Selective tetraspan–integrin complexes (CD81/α4β1, CD151/α3β1, CD151/α6β1) under conditions disrupting tetraspan interactions

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The tetraspans are molecules with four transmembrane domains which are engaged in multimolecular complexes (the tetraspan web) containing a subset of β1 integrins (in particular $\alpha3\beta1$, $\alpha4\beta1$ and $\alpha6\beta1$), MHC antigens and several unidentified molecules. The molecules associated with tetraspans are readily detected after immunoprecipitation performed in mild detergents such as Brij 97 or CHAPS. In this study we show that another classical mild detergent, digitonin, dissociated most of these associated molecules, including integrins, from the tetraspans CD9, CD37, CD53, CD63, CD82, Co-029, Talla-1 and NAG-2. In contrast, reciprocal immunoprecipitations from various cell lines demonstrated that two other tetraspans, CD81 and CD151, formed complexes with integrins not disrupted by digitonin. These complexes were CD81/$\alpha4\beta1$, CD151/$\alpha3\beta1$ and CD151/$\alpha6\beta1$. Furthermore, a new anti-CD151 monoclonal antibody (mAb), TS151r, was shown to have a restricted pattern of expression, inversely related to the sum of the levels of expression of $\alpha6\beta1$ and $\alpha3\beta1$. This mAb was unable to co-precipitate integrins in digitonin, suggesting that its epitope is blocked by the association with integrins. Indeed, the binding of TS151r to the cell surface was quantitatively diminished following $\alpha3\beta1$ overexpression. Altogether, these data suggest that, among tetraspans, CD81 interacts directly with the integrin $\alpha4\beta1$, and CD151 interacts directly with integrins $\alpha3\beta1$ and $\alpha6\beta1$. Because all tetraspan–tetraspan associations are disrupted by digitonin, it is likely that the other tetraspans interact indirectly with integrins, through interactions with CD81 or CD151.

Key words: adhesion, integrins, tetraspans.

INTRODUCTION

The β1 or very-late-antigen integrins are a subgroup within the integrin family comprising at least 10 members, each with a distinct α subunit non-covalently associated with the common β1 subunit. The β1 integrins function as cellular receptors for extracellular-matrix proteins, such as different types of collagen, laminin and fibronectin. They not only control cell adhesion and migration, but also regulate proliferation and differentiation [1].

Some of the β1 integrins have been shown to associate with molecules of the tetraspan (TM4) superfamily, which are characterized by a significant sequence similarity to each other and the existence of four transmembrane domains delimiting three short cytoplasmic domains and two extracellular regions of unequal size [2]. All TM4 molecules studied so far, CD9, CD53, CD63, CD81 and CD82, were found to associate with integrins $\alpha3\beta1$, $\alpha4\beta1$ and $\alpha6\beta1$ when co-expressed in the same cell lines [3–8]. An association of tetraspans with integrin $\alpha5\beta1$ as been reported [3,5,9–11] which may be both cell-type- and tetraspan-dependent [3,5]. Anti-TM4 monoclonal antibodies (mAbs), or transfection of TM4, did not modify the adhesion of cells to extracellular-matrix components such as fibronectin, laminin or collagen [3,6]. However, mAbs to integrin and tetraspan molecules produce similar effects such as homotypic aggregation or inhibition of migration [3,12–14]. That the TM4 molecules may play a role in integrin-mediated migration is further supported by experiments demonstrating that ectopic expression of CD9 in a lymphoid B cell line or of CD63 in a melanoma cell line were associated with a modification of cell motility on the integrins' substrates [15,16]. Moreover, several tetraspans, when transfected in tumour cell lines, provide a metastasis inhibitory signal [17–19], as do several integrins [20].

Cross-linking or preclearing experiments have suggested that each β1 integrin can associate with at least two tetraspans [5,6]. In fact, all the tetraspans tested so far have been shown to associate with any other tetraspan tested [5,6,21], and CD9 has been shown to associate with its murine equivalent in transfected cells, suggesting that several molecules of a given tetraspan can be present in the same complex [5]. Other molecules have been shown to associate with multiple tetraspans. For example, CD4 or CD8 associate with both CD81 and CD82 [22]. Several tetraspans associate with HLA-DR and CD19 in lymphoid B cells [5,21,23,24], which are themselves associated with β1 integrins in these cells [5,25]. Together, these data suggest that the tetraspans are components of large molecular complexes, building what we call the tetraspan web, and possibly a molecular network [5]. These molecules have been suggested to be surface organizers [5,24], or facilitators [2], that would connect different molecules of the cell surface, and therefore couple different functions.

Although several components of the tetraspan web are now identified, there is to date no information about its organization. We have demonstrated recently that CD19, a lymphoid B-cell co-stimulatory molecule, is associated on the cell surface with CD9 [24]. CD9 and CD81 were shown to interact with CD19 in different manners since the CD19/CD9 association was disrupted in the presence of digitonin, but the CD19/CD81 complex was not. Under these conditions, the CD9/CD81 interaction was also disrupted, which suggested that the use of digitonin could allow the identification of primary complexes independent from tetraspan–tetraspan interactions. We therefore compared, in digitonin extracts, the patterns of molecules co-precipitated with tetraspans in several cell lines offering a wide combination of possible tetraspan–integrin associations. Under these conditions,

Abbreviations used: mAb, monoclonal antibody; CHO, Chinese hamster ovary.

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which disrupt all tetraspan–tetraspan interactions, some of the tetraspan molecules still interact with other surface molecules, in particular integrins.

EXPERIMENTAL

mAbs

The anti-tetraspan mAbs used for this study were SYB-1 and ALB-6 (CD9) [3], BL14 (CD37), Z53 (CD53) [26], H5C6 (CD63) [27], Z81 (CD81) [26], γC11 (CD82) [28], 11B1.G4 (CD151) [29], AZM22.1 (Co-029) [26], AZM30.1 (Talla-1) [26] and 2E12 (NAG-2) [30]. The anti-β1 integrin used for this study was C9 [5] and the anti-α3 integrin was M-KID-2 [31]. mAbs to integrins α1 (HP2B6), α2 (Gi9), α4 (HP2/1), α5 (SAM-1) and α6 (GoH3) were obtained from Immunotech (Marseille, France). The anti-α6 integrin mAb 4F10 was purchased from Serotec (Oxford, U.K.). The anti-α4 integrin mAb 9F10, the anti-β4 integrin mAb 450-9D and the phycoerythrin-labelled anti-α3β1 mAb C3 II.1 were obtained from Pharmingen (San Diego, CA, U.S.A.). Biotinylation of mAbs with EZ-link-Sulpho-NHS-LC-biotin (Pierce, Rockford, IL, U.S.A.) was performed according to the manufacturer's instructions.

Generation of mAbs

BALB/c mice were injected intraperitoneally three times with 10^7 HeLa cells. Spleen cells were fused with P3 × 63AG8 mouse myeloma cells (5×10^7 and 3×10^7 cells, respectively) according to standard techniques and distributed on to 96-well tissue culture plates. After 2 weeks, hybridoma culture supernatants were harvested and tested for HeLa-cell staining by flow cytometry. Positive supernatants were then further characterized by immunoprecipitation. Three of these mAbs (all IgG1) were used in this study: MIR-12A12 recognizes a 60 kDa unidentified molecule not associated with tetraspans (results not shown); TS151r is an anti-CD151 mAb; and V5-vjf is an anti-α5β1 mAb.

Flow cytometry

For indirect immunofluorescence, cells were incubated on ice for 30 min in RPMI 1640 medium containing 10 % fetal calf serum, washed three times in the same solution and incubated with $F(ab')_2$ goat anti-mouse IgG coupled to fluorescein (Immunotech, Lumminy, France). The cells were washed three times, re-suspended in PBS containing 1 % formaldehyde and analysed using a Facscalibur (Becton-Dickinson, San Jose, CA, U.S.A.). For direct fluorescence, the cells were incubated with phycoerythrin-labelled anti-α3 integrin mAb and/or biotin-labelled mAbs (SYB-1, 11B1.G4 or TS151r) in the same conditions before washing three times with PBS containing 0.2 % BSA. They were then incubated with allophycocyanin-labelled streptavidin (Becton-Dickinson) and analysed using a Facscalibur equipped with a 635 nm red-diode laser.

Plasmids

A full-length cDNA for CD151 was provided by Dr. L. Ashman (Institute of Medical and Veterinary Sciences, Adelaide, Australia) [29] and a β1-integrin subunit cDNA by Dr. F. Altruda (University of Torino, Torino, Italy) [32]. These two plasmids were subcloned in the mammalian expression vector pCDNA3 (Invitrogen, Carlsbad, CA, U.S.A.). The integrin α3 subunit cDNA in the mammalian expression vector pFneo was provided by Dr. M. Hemler (Dana Farber Cancer Institute, Boston, MA, U.S.A.) [33].

Cell lines, cell culture and transfection

The megakaryocytic cell line HEL, the B cell line Raji, the pre-B cell line Nalm-6, and the T cell line Jurkat were cultured in RPMI 1640 medium (Eurobio, Les Ulis, France) supplemented with 10 % fetal calf serum, 2 mM glutamine and antibiotics (all from Life Technologies, Cergy-Pontoise, France). The adherent cell lines HeLa (cervical carcinoma), A431 (squamous carcinoma), LoVo (colon carcinoma) HT1080 (fibrosarcoma) and CHO (Chinese hamster ovary) were grown in Dulbecco's modified Eagle's medium (Eurobio). CHO and HeLa cells were electroporated as described previously [5] and the cells were analysed 2 days after transfection.

Cell labelling and immunoprecipitation

Surface labelling of cells with EZ-link-Sulpho-NHS-LC-biotin was performed as described previously [5]. Adherent cells were lysed directly in the tissue-culture flask (2 ml for a 75-cm² flask) and haematopoietic cells were lysed at the concentration of 2×10^7/ml in a lysis buffer containing 1 % detergent [Brij 97 (Sigma, St. Louis, MO, U.S.A.) or digitonin (Boehringer, Meylan, France)], 10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.02 % NaN_3, 1 mM PMSF, 0.5 μg/ml leupeptin, 1 μg/ml pepstatin A and 10 kallikrein-inhibitory units/ml aprotinin. Digitonin was first dissolved in methanol at a concentration of 10 % (w/v) and then diluted in lysis buffer without $CaCl_2$ and $MgCl_2$. After 30 min incubation at 4 °C, the insoluble material was removed by centrifugation at 10000 ***g*** and the cell lysate was precleared overnight by addition of an irrelevant mouse IgG mAb and Protein G–Sepharose beads (Pharmacia, Uppsala, Sweden). Proteins were then immunoprecipitated by adding to the supernatant 10 μg/ml mAb (or 1:200 ascites fluid) and 1:20 (v/v). Protein G–Sepharose beads. After 5 h incubation at 4 °C under constant agitation, the beads were washed five times in lysis buffer. For reprecipitation of integrin subunits, the molecules co-precipitated with tetraspans were eluted with 1 % Nonidet P-40 in lysis buffer and re-precipitated using specific anti-integrin antibodies.

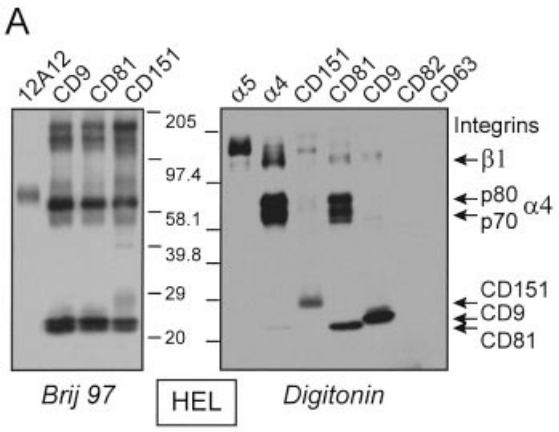
SDS/PAGE, blotting and quantification

The proteins were separated by SDS/PAGE (5–15 % gel) under non-reducing conditions to obtain a better separation of CD9 and CD81 on the one hand, and of the α and β chains of β1 integrins on the other hand. The proteins were transferred on to a PVDF membrane (NEN, Boston, MA, U.S.A.) in a buffer containing 25 mM Tris, 192 mM glycine and 20 % (v/v) methanol, and the membrane was blocked overnight in 5 % (w/v) non-fat dry milk in Tris-buffered saline containing 0.05 % Tween 20. Biotinylated surface proteins were probed by incubation with a 1:400 dilution of a streptavidin-biotinylated horseradish peroxidase complex (Amersham Pharmacia Biotech, Rainham, Essex, U.K.). Specific antigens were probed by a combination of biotin-labelled mAbs and the streptavidin-biotinylated horseradish peroxidase complex, and revealed by enhanced chemiluminescence (NEN). Densitometry analysis was performed with a personal densitometer (Molecular Dynamics, Evry, France). Only films in which the bands of interest were not saturated were used for this analysis.

RESULTS

Only CD81 and CD151 co-immunoprecipitate integrin-like molecules from digitonin lysates of haematopoietic cells

All tetraspan mAbs tested so far co-precipitate a number of molecules after lysing the cells in the mild non-ionic detergents of



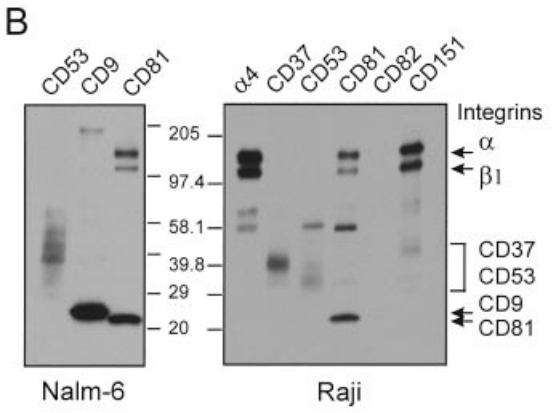
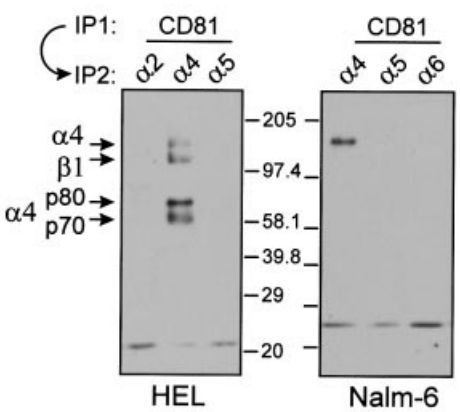


Figure 1 Pattern of proteins co-precipitated by anti-tetraspan mAbs in haematopoietic cells

Biotin-labelled cells were lysed in Brij 97 or digitonin and precipitations with anti-tetraspan or anti-integrin mAbs were performed as indicated on the top of each lane. (**A**) Immunoprecipitates collected from Brij 97 and digitonin lysates of HEL cells. (**B**) Immunoprecipitates collected from digitonin lysates of Raji and Nalm-6 cells. CD81 co-precipitated integrin heterodimers in all cell lines tested, and CD151 only in Raji cells. Note the co-precipitation of CD81 by the anti-integrin $\alpha 4$ mAb in HEL cells in digitonin. CD82 and CD63 did not appear on the blots because of the poor labelling by biotin. This also explains the absence of detection of CD151 in Raji cells at this exposure. The CD9 mAb was SYB-1, the $\alpha 4\beta 1$ mAb was 9F10 and the $\alpha 5\beta 1$ mAb was V5-vjf.



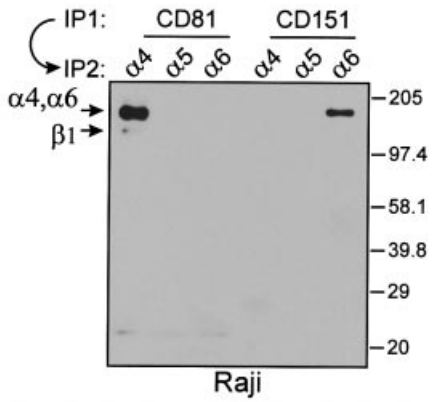


Figure 2 Identification of the molecules co-precipitated with CD81 and CD151 in digitonin lysates of haematopoietic cells

Biotin-labelled Nalm-6, HEL and Raji cells were lysed in digitonin before immunoprecipitation (IP1) of CD81 and CD151 complexes. The proteins co-precipitated were eluted with 1% Nonidet P-40 and reprecipitated with anti-integrin mAbs (Gi9, $\alpha 2$; HP2/1, $\alpha 4$; SAM-1, $\alpha 5$; GoH3, $\alpha 6$) as indicated (IP2). The integrin $\alpha 4\beta 1$, but not the other integrins, was present in the CD81 immunoprecipitates obtained from the three cell lines. In Raji cells, the CD151 immunoprecipitate contained the integrin $\alpha 6\beta 1$, but not $\alpha 4\beta 1$. Because of the low levels of integrins present on these cells, the $\beta 1$-integrin subunit, less labelled than the α subunits, is not always visible after the second precipitation.

Table 1 Expression of integrins by cultured cells

Cell-surface antigen expression was determined by flow cytometry. Representation of the mean fluorescence intensity compared with the control staining is based on the following scale: −, negative; ±, < 6; +, > 6, ⩽ 25; ++, > 25, ⩽ 100; +++, > 100, ⩽ 400; ++++, > 400. ND, not determined.

Integrin	Cell type...	HEL	Nalm-6	Raji	Jurkat	HeLa	A431	LoVo	HT1080
$\alpha 1$		−	−	−	−	+++	−	−	++
$\alpha 2$		+	−	−	−	+	+++	+++	++++
$\alpha 3$		−	−	−	−	++	++++	+	++++
$\alpha 4$		++	++	+	++	−	−	−	+
$\alpha 5$		++	++	−	+	+++	+++	+	+++
$\alpha 6$		±	+	+	−	+	+++	++++	+++
$\beta 4$		−	−*	−	−*	+	+++	++++	ND

* Data from Leucocyte Typing V [41].

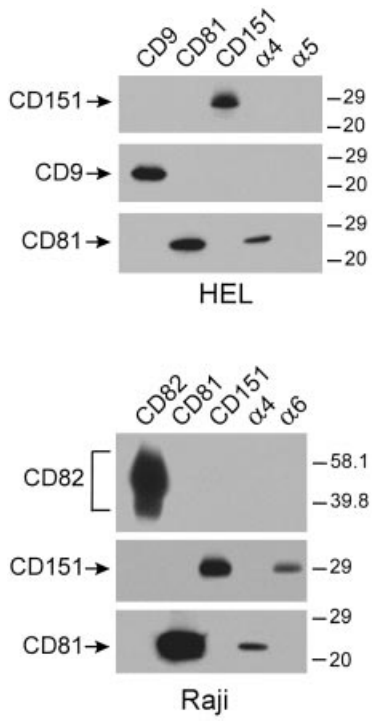


Figure 3 α4β1 co-precipitates CD81 and α6β1 co-precipitates CD151 in digitonin

Unlabelled HEL and Raji cells were lysed in digitonin and the solubilized proteins were immunoprecipitated as indicated on the top of each lane before Western-blot analysis with biotin-labelled anti-tetraspan mAbs. The anti-α4 mAb was 9F10, the anti-α5 mAb was V5-vjf and the anti-α6 was mAb 4F10. Note the presence of CD81 in the α4 immunoprecipitates and CD151 in the α6 immunoprecipitate.

the Brij series or in the zwitterionic detergent CHAPS [5,6,21]. As an example, the pattern of molecules co-precipitated from HEL cells by tetraspan mAbs, in the presence of Brij 97, is shown in Figure 1(A). Clearly, the same molecules are present in CD9, CD81 and CD151 immunoprecipitates. Among them, CD9 (24 kDa) and CD81 (23 kDa) can be easily identified. The patterns of co-precipitation obtained from Brij 97 extracts of Raji and NALM-6 cells have been described recently [24].

Most of the proteins co-precipitated with tetraspans were dissociated when the cells were lysed with digitonin (Figure 1). In particular, CD9 and CD81 were not co-precipitated with the other tetraspans under these conditions. CD9, CD37, CD53, CD63, CD82 (Figure 1) and Talla-1 (in Jurkat, results not shown) did not efficiently co-precipitate integrin-like molecules in the presence of digitonin. In contrast, CD81 co-precipitated molecules co-migrating with the α and β chains of integrins in all haematopoietic cell lines tested (Figure 1). In the megakaryocytic cell line HEL, the α4 subunit is cleaved into two polypeptides, non-covalently linked, of 80 and 70 kDa [3,34], which were apparently co-precipitated with CD81. CD151 strongly co-precipitated an integrin-like molecule from Raji cells. Interestingly, this is the only haematopoietic cell line studied here that expresses substantial levels of the α6 integrin subunit, probably associated with the β1 subunit since it is devoid of the β4 subunit (Table 1).

CD81 co-immunoprecipitates the α4β1 integrin and CD151 co-immunoprecipitates α6β1 integrin from digitonin lysates of haematopoietic cells

To identify the molecules co-precipitated with CD81 and CD151 after digitonin lysis, the proteins co-precipitated with these molecules were eluted in the presence of Nonidet P-40 before reprecipitation with anti-integrin mAbs (Figure 2). The integrin α4β1 was present in CD81 immunoprecipitates collected from all haematopoietic cell lines, but was not found in the CD151 immunoprecipitates of Raji cells (Figure 2). In contrast, in Raji cells, the integrin α6β1 was present in CD151 immunoprecipitates, but was not found in the CD81 immunoprecipitates. The integrins α2β1 and α5β1 could not be reprecipitated from the tetraspan immunoprecipitates.

α4β1 integrin only co-immunoprecipitates CD81 and α6β1 integrin only co-immunoprecipitates CD151 when cells are lysed in digitonin

Due to the fact that tetraspans are less biotin-labelled than integrins, the reciprocal immunoprecipitations were analysed by Western blotting (Figure 3). The integrin α4β1 co-precipitated a fraction of CD81 from HEL cells, but not CD9, CD151 (Figure 3, upper panel), CD63 or CD82 (results not shown). Similarly, it co-precipitated CD81 from Raji cells, but not CD151 or CD82 (Figure 3, lower panel). In contrast, the α6β1 integrin only co-precipitated CD151 (Figure 3, lower panel). These experiments

Table 2 Expression of tetraspans by cultured cells

Cell-surface antigen expression was determined by flow-cytometry. Representation of the mean fluorescence intensity compared with the control staining is based on the following scale: −, negative; ±, < 6; +, > 6, ⩽ 25; ++, > 25, ⩽ 100; +++, > 100, ⩽ 400; ++++, > 400. ND, not determined.

Antigen	Cell type...	HEL	Nalm-6	Raji	Jurkat	HeLa	A431	LoVo	HT1080
CD9		+++	++++	−	+	++++	++++	++++	+
CD37		+	−	+++	−	−*	−*	ND	ND
CD53		++	++	+++	+	−*	−*	ND	ND
CD63		++	++	+	±	+++	+	++	++
CD81		+++	+++	++++	++	+++	+++	++	+++
CD82		+++	+	++	++	+	+++	++	+++
CD151		+++	+	+++	+	+++	++++	++++	++++
Co-029		−	−	−	−	−	−	++++	−
Talla-1		−	−	−	++	−	ND	ND	ND
NAG-2		+	−	−	−	ND	ND	−	++

* Data from Leucocyte Typing V [41].

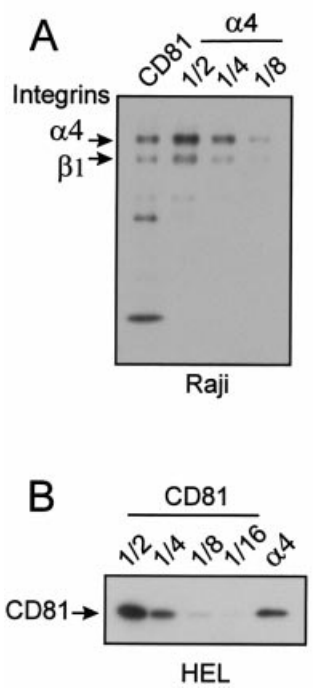


Figure 4 Quantification of the CD81/α4β1 association

(**A**) Serial dilutions of the α4 immunoprecipitate of biotin-labelled Raji cells are compared with the CD81 immunoprecipitate (see Figure 1). (**B**) Serial dilutions of the CD81 immunoprecipitate of unlabelled HEL cells are compared with the α4 immunoprecipitate after immunoblotting with a biotin-labelled CD81 mAb (see Figure 3).

further demonstrate that the tetraspans are not co-precipitated by other tetraspans in digitonin.

Stoichiometry of the CD81/α4β1 and CD151/α6β1 associations

The fractions of CD81 and integrin α4β1 associated together were estimated by densitometry both in HEL cells, which have a similar expression of the two molecules, and in Raji cells, which express 5–10 times more CD81 than α4β1 (Tables 1 and 2). Because the signal generated by enhanced chemiluminescence is not linear, serial dilutions of the immunoprecipitates giving the strongest signals were compared with the other immunoprecipitates. This analysis was possible because both the CD81 and the α4β1 mAbs immunoprecipitate most of the target antigens in our experimental conditions (results not shown). Figure 4(A) shows that CD81 co-precipitated ≈ 25% of α4β1 from Raji cells. Similar results were obtained in HEL cells (results not shown). Reciprocally, the α4β1 mAb co-precipitated ≈ 25% of CD81 in HEL cells (Figure 4B), but a smaller fraction in Raji cells (Figure 3), probably because of the large excess of CD81 in this cell line (Tables 1 and 2).

A similar analysis showed that α6β1 co-precipitated ≈ 30% of CD151 from Raji-cell digitonin extracts (results not shown). These data, together with the data obtained by flow cytometry (Tables 1 and 2), suggest that the majority of the α6β1 heterodimers were associated with CD151 in this cell line.

Only CD9, CD81 and CD151 co-immunoprecipitate high-molecular-mass molecules from digitonin lysates of adherent cells

The study of tetraspan associations conserved in the presence of digitonin was then extended to adherent cells in order to study

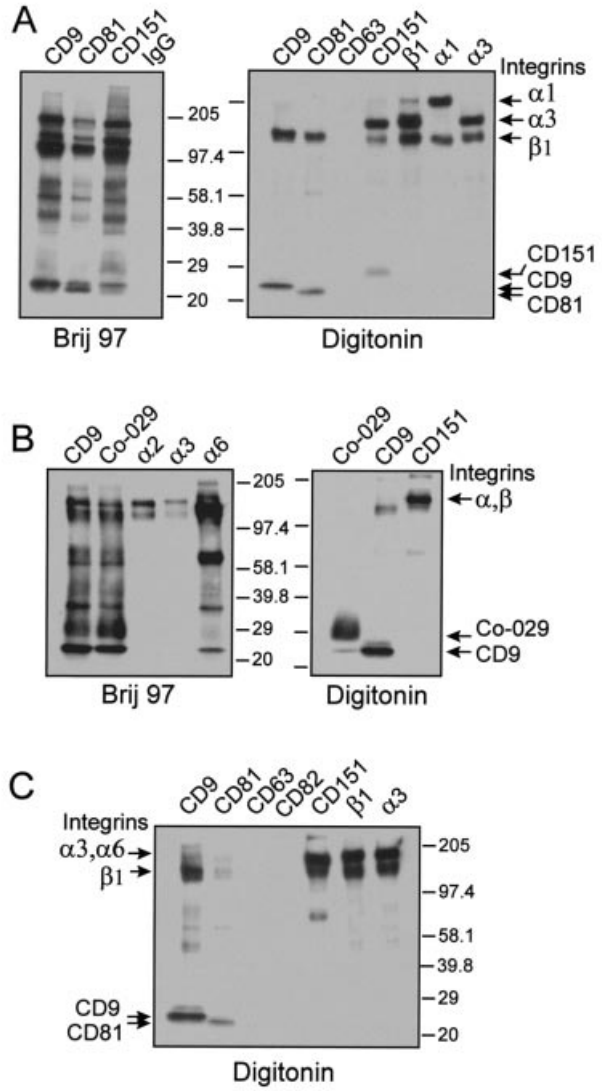


Figure 5 Pattern of proteins co-precipitated with tetraspans and integrins in adherent cells

Biotin-labelled cells [(**A**) HeLa; (**B**) LoVo; (**C**) A431] were lysed in Brij 97 or in digitonin and precipitations with anti-tetraspan or anti-integrin mAbs were performed as indicated on the top of each lane. CD82 and CD63 did not appear on the blots because of the poor labelling by biotin, but were immunoprecipitated efficiently as determined by Western blotting. A poor labelling also explains the absence of detection of CD151 in LoVo and A431 cells at these exposures. The anti-tetraspan mAbs used for these immunoprecipitations were SYB-1 (CD9), Z81 (CD81), 11B1.G4 (CD151), H5C6 (CD63), γC11 (CD82) and AZM-22 (Co-029). The anti-integrin mAbs were HP2B6 (α1), Gi9 (α2), M-KID-2 (α3) and GoH3 (α6)

tetraspans and integrins not expressed by haematopoietic cell lines (Tables 1 and 2). Among these tetraspans, the complexes formed by CD9, CD81 CD63 and CD82 have been studied extensively. Figures 5(A) and 5(B) show that CD151 and Co-029 co-precipitate from Brij 97 lysates the same molecules as the other tetraspans, among which integrins could be identified definitively by elution/reprecipitation experiments (results not shown).

Similar to the results obtained with haematopoietic cell lines, most of the proteins co-precipitated with tetraspans were dissociated when the cells were lysed with digitonin. Here again, CD9, CD81 and Co-029 were not co-precipitated with the other

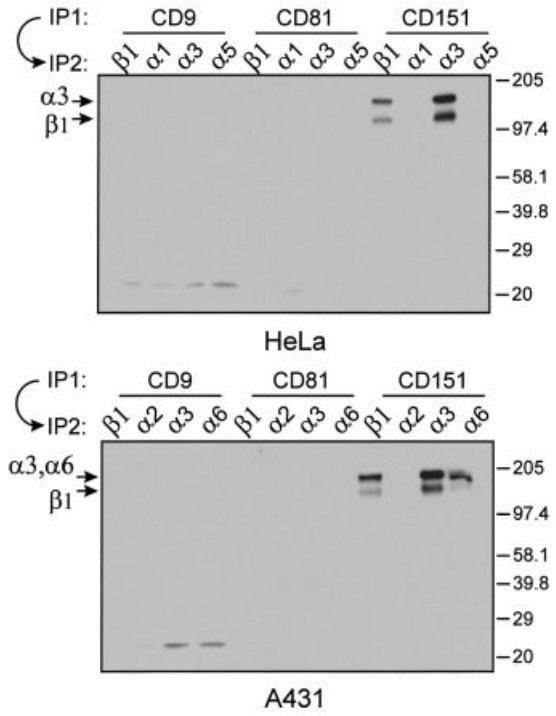


Figure 6 Identification of the molecules co-precipitated with CD151 in digitonin lysates of HeLa and A431 cells

Biotin-labelled HeLa or A431 cells were lysed in digitonin and precipitations (IP1) with CD9, CD81 and CD151 mAbs were performed. The proteins co-precipitated were eluted with 1% Nonidet P-40 and reprecipitated (IP2) with anti-integrin mAbs as indicated. In HeLa cells, the integrin $\alpha3\beta1$, but not $\alpha1\beta1$ or $\alpha5\beta1$, was present in the CD151 immunoprecipitate. In A431 cells, both integrins $\alpha3\beta1$ and $\alpha6\beta1$ were present in the CD151 immunoprecipitate, but not the integrin $\alpha2\beta1$. No integrin could be reprecipitated from CD9 and CD81 immunoprecipitates.

tetraspans in these conditions. A band co-migrating with CD9 was present in the Co-029 immunoprecipitate of LoVo cells (Figure 5A). This band was still observed after lysing the cells in the presence of Nonidet P-40 and SDS (results not shown) and was therefore likely to be a non-glycosylated form of Co-029. Indeed, a protein of this size is recognized by Western blotting [35].

In digitonin, CD151 co-precipitated integrin-like molecules from all cell lines and CD9 and CD81 co-precipitated a molecule the size of the $\beta1$ integrin ($\approx$ 125 kDa in non-reducing conditions, $\approx$ 135 kDa in reducing conditions). CD63, CD82, Co-029 (Figure 5) and NAG-2 (results not shown) did not co-precipitate other proteins from HeLa, A431, LoVo or HT1080 cells respectively.

CD151 co-immunoprecipitates both $\alpha3\beta1$ and $\alpha6\beta1$ integrins from digitonin extracts of epithelial cell lines

The molecules co-precipitated with CD151 were identified as the integrins $\alpha3\beta1$ and $\alpha6\beta1$ by elution/reprecipitation experiments (Figure 6). $\alpha1\beta1$, $\alpha2\beta1$ or $\alpha5\beta1$ were not present in the CD151 immunoprecipitates. No integrin could be reprecipitated from CD9 or CD81 immunoprecipitates (Figure 3), suggesting that the 125 kDa molecule co-precipitated with these tetraspans was not the $\beta1$ integrin subunit. Indeed, in Western-blotting experiments, this molecule was not recognized by a $\beta1$ integrin mAb, under conditions where the $\beta1$ integrin was clearly identified in the CD151 immunoprecipitate (results not shown).

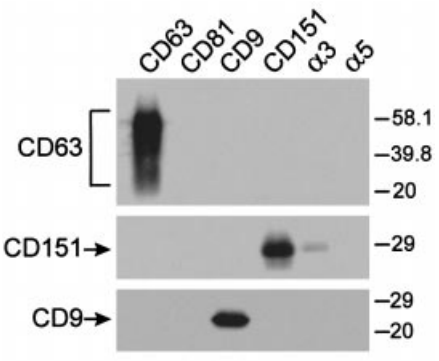


Figure 7 $\alpha3\beta1$ co-precipitates CD151 in digitonin but not other tetraspans

Unlabelled HeLa cells were lysed in digitonin and the solubilized proteins were immunoprecipitated as indicated on the top of each lane before Western-blot analysis using biotin-labelled anti-tetraspan mAbs. The mAbs used were H5C6 (CD63), Z81 (CD81), SYB-1 (CD9), 11B1.G4 (CD151), M-KID 2 ($\alpha3$) and V5-vjf ($\alpha5$).

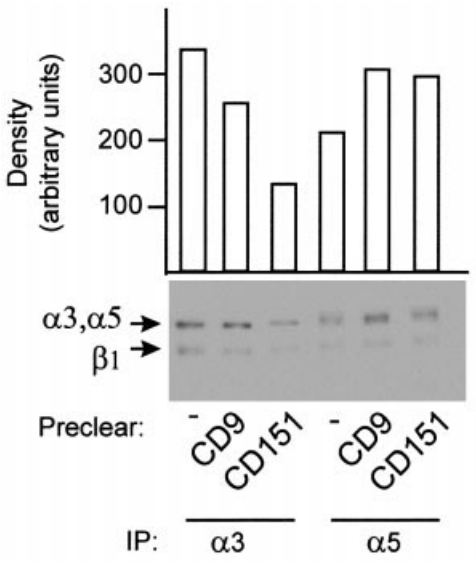


Figure 8 Quantification of the CD151/$\alpha3\beta1$ association

Biotin-labelled HeLa cells were lysed in digitonin and subjected to three rounds of preclearing with CD9 or CD151 mAbs before immunoprecipitation (IP) of integrins $\alpha3$ and $\alpha5$ with mAbs M-KID-2 and V5-vjf respectively. The proteins were then transferred to a PVDF membrane, probed with streptavidin peroxidase and revealed by chemiluminescence. The integrin $\alpha3$- and $\alpha5$-subunit bands were then quantified by densitometry.

$\alpha3\beta1$ and $\alpha6\beta1$ co-immunoprecipitate CD151 but not other tetraspans when the cells are lysed in digitonin

Longer exposures of the blots (results not shown) demonstrated the presence of a molecule co-migrating with CD151 in integrin $\alpha3\beta1$ and $\alpha6\beta1$ immunoprecipitates of digitonin-lysed cells. The presence of CD151 in these immunoprecipitates, but not in $\alpha5\beta1$ immunoprecipitates, was confirmed by Western blotting (Figure 7 and results not shown). Neither CD9 nor CD63 was co-precipitated with these integrins in HeLa cells. Moreover, these experiments confirm the dissociation of tetraspan–tetraspan complexes in digitonin.

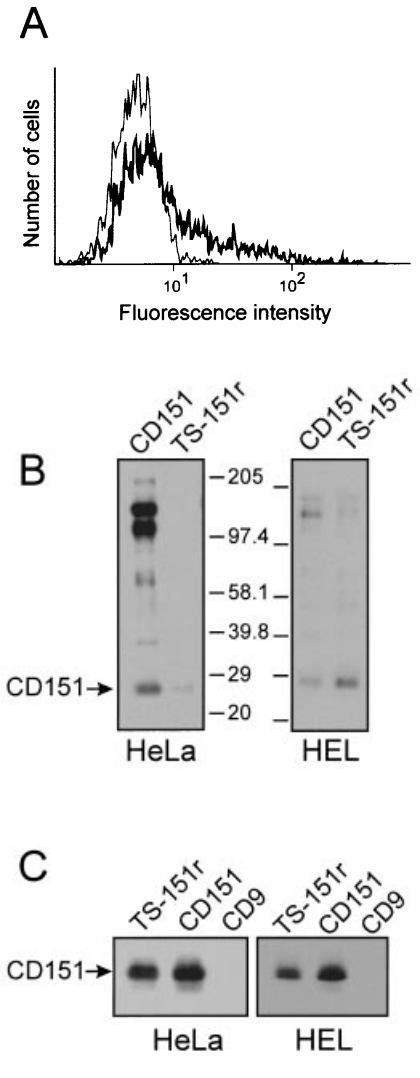


Figure 9 Characterization of a new anti-CD151 mAb

TS151r was produced after immunizing mice with HeLa cells. (**A**) CHO cells were transfected with a CD151 cDNA and analysed by flow cytometry. Thin line, control; thick line, TS151r staining. (**B**) Biotin-labelled HeLa or HEL cells were lysed in digitonin and immunoprecipitations with mAbs 11B1.G4 (CD151) and TS151r were performed. The faint band co-precipitated with TS151r from HEL extract is probably not a β1 integrin since its size was repeatedly too great and no β1 subunit was detected. (**C**) Unlabelled HeLa or HEL cells were lysed in digitonin and immunoprecipitations with mAbs 11B1.G4, TS151r or SYB-1 (anti-CD9) were performed. The presence of CD151 in the immunoprecipitates was assessed by Western blotting with the biotin-labelled CD151 mAb (11B1.G4).

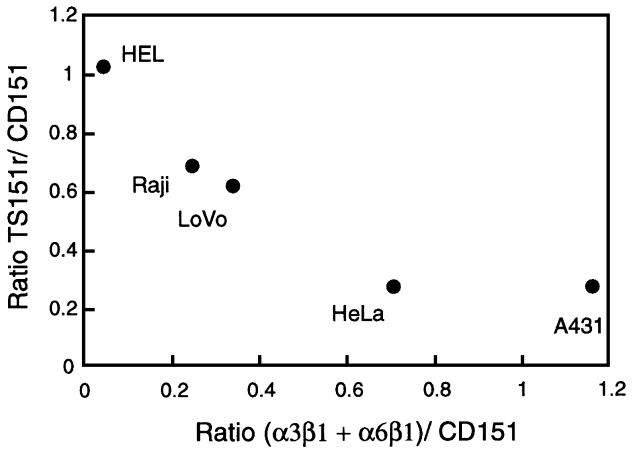


Figure 10 TS151r mAb has a restricted expression: inverse relationship with $\alpha3\beta1 + \alpha6\beta1$ expression

The expression of CD151, α6, α3 and β4 integrins was determined by indirect immunofluorescence with mAbs 11B1.G4, 4F10, M-KID 2 and 450–9D respectively, and by flow-cytometric analysis. The staining of mAb TS151r was measured similarly. The graph shows the ratio of TS151r/11B1.G4 mean fluorescence intensity (both CD151 mAb) as a function of the ratio ($\alpha3\beta1 + \alpha6\beta1$ expression)/CD151 expression where $\alpha3\beta1 + \alpha6\beta1$ expression is the sum of α3 and α6 mean fluorescence intensities from which is removed the integrin β4 mean fluorescence intensity.

Stoichiometry of the CD151/$\alpha3\beta1$ association

The stoichiometry of the CD151/$\alpha3\beta1$ relationship in HeLa cells was then assessed by preclearing the lysates with CD151 mAbs before precipitation of $\alpha3\beta1$, or of $\alpha5\beta1$ as a control. The CD151 preclearing reduced the amount of $\alpha3\beta1$ that could be immunoprecipitated by $\approx 60\,\%$, but had no effect on $\alpha5\beta1$ immunoprecipitation (Figure 8).

Identification of a new mAb recognizing an epitope of CD151 blocked by the association with the integrin $\alpha3\beta1$

TS151r was produced after immunizing mice with HeLa cells. After initial screening by indirect immunofluorescence, it was selected for further studies because it co-precipitated both CD9 and CD81. It was identified subsequently as a CD151 mAb based on the recognition of CD151-transfected CHO cells (Figure 9A) and cross-blocking with CD151 mAbs (results not shown). This mAb co-precipitates from digitonin lysates of both HEL and HeLa cells a biotinylated molecule co-migrating with CD151 (Figure 9B), recognized by Western blot using the CD151 mAb 11B1.G4 (Figure 9C).

TS151r did not co-precipitate any integrin in digitonin (Figure 9B and results not shown). It immunoprecipitated less CD151 than 11B1.G4 in HeLa cells, which express both $\alpha3\beta1$ and $\alpha6\beta1$, but not in HEL cells, which only express low levels of $\alpha6\beta1$ (Figure 9B). We therefore hypothesized that this mAb was only able to recognize CD151 when it was not associated with integrins. In favour of this hypothesis, it should be noted that there is an inverse relationship between the number of CD151 molecules recognized by TS151r and the expression of $\alpha3\beta1$ plus $\alpha6\beta1$ in various cell lines (Figure 10). For example, in Raji cells, which express $\alpha6\beta1$, the staining intensity of TS151r corresponds to the difference between 11B1.G4 staining and α6 staining. This tendency is also discernible in adherent cell lines expressing both $\alpha3\beta1$ and $\alpha6\beta1$ (Figure 10).

We reasoned that if the mAb TS151r was actually directed against a discrete epitope of CD151 molecules not associated with integrins, overexpression of $\alpha3\beta1$ should induce a decrease of the TS151r staining. For this purpose, HeLa cells were transiently transfected with plasmids encoding β1 and α3 integrins. The average level of $\alpha3\beta1$ was increased by approx. three times, as determined by indirect immunofluorescence, with some cells expressing up to 15-fold more than the mean level of mock-transfected cells. The gradual increase in $\alpha3\beta1$ integrin expression quantitatively decreased TS151r staining (Figure 11) without modification of CD151 expression, as determined by the lack of modification of 11B1.G4 staining. The staining of HeLa cells with the CD9 mAb SYB-1 was not modified (results not shown).

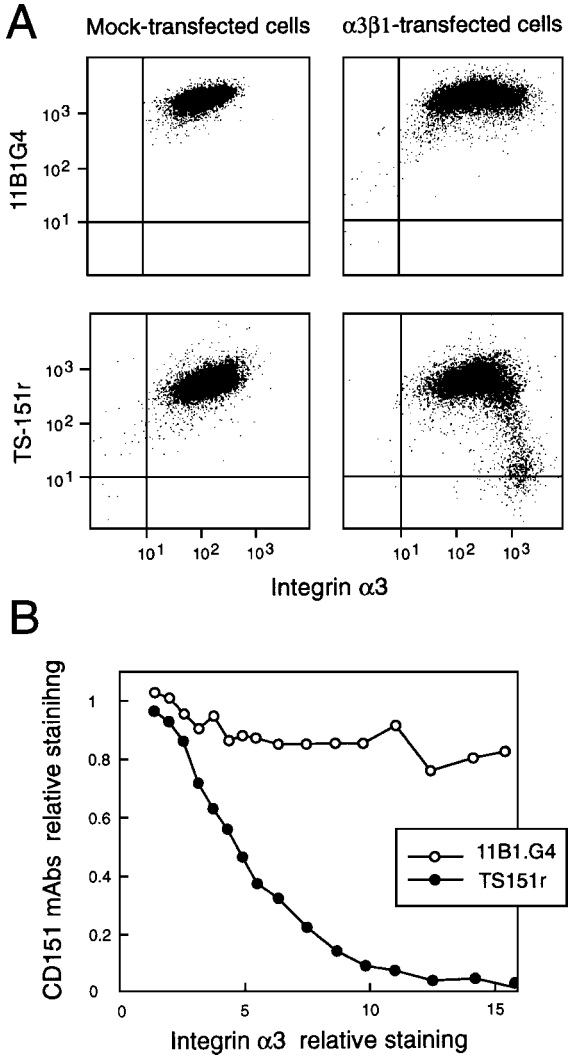


Figure 11 Effect of overexpression of integrin α3β1 on the binding of mAb TS151r

HeLa cells were transiently transfected with anti-cDNA encoding $\beta 1$ and $\alpha 3$ integrin subunits or mock-transfected. (**A**) After 48 h cells were harvested and analysed by two-colour flow cytometry for $\alpha 3\beta 1$ and 11B1.G4 (anti-CD151) or $\alpha 3\beta 1$ and TS151r staining. The binding of TS151r, but not of 11B1.G4, is decreased in cells overexpressing $\alpha 3\beta 1$. (**B**) Several region gates were generated along the *x* axis ($\alpha 3$ staining) every 100 arbitrary units (below 1000 units) or 200 units (above 1000 units), and the mean values obtained in each gate for $\alpha 3$ (*x* axis), 11B1.G4 or TS151r (*y* axis) staining were determined. The relative intensities correspond to the ratio of these values to the mean fluorescence intensity of mock-transfected cells.

DISCUSSION

A striking feature of the tetraspans is their lack of apparent specificities, since tetraspan mAbs produce on the same cell types similar effects such as homotypic aggregation, co-stimulation of T cells and inhibition of cell migration [12]. This is thought to be related to their association in multimolecular complexes (the tetraspan web) containing several tetraspans and $\beta 1$ integrins, such as $\alpha 3\beta 1$, $\alpha 4\beta 1$ and $\alpha 6\beta 1$ [3–7], which may provide an explanation of their effect on migration.

This study demonstrates that selective tetraspan–integrin associations can be observed after lysing the cells in digitonin, a detergent that disrupts tetraspan–tetraspan interactions. Under these conditions, only two tetraspan molecules co-precipitated integrins: CD81 selectively co-precipitated the $\alpha 4\beta 1$ integrin in all cells expressing this molecule whereas CD151 co-precipitated $\alpha 3\beta 1$ and $\alpha 6\beta 1$, but not $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 4\beta 1$ or $\alpha 5\beta 1$. Reciprocally, an anti-$\alpha 4\beta 1$ mAb selectively co-precipitated CD81 whereas anti-$\alpha 3\beta 1$ and anti-$\alpha 6\beta 1$ mAbs co-precipitated CD151. These anti-integrin mAb did not co-precipitate other tetraspans.

The selective association of a restricted subset of integrins with either CD81 or CD151 suggests a tight association of these molecules. Because in most cases no other molecule is co-precipitated with CD81 or CD151, their association with integrins is likely to be direct. The other tetraspans, which do not co-precipitate integrins in the presence of digitonin, probably associate indirectly with the integrin heterodimers. The fact that the tetraspan interactions are disrupted by digitonin suggests that this indirect association is mediated through CD81 or CD151.

The distinctive association of CD151 with $\alpha 3\beta 1$ was strengthened by the finding that ectopic expression of $\alpha 3\beta 1$ integrin quantitatively decreased the binding of a new mAb, TS151r, recognizing a discrete epitope of CD151. This, together with the fact that this mAb does not co-precipitate integrins in digitonin, suggests that its target epitope is masked by the association with $\alpha 3\beta 1$ or $\alpha 6\beta 1$ integrins. Accordingly, there is an inverse relationship between the number of CD151 molecules recognized by TS151r and the expression of $\alpha 3\beta 1$ plus $\alpha 6\beta 1$ in various cell lines: there is no difference between 11B1.G4 (anti-CD151) staining and TS151r staining in HEL cells ($\alpha 3\beta 1$-negative and weakly $\alpha 6\beta 1$-positive), and this difference corresponds to the staining of $\alpha 3\beta 1$ plus $\alpha 6\beta 1$ in Raji and LoVo cells. The difference between 11B1.G4 (CD151) staining and TS151r staining may therefore be a measure of $\alpha 3\beta 1$/CD151 complexes. In A431 cells, which express similar levels of CD151 and $\alpha 3\beta 1$, 70–80% of CD151 and $\alpha 3\beta 1$ may be complexed. In HeLa cells, which express a higher level of CD151 than integrins, this difference corresponds to approx. two thirds of $\alpha 3\beta 1$ staining, suggesting that in this cell line not all $\alpha 3\beta 1$ integrin is associated with CD151. This is validated by the fact that preclearing HeLa digitonin extracts with the CD151 mAb 11B1.G4 removed $\approx 60\%$ of $\alpha 3\beta 1$. The level of CD81/$\alpha 4\beta 1$ association was also quantified, after immunoprecipitations. CD81 co-precipitates ≈ 30–50% of this integrin in HEL cells, which express similar levels of CD81 and $\alpha 4\beta 1$, and $\approx 25\%$ in Raji cells, which express much more CD81 than $\alpha 4\beta 1$. Whether some disruption of the complexes occurs during cell lysis remains to be determined.

As shown in Figure 1, the $\beta 1$ integrins are not the only molecules to be co-precipitated with tetraspans in Brij 97 [5,6]. Most of these tetraspan-associated molecules were dissociated from tetraspans in the presence of digitonin. Moreover, CD37, CD53, CD63, CD82, Talla-1, NAG-2 and Co-029 did not co-precipitate any other molecules in digitonin lysates. That they do not co-precipitate associated molecules in the presence of digitonin may signify that they interact directly only with other tetraspans. Alternatively, they may associate with molecules that are not labelled, possibly because they are intracellular or do not have a large extracellular domain. One of the tetraspans may for example connect phosphatidylinositol 4-kinase to the tetraspan web [36]. It is also possible that they associate with partner molecules in a way that is disrupted by digitonin. In this respect, it should be noted that HLA-DR antigens, which are clearly co-precipitated with CD9, CD37, CD53, CD81 and CD82 in lymphoid B cell lines (including Raji cells) [5,21,23], could not be found in any of the tetraspan immunoprecipitates obtained from digitonin lysates of Raji cells (Figure 3). Energy-transfer experiments have confirmed that the tetraspans associate with HLA-DR and have suggested that CD53 was closer to HLA-DR than CD81 and CD82 [37].

Finally, the tetraspans that do not co-precipitate associated molecules in digitonin may contribute to other properties of the tetraspan web. This may be especially the case for CD63 which has a Gly-Tyr lysosomal targeting signal in its short C-terminal cytoplasmic domain [27]. CD63 is indeed localized in lysosomes in platelets, but also in intracellular granules in multiple cell types such as neutrophils, lymphocytes and endothelial cells [27,38–40]. CD63 may associate intracellularly to granular proteins and may allow the connection of these proteins to the tetraspan web upon stimulation.

In conclusion, our data provide evidence that among the tetraspans, CD81 is associated preferentially with the $\alpha 4\beta 1$ integrin, and CD151 with both $\alpha 3\beta 1$ and $\alpha 6\beta 1$. These two tetraspans are likely to be responsible for the connection of these integrins to other tetraspans, through tetraspan–tetraspan interactions. Other tetraspans may similarly link other molecules to the whole set of tetraspans. The organization of the tetraspans in a tetraspan web may allow the crosstalk of different kinds of associated molecules on the cell surface.

We are grateful to Dr. F. Altruda, Dr. L. Ashman, Dr. F. Berditchevski, Dr. J. Bréard, Dr. J. Brochier, Dr. H. Conjeaud, Dr. M. Hemler, Dr. P. Natali and Dr L. Zardi for the generous gifts of cDNA and antibodies. This work was supported by the Association Nouvelles Recherches Biomédicales, by the Association pour la Recherche contre le Cancer and by the Institut de Cancérologie et d'Immunogénétique.

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Received 13 November 1998/18 January 1999; accepted 17 February 1999