Expression of the Palmitoylation-deficient CD151 Weakens the Association of \( \alpha_3\beta_1 \) Integrin with the Tetraspanin-enriched Microdomains and Affects Integrin-dependent Signaling*

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Transmembrane proteins of the tetraspanin superfam-ily are assembled in multimeric complexes on the cell surface. Spatial orientation of tetraspanins within these complexes may affect signaling functions of the associated transmembrane receptors (e.g. integrins, receptor-type tyrosine kinases). The structural determinants that control assembly of the tetraspanin complexes are unknown. We have found that various tetraspanins and the \( \alpha_3 \) integrin subunit are palmitoylated. The stability and molecular composition of the palmitoylated \( \alpha_3\beta_1 \)-tetraspanin complexes are not affected by adhesion. To assess the significance of palmitoylation in the function of the \( \alpha_3\beta_1 \)-tetraspanin complexes we mapped the sites of palmitoylation for CD51. Mutation of six cysteines, Cys11, Cys15, Cys79, Cys80, Cys242, and Cys243 was necessary to completely abolish palmitoylation of CD51. The association of the palmitoylation-deficient mutant of CD51 (CD51Cys8) with CD81 and CD63 was markedly decreased, but the interaction of the \( \alpha_3\beta_1 \)-CD51Cys8 complex with phosphatidylinositol 4-kinase was not affected. Ectopic expression of CD51Cys8 in Rat-1 cells impaired the interactions of the endogenous CD63 and CD81 with the \( \alpha_3\beta_1 \) integrin. Although the expression of the palmitoylation-deficient CD51 does not change cell spreading on the extracellular matrix, the number of focal adhesions increased. Adhesion-induced phosphorylation of PKB/c-Akt is markedly increased in cells expressing a palmitoylation-deficient mutant, thereby providing direct evidence for the role of the tetraspanin microdomains in regulation of the integrin-dependent phosphatidylinositol 3-kinase signaling pathway. In contrast, activation of FAK and ERK1/2 were not affected by the expression of CD51Cys8. Our results demonstrate that palmitoylation of tetraspanins is critical not only for the organization of the integrin-tetraspanin microdomains but also has a specific role in modulation of adhesion-dependent signaling.

Tetraspanins constitute a large family of widely expressed four-transmembrane domain proteins (1). Although the biochemical function of tetraspanins remains unclear, recent gene knock-out experiments and numerous in vitro data strongly indicate that tetraspanins play an important role in generating immune responses, membrane dynamics and fusion, cell migration, and invasion (2–4).

An important biochemical feature of tetraspanins is their ability to aggregate with one another and with various other transmembrane receptors into multimeric clusters often referred to as the “tetraspanin web” or tetraspanin-enriched microdomains (or TERM) (2, 3). It has been proposed that within these microdomains tetraspanins may regulate spatial orientation and lateral interactions of the receptors thereby affecting their biological activities (3, 4). The molecular mechanisms that govern the recruitment of tetraspanins into TERM and hierarchy of their interactions within these microdomains are not well established. It has been proposed that although each of the tetraspanin components of TERM has a specific proximal non-tetraspanin partner, no preferential partnerships are formed between the tetraspanins themselves (2). Thus, the receptor cross-communication within TERM may be controlled by a variety of tetraspanin-tetraspanin interactions.

Recent reports have established that while the association with a non-tetraspanin partner is controlled by the large extracellular loop of tetraspanins (5–7), this part of the protein is not required for the interaction of tetraspanin with one another (7). The association between the tetraspanins occurs at the later stages during the protein biosynthesis (presumably, in Golgi or post-Golgi compartments (7)). This suggests the following possibilities: (i) the interaction requires a post-translational modification of the proteins (e.g. glycosylation, lipidation); (ii) there may be sorting mechanisms that compartmentalize tetraspanins in proximity to one another thereby allowing the interaction to occur. Although glycosylation may play an important role in association of tetraspanins with non-tetraspanin partners and their biological activities (8), it is unlikely to contribute significantly to the tetraspanin-tetraspanin interactions. First, the sequence analysis predicts that in most tetraspanins sites of glycosylation are located within the large extracellular loop. Second, CD81, a tetraspanin that does not have consensus glycosylation motifs, is readily incorporated into TERM (9). Protein lipidation (prenylation, myristoylation, and palmitoylation) is one of the most common covalent modifications of cellular proteins (10, 11). It has been shown that acylation may

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1 The abbreviations used are: TERM, tetraspanin-enriched microdomains; PI, phosphatidylinositol; PKB, protein kinase B; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; DMEM, Dulbecco’s modified Eagle’s medium; ECM, extracellular matrix; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; 2BP, 2-bromopalmitate; mAb, monoclonal antibody; Ab, antibody.
Fig. 1. Palmitoylation of tetraspanins. MDA-MB-231 cells were metabolically labeled overnight with \([3H]\)palmitic acid. The cells were lysed in buffer containing 1% Triton X-100 (A) or 1% Brij98 (D), and protein complexes were immunoprecipitated using specific anti-tetraspanin or anti-integrin mAbs. The mAbs were ALB-6 to CD9, 6H1 to CD81, M38 to CD81, M104 to CD82, 5C11 to CD151, P1B5 to \(\alpha_2\) integrin subunit, A2-VIIc8 to \(\alpha_2\) integrin subunit, 187.1 as a negative control. B, the cells were lysed in buffer containing Triton X-100, and the CD151 complex was immunoprecipitated with 5C11 mAb. The complex was dissociated with 0.1% SDS at 85 °C, and the eluate was subjected to re-precipitation with polyclonal Abs to the \(\alpha_2\) integrin subunit and CD151.

Influence protein stability, subcellular localization, and can play an important role in overall re-distribution of proteins at the plasma membrane (12, 13). In turn, this compartmentalization of acylated proteins into microdomains can facilitate protein-protein interactions and affect their biological functions (11, 15).

Earlier studies have shown that tetraspanins CD9 and CD81 are linked to saturated acyl chains (16–18). Given their intrinsic propensity to assemble into tightly packed microdomains (19), saturated acyl chains may stabilize and control spatial orientation of proteins within the tetraspanin complexes. Indeed, recent reports (20, 21) and our present study demonstrate that mutations of the palmitoylation sites in CD9 and CD151 affect the association of the tetraspanins with one another within TERM. Importantly, here we show for the first time that (i) palmitoylation of tetraspanins is crucial for the recruitment of the associated non-tetraspanin partners into TERM, and (ii) palmitoylation-dependent localization of the \(\alpha_3\beta_1\) integrin in TERM selectively affects its signaling potential.

MATERIALS AND METHODS

Cells, Antibodies, and Reagents—MDA-MB-231 cells were maintained in Leibovitz L-15 medium supplemented with 10% fetal calf serum. Rat-1 and Rat-1 transfectants were routinely maintained in DMEM supplemented with 10% fetal calf serum. The Rat-1/CD151wt, Rat-1/CD151Cys, Rat-1/CD151Cys2, Rat-1/CD151Cys3, and Rat-1/CD151Cys8 cell lines were generated by transfecting pZeosSV/CD151, pZeosSV/CD151Cys, pZeosSV/CD151Cys2, pZeosSV/CD151Cys3, and pZeosSV/CD151Cys8 plasmids, respectively, into Rat-1 cells. Zeocin-resistant colonies in each transfection experiment were pooled together (30–40 individual colonies). To obtain the cells that homogeneously express the variants of CD151, the pool of the zeocin-resistant colonies was subjected to two cycles of panning using an immobilized mixture of anti-CD151 mAbs, 5C11 (22) and 11G1B4. Antibodies to CD151 (mouse mAb 11G1B4 and rabbit polyclonal sera) were kindly provided by Dr. L. Ashman (University of Newcastle, Australia). Mouse mAb against the cytoplasmic tail of the \(\alpha_2\) integrin subunit was from Dr. A. Sonnenberg (Netherlands Cancer Institute, Amsterdam). Rabbit polyclonal sera directed against the cytoplasmic tail of the \(\alpha_2\) integrin subunit was from Dr. F. Watt (Imperial Cancer Research Fund, London). Mouse anti-CD81 rat mAb was kindly provided by Dr. E. Geisert, Jr. (University of Tennessee). Mouse anti-\(\alpha_2\) rat mAb was purchased from the Development Study Hybridoma Bank. Hamster mAb against rat \(\beta_1\) integrin subunit was purchased from BD PharMingen. Hamster mAb to rat CD81 was purchased from Serotec. Rabbit anti-phospho-specific Abs to FAK were from BioSource International. Rabbit anti-FAK Ab was purchased from Autogen Bioclear. All antibodies to PKB were purchased from New England Biolabs. All other reagents were purchased from Sigma.

Construction of CD151 Mutants—The CD151 mutants were engineered by a standard two-step PCR protocol on the pZeoCD151 template. The PCR fragments were subcloned (HindIII-EcoRI) into the pZeosSV plasmid.

Immunoprecipitation—Cells were labeled overnight with \([9,10^{-}\text{H}]\)palmitic acid (PerkinElmer Life Sciences) in DMEM (or L-15 medium) supplemented with 2% fetal calf serum. The proteins were solubilized into the immunoprecipitation buffer containing 1% Brij98/PBS (or 1% Triton X-100/PBS), 2 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin for 2–16 h at 4 °C. The insoluble material was pelleted at 12,000 rpm for 10 min, and the cell lysates were precleared by incubation for 4 h at 4 °C with agarose beads conjugated with goat anti-mouse antibodies (Sigma). Immune complexes were collected on the agarose beads prebound with mAbs, followed by four successive washes with the immunoprecipitation buffer. Immune complexes were eluted from the beads with Laemmli sample buffer, and proteins were resolved in 11% SDS-PAGE. The gels were dried and exposed to the x-ray Kodak film for 14–42 days.

Analysis of Activation of FAK and ERK1/2—The serum-starved cells
genates were mixed with 2 volumes of 2M sucrose, 20 mM Tris-HCl, the MICROSON sonicator (Misonix) on power setting 10. The homogenates were sonicated (4 times) with a 25-gauge needle. The homogenates were sonicated (4 times) with a 25-gauge needle. The immunoprecipitated proteins were resolved in 8% SDS-PAGE, transferred to the nitrocellulose membrane, and probed with appropriate phospho-specific Ab according to the manufacturer’s instructions.

Analysis of Activation of PKB-Ab and PI 4-Kinase—Serum-starved cells plated on the laminin-5 matrix were scraped into Triton X-100 (or Brij98)–based lysis buffer, and the immune complexes were isolated as described above. For the analysis of PKB activation equal aliquots of the immunoprecipitated proteins were resolved in 8% SDS-PAGE, transferred to the nitrocellulose membrane, and probed either with phospho-specific (anti-Ser173) Ab or with the polyclonal Ab recognizing total PKB. For the analysis of PI 4-kinase activation, the intact or CD151 complexes were immunoprecipitated from the equalized amounts of the protein lysates, and the activity of the associated kinase was examined as described earlier (24).

Immunofluorescence Staining—For immunofluorescence analysis cells were grown on glass coverslips for 16–24 h. Cells were fixed for 7–10 min with 2% paraformaldehyde, in PBS, containing 5% sucrose and 0.1 mM MgCl2, and then treated with 1% Brij98 in PBS for 2 min. In some experiments, fixed cells were permeabilized with 0.1% Triton X-100 for 30 s. Coverslips were blocked for 1 h with 20% heat-inactivated normal goat serum in PBS. Cells were then stained with primary mAbs diluted in 20% heat-inactivated normal goat serum in PBS. Staining was subsequently visualized with FITC-conjugated goat antimouse serum (Sigma) before the coverslips were mounted with FluorSave (Calbiochem), and immunofluorescence was examined using a Zeiss AxioScope. Serial Z-sections (0.2 μm) of stained cells captured with the Coolview CCD camera (Photonic Sciences) were digitally saved by using the Biovision software package (Bio-Rad). The images were further processed by using a digital deconvolution module of the OpenLab software package (Improvement).

Sucrose Gradient Fractionation—Cells were scraped into PBS, pH 7.4, supplemented with protease and phosphatase inhibitors (1 mM Na3VO4, 1 mM NaF, 10 mM sodium pyrophosphate, 2 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 10 μg/ml leupeptin). The cells were washed twice with the PBS (containing the inhibitor mixture) and resuspended in 100 mM Na2CO3, pH 11.0 (containing the inhibitor mixture). The cells were then broken by passing 20 times through a MICROSON sonicator (Misonix) on power setting 10. The homogenates were mixed with 2 volumes of 2 M sucrose, 20 mM Tris-HCl, pH 7.6, 100 mM NaCl containing the protease inhibitors and overlaid with solutions containing decreasing concentrations of sucrose (0.2–7 M) of stained cells captured with the Coolview CCD camera (Photonic Sciences) were digitally saved by using the Biovision software package (Bio-Rad). The images were further processed by using a digital deconvolution module of the OpenLab software package (Improvement).

Flow Cytometry—Cells were incubated with saturating concentrations of primary mouse mAbs for 45 min at 4 °C, washed twice, and then labeled with FITC-conjugated goat anti-mouse immunoglobulin. Stained cells were analyzed on a FACScan (BD Pharmingen).

RESULTS

Tetraspanins Are Palmitoylated—It has been previously shown that the tetraspanin CD9 is palmitoylated in platelets (17). To investigate whether other members of the tetraspanin superfamily undergo similar modification, MDA-MB-231 cells were metabolically labeled with [3H]palmitic acid and subjected to the immunoprecipitation analysis. The cells were lysed using Triton X-100 and the tetraspanin complexes precipitated using specific mAbs to CD9, CD63, CD81, CD82, and CD36. The precipitates were analyzed on a second round of immunoprecipitation experiments. The CD151 complex isolated from the prelabeled MDA-MB-231 cells was first dissociated with 0.2% SDS at 85 °C and subsequently subjected to a second round of immunoprecipitation using polyclonal antibodies either to the cytoplasmic tail of the α3 subunit or to CD151. As shown in Fig. 1C, the anti-α3 Ab precipitated only a 150-kDa protein (lane 2), thus indicating that the α3 subunit is indeed palmitoylated. Surprisingly, the anti-CD151 Ab precipitated not only the 27-kDa protein but also a 210-kDa protein. We concluded that the α3 subunit was palmitoylated.
kDa protein (lane 3). These results indicate that the 210-kDa protein may represent a homomultimer of CD151 or a covalently linked complex between CD151 and another cellular protein.

To examine the association of palmitoylated tetraspanins with one another and to investigate which other palmitoylated proteins might be associated with the tetraspanin network, we carried out the immunoprecipitation experiments using conditions that do not interfere with the tetraspanin-tetraspanin interactions (e.g., in the presence of 1% Brij98) (26). The patterns of 3H-labeled proteins co-immunoprecipitated with different anti-tetraspanin and anti-α5 mAbs were comparable: six major protein bands with calculated molecular mass values of 150, 46–60, 27, 24, 22, and 16 kDa were detected (Fig. 1D, lanes 2–5). No labeled proteins were immunoprecipitated using the anti-α5 or negative control mAbs (Fig. 1D, lanes 1 and 6). These data not only indicate that palmitoylation does not affect the tetraspanin-tetraspanin and tetraspanin-α5β1 interactions but also suggest that acylated tetraspanins are combined in large protein aggregates. Given the results of immunoprecipitation experiments carried out in the presence of Triton X-100 (Fig. 1A), we surmised that 150-, 46–60, 27-, 24-, 22- and 16-kDa proteins correspond to the α5 integrin subunit, CD63, CD151, CD9, and CD81, respectively. The identity of the 16-kDa protein is currently being investigated. In addition, the anti-CD81 and anti-CD151 mAbs immunoprecipitated the proteins of 40 and 210 kDa that were absent from the other immunoprecipitates (Fig. 1D, lanes 4 and 5). Thus, the CD81–40kD and CD151–210kD complexes are unlikely to be a part of the tetraspanin microdomains.

The Effect of Cell Adhesion on the Palmitoylation of Tetraspanins and α5β1 Integrin—It has been previously shown that ligand binding modulates palmitoylation of G protein-coupled receptors (27). To investigate whether interaction with the extracellular matrix (ECM) affects palmitoylation of α5β1 and tetraspanins, we compared the patterns of protein palmitoylation in complexes purified from the detached cells and from the cells that were plated on collagen type I and laminin-5-containing ECM for 60 min. The patterns of palmitoylated proteins immunoprecipitated with either anti-α5 or anti-CD151 mAbs were similar for all experimental conditions (Fig. 2). In additional experiments we found no changes in the overall composition of the complexes in cells plated on the ECM ligands for 30 min or 2 h (results are not shown). These results indicate that integrin-mediated cell adhesion does not grossly influence interaction of palmitoylated tetraspanins with one another and with α5β1 integrin.

The Role of Palmitoylation in the Cellular Distribution and Compartmentalization of Tetraspanins—Fatty acylation plays an important role in membrane compartmentalization of many cellular proteins (11). Furthermore, lipidation may influence interactions between proteins within membrane compartments (15). 2-Bromopalmitate (2BP), a potent inhibitor of protein palmitoylation, was used to examine whether palmitoylation plays a role in cellular distribution of tetraspanins and their association with each other. First, we established that the treatment of MDA-MB-231 cells with 25 μM 2BP for up to 36–40 h (e.g., prior and during the labeling) completely abolished labeling of tetraspanins and the α5 integrin subunit with [3H]palmitate (Fig. 3A). As indicated in Fig. 3B the treatment with 2BP had only a minor effect on the association of CD9 with CD151 and the α5β1 integrin; densitometric analysis has shown that the amounts of CD9-associated CD151 and α5β1 decreased by ~30% (compare lanes 1 and 2 and lanes 4 and 5). In contrast, incubation with 2BP had no influence on the stability of the α5β1–CD151 complex (Fig. 3B, lanes 2 and 5 on the upper panel).

Immunofluorescence analysis has shown that the distribution of the complexes in MDA-MB-231 cells pretreated with 2BP is comparable with that seen in non-treated cells (Fig. 4A); in agreement with earlier studies (28) we found that the complexes were abundant at the cell periphery and on intracellular vesicles. It has been recently shown that the tetraspanin-enriched microdomains are co-fractionated into the light
The effect of 2BP on the cellular distribution and compartmentalization of the α3β1-tetraspanin complexes. A, MDA-MB-231 cells pretreated with 2BP (or Me2SO) for 48 h were detached and replated on glass coverslips precoated with the laminin-5-containing ECM for 1 h in the presence of 2BP (or Me2SO). Cells were fixed with paraformaldehyde and permeabilized with 1% Brij98. Indirect immunofluorescence staining was carried out using mAbs to CD81 (M38) and the α3 integrin subunit (29A3). Staining was visualized using FITC-conjugated goat anti-mouse IgG. B, MDA-MB-231 cells were treated as described in the legend to A. The cells were scraped from the plates, and protein homogenates derived were fractionated using discontinuous 0.2–0.9 M sucrose gradient as described under “Materials and Methods.” The protein content of each fraction was adjusted to equal concentrations. Distribution of proteins in the fractions was assessed by immunoblotting. The Abs used were: either rabbit polyclonal Abs (CD151, α3 integrin subunit, caveolin) or mouse mAb Syb-1 (CD9). The experiments were carried out twice with a similar outcome.
Expression of the Palmitoylation-deficient CD151

A. CD151/Cys

Cys15 → Ala
Cys11 → Ala

CD151/Cys2

Cys15 → Ala
Cys242 → Ala

CD151/Cys3

Cys79 → Ala
Cys243 → Ala

CD151/Cys8

Cys15 → Ala
Cys79 → Ala
Cys242 → Ala
Cys243 → Ala

B. 3H-palm.

WB:CD151

CD151/Cys

Cys15 → Ala
Cys11 → Ala

CD151/Cys2

Cys15 → Ala
Cys242 → Ala

CD151/Cys3

Cys79 → Ala
Cys243 → Ala

CD151/Cys8

Cys15 → Ala
Cys79 → Ala
Cys242 → Ala
Cys243 → Ala

D. IP:

CD63
CD81

WB:CD151

PB:α3

anti-α3

anti-CD151

anti-CD151

anti-CD151

anti-CD151

anti-CD151

anti-CD151

anti-CD151

anti-CD151

Fig. 5. Mapping the sites of palmitoylation in CD151. A, schematic diagram showing mutation variants of CD151 used in the transfection experiments. B and C, Rat-1 transfectants were labeled with [3H]palmitic acid overnight. The cells were lysed in buffer containing 1% Triton X-100 (B) or 1% Brij98 (C), and the CD151 complex was immunoprecipitated using 5C11 mAb. The amounts of precipitated proteins were verified by Western blotting with the anti-CD151 rabbit polyclonal Ab. D, the tetraspanin complexes were immunoprecipitated from the Brij98 lysates and then resolved in 11% SDS-PAGE under either non-reduced (for detection of CD81 and CD151) or reduced (for detection of α3 integrin subunits) conditions. The proteins were transferred to a nitrocellulose membrane which were probed with rabbit polyclonal Abs to CD151 and α3 or mAb to CD81. E, the protein complexes were purified from the Brij98 lysates using anti-β3 integrins and anti-CD151 mAbs. The phosphoinositide kinase activity in the immunoprecipitates was assayed as described in Ref. 24. The results of a representative of three experiments are shown.
they also demonstrate that CD151 plays a critical role in linking other tetraspanins to the \(\alpha_\beta_1\) integrin.

We have previously demonstrated that \(\alpha_\beta_1\)-tetraspanin complexes are associated with PI 4-kinase (24, 30). Furthermore, it has recently been reported that this enzyme can be palmitoylated (31). To examine whether palmitoylation contributes to the association of PI 4-kinase with the CD151-\(\alpha_\beta_1\) integrin complexes, we performed a lipid kinase assay on the immunoprecipitated complexes purified from Rat-1/CD151wt and Rat-1/CD151Cys8 cells (Fig. 5E). In these experiments we also examined whether adhesion to the ECM affects the association (or activation) of the enzyme with the tetraspanin complexes. Fig. 5E illustrates that anti-CD151 and anti-\(\alpha_\beta_1\) mAbs immunoprecipitated similar amounts of the enzymatic activity from the transfectants. These results not only indicate that adhesion does not affect the activity of PI 4-kinase associated with the \(\alpha_\beta_1\)-CD151 complex but also show that palmitoylation has no role in this interaction.

The Role of Palmitoylation in Signaling Mediated by the \(\alpha_\beta_1\)-Tetraspanin Complexes—The fact that the expression of the palmitoylation-deficient mutant of CD151 profoundly decreases the association of \(\alpha_\beta_1\) with other tetraspanins allowed us to assess the contribution of the tetraspanin microdomains in integrin-dependent signaling. First, we compared cellular compartmentalization of the ectopically expressed CD151wt and CD151Cys8 proteins in Rat-1 cells. The immunofluorescence staining showed that cellular distribution of the palmitoylation-deficient CD151 was similar to that of the wild-type protein (Fig. 6A); the proteins were detected on the cell surface and intracellular vesicles. Similarly, no differences were found in the distribution of \(\alpha_\beta_1\) integrin in these cells (results are not shown). Furthermore, we established that CD151wt and CD151Cys8 proteins have identical flotation properties in the sucrose gradient (Fig. 6B). Thus, these experiments confirmed that blocking of palmitoylation has no gross effect on the cellular distribution of the \(\alpha_\beta_1\)-CD151 complexes. Next, we examined how dissociation of the \(\alpha_\beta_1\)-CD151 complex from TERM affects the biological activities of the integrin. Fig. 7 illustrates that, although adhesion and spreading of Rat-1 cells expressing the mutated and wild-type CD151 were comparable, assembly of focal adhesions was somewhat more pronounced in cells expressing CD151Cys8. Not only were the total number of focal adhesions significantly increased but they were also more evenly spread throughout the basal surface of the cells (Fig. 7B, left panels, Rat-1/CD151wt; right panels, Rat-1/CD151Cys8). To understand the biochemical basis of these differences we examine how plating of the cells on the ECM affects various adhesion-dependent signaling pathways. To this end serum-starved cells were plated on the laminin-5-containing ECM for varying lengths of time and activation of various adhesion-dependent signaling pathways was examined using activation specific Ab. As shown in Fig. 8A, the kinetics and amplitude of the adhesion-dependent phosphorylation of FAK and Erk1/2 were comparable for both cell lines. On the other hand, we observed that the levels of phosphorylation of PKB/e-Akt in the attached Rat-1/CD151Cys8 cells was significantly higher than in Rat-1 cells expressing wild-type CD151 (Fig. 8B). These data demonstrate that localization of the \(\alpha_\beta_1\)-CD151 complex in the tetraspanin-enriched microdomains has a specific effect on the adhesion-dependent modulation of the PI 3-kinase signaling pathway.

**DISCUSSION**

Multiplicity and apparent redundancy of protein-protein interactions involving tetraspanins pose a major problem in assessing the contribution of an individual tetraspanin to a par-
ticular biological phenomenon such as cell migration, cell-cell adhesion, and co-stimulatory processes in T- and B-cells. Here we have shown that tetraspanins are palmitoylated and palmitoylation is important for the assembly of the tetraspanin-enriched microdomains. It has to be emphasized that although palmitic chains facilitate co-aggregation of different tetraspanins, their role in establishing and maintaining direct protein-protein contacts within TERM is less apparent. Indeed, treatment of cells with 2-bromopalmitate under the conditions that completely block incorporation of \([3H]\)palmitate into cellular proteins has only a modest effect on the tetraspanin-tetraspanin interactions. Thus, we propose a two-step model of the assembly of TERM. First, palmitoylated tetraspanins (each in association with a specific non-tetraspanin partner) are aggregated, possibly as a consequence of preferential coalescence of saturated acyl chains (19, 32). Second, direct contacts between tetraspanin polypeptides are established that may lead to subsequent re-organization of the tetraspanin aggregates.

Previous biochemical studies suggested that CD151 has a pivotal role in linking the \(\alpha_\beta_1\) integrin to other tetraspanins (5, 7). Not only does this study provide direct experimental support for this idea, but we also demonstrate that palmitoylation of CD151 is critical for its function in sorting of \(\alpha_\beta_1\). It has been recently proposed that CD36, an unrelated palmitoylated membrane protein, is also implicated in surface compartmentalization of \(\beta_1\) integrins (34). Specifically, it was shown that the ectopic expression of CD36 increases the amounts of \(\beta_1\) integrin (mainly, \(\alpha_\beta_1\) and \(\alpha_\beta_1\)) in the Triton X-100-resistant lipid rafts (34). Although additional studies are required to establish the relationship between CD36 and the \(\alpha_\beta_1\)-CD151 complex, one possibility is that CD36 controls distribution of \(\beta_1\) integrins between TERM and cholesterol-rich lipid rafts. Indeed, although a major portion of the \(\alpha_\beta_1\)-tetraspanin complexes are sensitive to extraction with Triton X-100, small amounts can be found in the Triton X-100-insoluble fractions of sucrose gradient (29).

Site-directed mutagenesis has revealed that CD151 can be palmitoylated at multiple sites; simultaneous mutation of six cysteines (Cys\(^{11}\), Cys\(^{15}\), Cys\(^{79}\), Cys\(^{80}\), Cys\(^{242}\), and Cys\(^{243}\)) is required to eliminate palmitoylation. Multiple palmitoylation sites were also identified in the tetraspanin CD9 (20). However, in contrast to CD151 corresponding cysteines within the second transmembrane domain of CD9 do not seem to function as acceptor sites for palmitic acid. Why do tetraspanins need multiple acyl chains attached to their polypeptide backbone? Not only would this make the membrane sorting of tetraspanins more efficient (see above), but multiple palmitates may also stabilize intramolecular interactions between their various domains. For example, tight packing of saturated acyl chains within the inner leaflet of the surface membrane may facilitate the alignment of transmembrane domains of tetraspanins rel-

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**Fig. 7. Expression of a palmitoylation-deficient mutant of CD151 affects assembly of focal adhesions.** Serum-starved cells were plated on the laminin-5-containing ECM for 1 h. Cells were fixed with paraformaldehyde and permeabilized with 0.1% Triton X-100. Indirect immunofluorescence staining (B) was carried out using mAb to vinculin (hvin-1). Staining was visualized using FITC-conjugated goat anti-mouse IgG. Images of parallel cultures of fixed non-permeabilized cells (A) were taken using the Hitachi CCD camera. Left panels are Rat-1/CD151wt cells; right panels are Rat-1/CD151Cys8 cells.
A number of other palmitoylated cellular proteins are co-immunoprecipitated with tetraspanins (Figs. 1D and 5C). One of these proteins has been identified as the α5 integrin subunit. To our knowledge this is a first report describing palmitoylation of any integrin subunit. Although the functional significance of the integrin palmitoylation remains to be established, our results show that it is dependent on the palmitoylation of CD151; the level of palmitoylation of the integrin in the complex with CD151Cys8 is significantly reduced when compared with the α5β1-CD151wt complex. One possibility is that palmitoylation of α5 is more efficient within the tetraspanin-enriched microdomains. Recent results have shown that palmitoyltransferase activity is compartmentalized on the surface membrane (35). Alternatively, palmitoylation-deficient CD151 may hinder the accessibility of the α5 transmembrane cysteine for the enzyme. Although the identity of other palmitoylated proteins associated with the α5β1-tetraspanin complex remains unknown, we were able to exclude two of the potential candidates. It has been reported that the α5β1 integrin is associated with caveolin, the principal structural component of caveolae (36). Although caveolin is palmitoylated in MDA-MB-231 cells, it does not associate with the α5β1-tetraspanin complexes (results are not shown). Furthermore, in these cells we found no evidence for the association of the complexes with palmitoylated Fyn, a tyrosine kinase that is known to be abundant in the cholesterol-rich microdomains (results are not shown). The lack of the association between α5β1-tetraspanin complexes with Fyn and caveolin further supports the idea that palmitoylated tetraspanins are sorted into membrane microdomains with a distinct repertoire of the associated proteins.

Recent observations demonstrate that membrane compartmentalization into cholesterol-rich lipid rafts controls activation state and signaling potency of integrins (37–39). Here we found that adhesion-dependent signaling is controlled by compartmentalization of integrins into microdomains, which lay outside of cholesterol-rich lipid rafts; uncoupling of the α5β1-CD151 complex from the endogenous tetraspanins potentiates assembly of focal adhesions and has a positive effect on activation of PI 3-kinase-dependent signaling pathway. These results suggest that signaling heterogeneity of integrins is even more complex than previously thought i.e. localization into cholesterol-rich lipid rafts → activation of integrins → potentiation of adhesion-dependent signaling) (38). Furthermore, our data not only directly links TERM to regulation of a specific signaling pathway(α5β1) triggered by α5β1, but also implies that the association with these microdomains negatively affects its signaling potential.

Although in this study we did not specifically examine the link between PI 3-kinase and focal adhesions, several earlier reports placed PI 3-kinase upstream of RhoA GTPase, a protein that is known to play a key role in the assembly of focal adhesions (40–42). The mechanisms of the adhesion-dependent activation of PI 3-kinase are not fully understood and may involve complementary signals from various small GTPases and tyrosine kinases (43). It remains to be established which signaling pathway connects the α5β1-CD151 complex with PI 3-kinase. Earlier studies linked integrin-dependent activation of PI 3-kinase with FAK (44, 45). Specifically, it was found that phosphorylation of FAK on tyrosine 397 induces assembly of the FAK-PI 3-kinase (class I) complex and activation of the kinase. However, our results show that adhesion-induced phosphorylation of Tyr397 of FAK was similar in cells expressing the wild-type and palmitoylation-deficient mutant of CD151. Furthermore, we found that the association of PI 3-kinase (class I) and FAK was comparable in the Rat-1 transfection.
The sites does not affect the activity of PI 4-kinase associated with cells. PI 4-kinase catalyzes synthesis of phosphatidylinositol 4-phosphate, a substrate for PI 3-kinase to generate phosphatidylinositol 3,4-bisphosphate, which, in turn, activates PKB. However, our results show that mutation of palmitoylation sites does not affect the activity of PI 4-kinase associated with the αβ2-CD151 complex.

In summary, we have demonstrated that palmitoylation is an important step in the assembly of the tetraspanin-enriched microdomains. Importantly, using a palmitoylation-deficient mutant of CD151, we have provided clear evidence that this tetraspanin has a pivotal role in the compartmentalization of the αβ2 integrin into TERM. Finally, our data indicate that CD151-induced entry into TERM selectively negates certain signaling pathways triggered by αβ2 integrin.

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