments with an irrelevant isotype-matched control (Figure 7G) or with several other mAbs that bound to the cell surface, e.g. PHM2 (anti- α_5), 15E8 (anti- β_1), OKM1 (anti- $\alpha_{IIb}\beta_3$) and m10E5 (anti- $\alpha_{IIb}\beta_3$). Similar results were obtained with K562 cells (results not shown). The aggregation of HEL cells was inhibited by the addition of cytochalasin D, demonstrating that cytoskeletal interactions were required (Figure 7H). Further-

Figure 8 Anti-CD151 mAb 11B1.G4 fails to modulate the adhesion of M07e, HEL or K562 cells to ECM

Cells were labelled with ^{51}Cr , then stimulated with $1\ \mu\text{g/ml}$ IgG_{2a} control 1D4.5 F(ab')₂ fragments, $1\ \mu\text{g/ml}$ anti-CD151 mAb 11B1.G4 F(ab')₂ fragments or $2\ \mu\text{g/ml}$ anti- β_1 mAb 8A2 for 15 min on ice. Cells were then incubated in 96-well trays coated with $2\ \mu\text{g}$ of fibronectin (Fn), laminin (Ln), collagen type IV (Col IV), collagen type I (Col I) or fibrinogen (Fg) per well for 30 min at 37°C . Non-adherent cells were removed from the wells by vigorous washing, then adherent cells were lysed and counts were determined with a γ -counter. Percentage adhesion was determined by dividing the counts from adherent cells by the total counts loaded per well and multiplying by 100. Error bars indicate S.E.M. for each determination in quadruplicate.

more, the incubation of HEL cells with sodium azide and 2-deoxyglucose also blocked aggregation, demonstrating that the process was energy-dependent (results not shown). In an attempt to determine the nature of the cellular ligands, we preincubated HEL cells with antibodies against a number of adhesion molecules, and with peptides containing the RGD sequence, which block integrin-dependent cell adhesion to the ECM. The inclusion of function-blocking mAbs against β_1 integrins (P5D2, P4C10), β_2 integrins (HB203, 5A4.C5), β_3 integrin (PMG/13), α_5 integrin (PHM2), CD31 (58-9H6) or ICAM-1 (QE2-1B4), or a polyclonal

antibody against P-selectin, failed to inhibit 11B1.G4-induced homotypic adhesion (results not shown). These results suggest that homotypic cell attachment was not mediated directly by these integrins and provisionally exclude several other adhesion systems. Interestingly, the inclusion of GRGDSP peptides, ranging in concentration from 1.5 to $500\ \mu\text{M}$, augmented homotypic adhesion (compare Figures 7B and 7F). This response was seen early in the aggregation process (1 h); no difference was seen after 24 h. GRGDSP peptides alone or in the presence of the control mAb failed to cause aggregation, and control GRGESP peptides failed to augment aggregation in the presence of 11B1.G4 (results not shown).

Perturbation of CD151 fails to modulate cell adhesion to the ECM

In accordance with the modulation of cell-cell adhesion by CD151 mAb, we next addressed the question of whether anti-CD151 mAbs would modulate the binding of M07e, HEL or K562 cells to immobilized ECM. Cells were labelled with ^{51}Cr and adhesion assays were performed on β_1 and β_3 ligands laminin, collagen I, collagen IV, fibronectin and fibrinogen [43] in the presence of an isotype-matched negative control mAb 1D4.5, anti-CD151 mAb 11B1.G4 or a positive control pan-activating β_1 mAb 8A2. Incubation of cells with 8A2 resulted in a marked increase in adhesion to the ECM as compared with basal adhesion levels observed in the presence of the control mAb 1D4.5 (Figure 8). In contrast, anti-CD151 mAb 11B1.G4 did not appreciably augment or inhibit cell adhesion to ECM components, although minor but reproducible augmentation of HEL cell adhesion to fibronectin and collagen type I was observed (Figure 8).

DISCUSSION

Using a combination of cell-surface labelling and immunoprecipitation, we have demonstrated that CD151 forms complexes on the cell surface of human haemopoietic cell lines with $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$ and $\alpha_{\text{IIb}}\beta_3$ integrins. The association of TM4SF molecules with both $\alpha_4\beta_1$ and $\alpha_6\beta_1$ has been reported previously in a number of cell systems [44]. Similarly, the association of CD9 with $\alpha_{\text{IIb}}\beta_3$ has been reported previously [38,39]. In contrast, the association of TM4SF members with $\alpha_5\beta_1$ is restricted to the CD9- $\alpha_5\beta_1$ complex observed on NALM-6 cells [19,41]. This complex seems functionally relevant, as CD9 mAbs induced the adhesion of NALM-6 cells to bone-marrow fibroblasts by up-regulating both $\alpha_4\beta_1$ and $\alpha_5\beta_1$ avidity for bone-marrow fibroblast-associated fibronectin [21]. In this study we have demonstrated that CD151 also complexes with $\alpha_5\beta_1$ in three different cell lines with myeloid/megakaryocytic properties. The association was detergent-sensitive in a manner similar to that observed for the association of CD53, CD63, CD81 and CD82 with $\alpha_4\beta_1$ in lymphoid cells [20]. Differential sensitivity of TM4SF-integrin complexes to detergents has also been demonstrated for CD9 and $\alpha_{\text{IIb}}\beta_3$, in which complexes were preserved in detergents that maintain hydrophobic interactions [38]. Thus the detergent sensitivity of TM4SF-integrin complexes could be indicative of the nature and/or the affinity of the association. With this in mind it seems that the association of CD151 with $\alpha_6\beta_1$ is of high affinity, given the stability of this complex in NP40.

At present we have limited information on the extent of CD151- $\alpha_5\beta_1$ complexes in other cell systems, although we have detected this complex on the surface of endothelial cells (P. M. Sincock, S. Fitter and L. K. Ashman, unpublished work). Despite

rigorous attempts by other workers to demonstrate TM4SF- $\alpha_5\beta_1$ complexes in other systems, no associations of TM4SF molecules with α_5 were reported [15–17], possibly owing to the detergent specificity of the association and/or the cell type specificity, because α_5 co-precipitated with CD9 from NALM-6 cells but not from HEL cells [19]. CD151 might also associate with $\alpha_5\beta_1$ in the absence of other TM4SF molecules. Indeed, we were unable to detect CD9, CD63 or CD81 complexed with CD151 in K562 cells (Figure 6A), which express only $\alpha_5\beta_1$, whereas these complexes were evident in M07e cells. The CD151–TM4SF associations observed on M07e cells might be due to the expression of $\alpha_6\beta_1$ in these cells, because CD9, CD63 and CD81 have previously been shown to complex with $\alpha_6\beta_1$ in epithelial cells [15,17]. It is possible that tertiary complexes of CD151, $\alpha_6\beta_1$ and other TM4SF molecules exist on M07e cells, although this has not been demonstrated formally.

The presence of substantial intracellular pools of CD151 and CD63, but not of CD9, was demonstrated by immunofluorescence/flow cytometry (Figure 5A) and by confocal microscopy (Figure 5B) on permeabilized M07e cells. CD63 has been shown to reside in platelet α -granules [45] and to localize in multivesicular bodies in megakaryocytes and megakaryoblastic cell lines [46]. CD151 co-localizes with CD63 and transferrin receptor in endosomes and/or lysosomes in endothelial cells (P. M. Sincock, S. Fitter and L. K. Ashman, unpublished work), suggesting that CD151 might reside in similar structures in haemopoietic cells. The stronger co-precipitation of CD151 with CD63, as compared to with that of CD9 (Figure 6B), might result from CD151–CD63 associations in intracellular membranes. Interestingly, we detected the non-glycosylated form of CD151 in integrin and TM4SF immunoprecipitates from M07e cells (Figures 3 and 6). However, it remains to be determined whether this form is expressed on the cell surface or whether it corresponds to the CD151 precursor in the endoplasmic reticulum and/or Golgi. That integrin–TM4SF associations can occur before export to the cell surface has been demonstrated by co-immunoprecipitation of precursor β_1 chain with CD9 in a number of cell systems [36]; however, the association of TM4SF proteins with intracellular molecules remains to be examined more thoroughly.

We went on to examine the functional consequences of CD151–integrin associations. We considered, a priori, that association with the TM4SF protein might mediate the regulation of integrin avidity ('inside-out' signalling) [40]. M07e cells provided a convenient tool for examining this, because stimulation with cytokines (interleukin 3, GM-CSF or SCF) results in a transient increase in the adhesive function of $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrin for fibronectin [35]. To examine whether the association of CD151 with β_1 integrins is modulated by stimuli that regulate integrin avidity, we performed quantitative immunoprecipitation and Western blotting experiments on starved and cytokine-treated cells. These treatments failed to alter the amount of CD151 that co-precipitated with $\alpha_4\beta_1$, $\alpha_5\beta_1$ or $\alpha_6\beta_1$ integrins, so we consider that the association is likely to be constitutive in these cells. In contrast, CD9 can be induced to associate with integrin $\alpha_{IIb}\beta_3$ in platelets by cross-linking with anti-CD9, resulting in platelet activation [39], although more recent results suggest that this association occurs in resting platelets independently of stimulation with mAb [38].

Further evidence that CD151 does not modulate integrin avidity came from studies with F(ab')₂ fragments of the anti-CD151 mAb 11B1.G4, which have agonist activity in that they effectively induce cell–cell adhesion (Figure 7) and platelet activation (P. M. Sincock and L. K. Ashman, unpublished work). These F(ab')₂ fragments were tested for their ability to modulate

the adhesion of the three cell lines to the ECM components laminin, collagen, fibronectin and fibrinogen, which are known ligands for $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$ and $\alpha_{IIb}\beta_3$ integrins. Although the activating anti- β_1 mAb 8A2 [30] effectively up-regulated adhesion to β_1 ligands, the CD151 mAb had no appreciable effect on cell adhesion to any ECM (Figure 8). Similarly, mAbs against CD9, CD37, CD53, CD81 and CD82 failed to modulate the adhesion of lymphoid cell lines to purified cellular fibronectin, laminin and collagen [19,20], suggesting that TM4SF molecules in general are unable to modulate integrin avidity. In contrast, several examples exist in which the perturbation of TM4SF members with agonistic mAb induced integrin-dependent cell adhesion. For example, anti-CD81 mAbs induced the adhesion of B cells to interfollicular stroma by modulating $\alpha_4\beta_1$ adhesion to stromal fibronectin [24]. Similarly, anti-CD9 mAbs induce pre-B-cell adhesion to bone-marrow fibroblasts by up-regulating $\alpha_4\beta_1$ and $\alpha_5\beta_1$ avidity for bone marrow fibroblast-bound fibronectin [21]. In this study, anti-CD9 mAbs failed to modulate the adhesion of pre-B cells to purified cellular fibronectin, suggesting the requirement for further cellular interactions to facilitate adhesion.

Despite the association of CD151 with integrins on the cell surface of HEL and K562 cells, the ability of CD151 mAb to modulate cell–cell adhesion did not seem to be mediated directly by β_1 or β_3 integrins. Homotypic adhesion was not blocked by function-inhibiting mAbs against β_1 , β_3 or α_5 integrin chains, or by RGDS peptide, which inhibits the binding of $\alpha_5\beta_1$ to fibronectin. mAbs against several other TM4SF members have also been shown to induce homotypic aggregation. In some lymphoid cell lines this is mediated by LFA-1 ($\alpha_L\beta_2$)-dependent mechanisms [25,47], although LFA-1-independent mechanisms have also been described in both lymphoid and non-lymphoid cells [19,23,48,49]. As observed for CD151 mAbs, LFA-1-independent homotypic adhesion required energy and cytoskeletal integrity; furthermore, it could be modulated by cations, which is indicative of the involvement of integrin. Also consistently with our observations for CD151, blocking β_1 mAbs and RGDS peptides failed to inhibit CD9-induced B-cell homotypic adhesion despite the surface association of CD9 with $\alpha_4\beta_1$ and $\alpha_5\beta_1$ [21,23]. Recently, the perturbation of rat CD53 with mAb was shown to induce the LFA-1-independent homotypic adhesion of B cells [50]. Ultrastructural analysis of aggregated cells revealed small high-density zones of cell–cell contact reminiscent of gap junctions, suggesting a novel adhesion mechanism that might be a general phenomenon for TM4SF molecules.

Cell–cell adhesion initiated by mAbs against CD151 and other TM4SF proteins seems to occur by a mechanism distinct from the up-regulation of ligand binding by the associated integrins. Ligation and clustering of integrins is known to initiate transmembrane signalling ('outside-in' signalling), leading to multiple effects on cellular function [43]. We propose that mAbs against CD151 might act by co-aggregation of β_1 and/or β_3 integrin molecules, thereby stimulating integrin 'outside-in' signalling pathways and resulting in indirect effects on cell–cell adhesion. RGDS peptides have been reported to induce the conformational activation of $\alpha_{IIb}\beta_3$ integrin in platelets and to act as partial agonists [51]. The enhancement of 11B1.G4-induced homotypic adhesion of HEL cells with RDGS peptides (Figure 7) is consistent with the view that the ligation of CD151 stimulates integrin-dependent signalling pathways. TM4SF proteins might be important components of the integrin signal-transducing mechanism, because they have been shown to associate with phosphatidylinositol 4-kinase [52] and small GTP-binding proteins [53].

In conclusion, we have demonstrated that CD151 co-precipitates with several integrins from human myeloid/mega-

karyoblastic cell lines. The ligation of CD151 with mAbs induced cell-cell adhesion that was not blocked by inhibitors of integrin-ECM interactions. CD151 mAbs did not modify β_1 or β_3 integrin-mediated adhesion to ECM and, at least in M07e cells, the association between CD151 and β_1 integrin seemed to be constitutive rather than modulated by cytokines that regulate integrin avidity. mAb against CD151 might induce cell-cell adhesion by activating 'outside-in' signalling pathways that indirectly up-regulated other adhesive mechanisms.

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