Palmitoylation supports assembly and function of integrin–tetraspanin complexes

Xiwei Yang, Oleg V. Kovalenko, Wei Tang, Christoph Claas, Christopher S. Stipp, and Martin E. Hemler
Dana-Farber Cancer Institute and Department of Pathology, Harvard Medical School, Boston, MA 02115

As observed previously, tetraspanin palmitoylation promotes tetraspanin microdomain assembly. Here, we show that palmitoylated integrins (α3, α6, and β4 subunits) and tetraspanins (CD9, CD81, and CD63) coexist in substantially overlapping complexes. Removal of β4 palmitoylation sites markedly impaired cell spreading and signaling through p130Cas on laminin substrate. Also in palmitoylation-deficient β4, secondary associations with tetraspanins (CD9, CD81, and CD63) were diminished and cell surface CD9 clustering was decreased, whereas core α6β4–CD151 complex formation was unaltered. There is also a functional connection between CD9 and β4 integrins, as evidenced by anti-CD9 antibody effects on β4-dependent cell spreading. Notably, β4 palmitoylation neither increased localization into “light membrane” fractions of sucrose gradients nor decreased solubility in nonionic detergents—hence it does not promote lipid raft association. Instead, palmitoylation of β4 (and of the closely associated tetraspanin CD151) promotes CD151–α6β4 incorporation into a network of secondary tetraspanin interactions (with CD9, CD81, CD63, etc.), which provides a novel framework for functional regulation.

Introduction

The α6β4 integrin appears on epithelial and other types of cells, acts as a receptor for basement membrane laminin-5 and related laminin isoforms, and plays a key role during cell migration and tumorigenesis (Belkin and Stepp, 2000; Mercurio et al., 2001). In response to EGF receptor (EGFR) stimulation, α6β4 disconnects from the intermediate filament cytoskeleton and becomes associated with the actin cytoskeleton in lamellipodia and membrane ruffles (Mercurio et al., 2001). During this process, EGFR signaling might activate the Src family kinase fyn, leading to phosphorylation of β4 on tyrosine (Maimiero et al., 1996; Mariotti et al., 2001), or might activate conventional PKC, leading to β4 phosphorylation on serine (Rabinovitz et al., 1999). Consistent with cooperative signaling between α6β4 and growth factor receptors, α6β4 has been suggested to physically associate with fyn (Mariotti et al., 2001), EGFR (Mariotti et al., 2001), ErbB2 (Gambaletta et al., 2000; Hintermann et al., 2001), c-met (Trusolino et al., 2001), and Ron (Santoro et al., 2003).

The laminin-binding integrins (α6β4, α3β1, α6β1, and α7β1) not only form a distinct subgroup among integrins in terms of amino acid sequence similarity, but also show robust association with tetraspanin proteins (Hemler, 1998; Berditchevski, 2001). There are 32 mammalian tetraspanins, and at least a few of these are abundantly present on nearly all cell and tissue types. Tetraspanin proteins regulate cell motility, morphology, fusion, and signaling in the brain and immune system, on tumors, and elsewhere (Levy et al., 1998; Boucheix and Rubinstein, 2001; Hemler, 2001; Stipp et al., 2003b). Tetraspanins CD151, CD81, and CD9 can modulate α3β1 and α6β1 integrin-dependent neurite outgrowth, cell migration, and/or cell morphology (Vázquez-Mó et al., 1998; Yau et al., 1998; Stipp and Hemler, 2000; Kazarov et al., 2002; Zhang et al., 2002). Of particular relevance here, CD151 associates with α6β4 to regulate kidney epithelial cell morphology (Yang et al., 2002), whereas CD9–α6β4 complexes may affect primary keratinocyte cell motility (Jones et al., 1996; Baudoux et al., 2000).

Associations of tetraspanins with each other are at least partly stabilized by palmitoylation. Mutation of CD9 palmitoylation sites impaired associations with tetraspanins CD81 and CD53 (Charrin et al., 2002), and loss of CD151 palmitoylation decreased association with other tetraspanins (CD81, CD63, and CD9), without affecting integrin α3β1 association (Berditchevski et al., 2002; Yang et al., 2002). Palmitoylation of CD151 contributes to cell signaling (Berditchevski et al., 2002). In some proteins (e.g., G proteins and Src family kinases), palmitoylation leads to the diminished detergent solubility and lower protein density characteristic of lipid raft association (Dunphy and Linder, 1998; Resh, 1999). However, palmitoylation...
of tetraspanins CD9 and CD151 causes neither decreased protein density in sucrose gradients nor decreased detergent solubility (Berditchevski et al., 2002; Charrin et al., 2002; Yang et al., 2002).

The α6β4 integrin, like other laminin-binding integrins, associates strongly with CD151 (Sterk et al., 2000, 2002). CD151 association with laminin-binding integrins is direct, occurs early in biosynthesis, and is resistant to disruption by non-ionic detergents (Yauch et al., 2000; Berditchevski et al., 2001; Kazarov et al., 2002). Removal of CD151 palmitoylation sites did not disrupt the CD151–α6β4 complex in epithelial cells, but did strongly influence α6β4 integrin–dependent cell morphology (Yang et al., 2002). In contrast to the primary (i.e., direct) associations of α3 and α6 integrins with CD151, there is an extended network of secondary (i.e., most likely indirect) associations with other tetraspanins (e.g., CD9, CD81, and CD63) that occur later in biosynthesis and are more sensitive to non-ionic detergents (Berditchevski et al., 2001; Kazarov et al., 2002). These secondary-type associations are impaired upon removal of CD151 or CD9 palmitoylation sites (Berditchevski et al., 2002; Charrin et al., 2002; Yang et al., 2002).

The integrin β4 subunit was recently shown also to be palmitoylated (Gagnoux-Palacios et al., 2003). Here, we provide evidence for palmitoylation of integrins α3, α6, and β4, and show striking parallels between β4 integrin and CD151 palmitoylations, in terms of effects on (1) core complex (CD151–α6β4) formation, (2) secondary complex formation (involving CD9, CD81, and CD63), (3) integrin density, (4) integrin solubility, (5) integrin-dependent cell morphology, and (6) integrin signaling. Our results are in sharp contrast to previous suggestions that β4 palmitoylation promotes lipid raft and Src family kinase association (Mariotti et al., 2001; Gagnoux-Palacios et al., 2003). We suggest that integrin palmitoylation promotes, instead of lipid rafts, assembly of a novel type of signaling platform enriched for palmitoylated tetraspanins.

**Results**

A network of palmitoylated integrins and palmitoylated tetraspanins

While studying palmitoylated CD151 (Yang et al., 2002), we noticed that it associates with palmitoylated proteins resembling the β4, α3, and α6 integrin subunits (Fig. 1 A, lanes 3 and 4). [3H]palmitate-labeled integrin subunits from [3H]palmitate-labeled MDA-MB-231 breast carcinoma cells were more abundant on the cell surface (Fig. 1 A, lane 3) than intracellularly (Fig. 1 A, lane 4), and were not present in tetraspanin CD82 immunoprecipitations (Fig. 1 A, lanes 1 and 2). Integrin palmitoylation was confirmed by recovery of [3H]palmitate-labeled α3 (Fig. 1 B, lane 5), α6 (Fig. 1 B, lanes 6 and 8), and β4 (Fig. 1 B, lane 6), but not α2 (Fig. 1 B, lanes 4 and 7), from stringent detergent (RIPA) lysates of A431 cells or B12 kidney epithelial cells. In A431 cells, α2, α3, and α6 integrin subunits were present at comparable levels, as indicated by cell surface labeling (Fig. 1 B, lanes 1–3), and in B12 cells, α2 and α6 were again at comparable levels (not depicted).

When A431 cells were pulse labeled with [3H]palmitate, followed by a 6-h chase, the palmitate label was retained fully on the β4 subunit (Fig. 1 C, top left), and almost completely on CD151 (Fig. 1 C, top right). Blotting of α6 and α3 (Fig. 1 C, bottom) revealed similar levels of total α6β4 and α3β1–CD151 complexes throughout the experiment. Palmitoylations of β4 and CD151 were almost completely inhibited by a protein synthesis inhibitor (cycloheximide) and by a Golgi-disrupting agent (brefeldin A) (unpublished data). Hence, CD151 and β4 palmitoylations both occur early in biosynthesis and undergo little subsequent turnover.

Results shown in Fig. 1 A suggested that additional palmitoylated proteins, including CD9, associate with palmitoylated CD151 and palmitoylated integrins. Indeed, the profiles of palmitoylated proteins associated with tetraspanins (CD9 and CD151) and integrins (α6β4 and α3β1) from MDA-MB-231 cells are strikingly similar (Fig. 2 A). No [3H]palmitate-labeled proteins associated with α2 integrin, which itself is not palmitoylated (Fig. 2 A, lane 1). Palmitoylated protein profiles for CD9, CD151, α3β1, and α6β4 were similarly congruent in two additional cell lines (MCF-10A and A431; unpublished data). Immunoprecipitation of CD9, CD151, α3, and α6 from MDA-MB-231 cells (Fig. 2 B) or MCF-10A cells (Fig. 2 D) yielded, in each case, β4, β1, and...
CD9, as indicated by blotting. Immunoprecipitation of α3, α6, and CD9 from A431 cells again yielded, in each case, both β4 and β1 (Fig. 2 C). Immunoprecipitation of α2 yielded β1 (as expected), but not β4 or CD9 (Fig. 2, B and C). Likewise, immunoprecipitation of CD147 (a highly expressed control cell surface protein) did not yield β1, β4, or CD9 (Fig. 2 D). Results shown in Fig. 2 indicate that complexes containing palmitoylated CD9, CD151, α6β4, and α3β1 are substantially overlapping in three different cell lines.

Removal of integrin palmitoylation sites

The membrane-proximal region of the β4 cytoplasmic tail contains seven potential cysteine palmitoylation sites (Fig. 3 A).

Because palmitoylation often occurs on multiple clustered cysteines in the same molecule (Gundersen et al., 1994; Chapman et al., 1996; Berditchevski et al., 2002; Charrin et al., 2002; Yang et al., 2002), we prepared a “7C/S” β4 mutant, replacing all seven cysteines with serines. When stably expressed in MDA-MB-435 cells, wild-type β4, but not the 7C/S mutant, incorporated [3H]palmitate (Fig. 3 B, left). Although β4 palmitoylation was lost, the 7C/S mutant retained association with palmitoylated α6 (Fig. 3 B, left). Wild-type and mutant β4 were present at similar levels, as detected by anti-β4 immunoblotting (Fig. 3 B, right; and Fig. 4 B, middle), flow cytometry (Fig. 4 A), cell surface biotinylation (Fig. 4 B, top), and by [35S]methionine labeling (not depicted). Mutant

![Image 50x607 to 290x732](image)

Figure 2. Palmitoylated integrins associate with each other and with multiple palmitoylated tetraspanin proteins. [A] MDA-MB-231 cells were pulsed with [3H]palmitate, lysed in 1% CHAPS, and then integrins (α2, α6, and α3) and tetraspanins (CD9 and CD151) were immunoprecipitated using mAbs A2-III-E10, GoH3, A3-X8, Du-All, and 5C11, respectively. Question mark indicates unknown protein. (B) MDA-MB-231 cells were immunoprecipitated as in A, and then samples were blotted for β4 (rabbit pAb), β1 (rabbit pAb), and CD9 (C9B8), as indicated. (C) A431 cells were lysed and immunoprecipitated as in A, and then samples were blotted for β4 and β1 (as in B). Note that the band in the α2 lanes migrating slightly below the β4 band (B and C) is an Ig background band, which does not appear in the α2 lane when [3H]palmitate labeling is used (A). (D) Indicated proteins were immunoprecipitated from MCF-10A cells, as in A, and then blotted as in B. A as a negative control, abundant cell surface protein CD147 was immunoprecipitated using mAb 8G6. White lines (A and D) indicate that intervening lanes have been spliced out.

![Image 340x603 to 520x732](image)

Figure 3. Identification of integrin palmitoylation sites. [A] Membrane-proximal regions containing candidate palmitoylation sites are shaded gray. The first lysine defines a putative transmembrane interface. A and B designate alternatively spliced forms of integrin cytoplasmic tails. (B) MDA-MB-435 cells stably expressing mutant or wild-type β4, or vector control, were [3H]palmitate labeled and lysed in 1% Brij 96. The α2 integrin was immunoprecipitated from vector control cells, and α6β4 was immunoprecipitated (using anti-α6 mAb GoH3) from β4-transfected cells. Shown are proteins labeled with [3H]palmitate (left) or blotted with anti-β4 antibody (right). (C) Murine B12 cells, stably expressing human integrin α subunits, were [3H]palmitate labeled and lysed in 1% RIPA. Integrins were immunoprecipitated using anti–human α2 (lane 1) and α3 (lanes 2–5) antibodies (A2-III-E10 and A3-X8) and resolved by SDS-PAGE, and [3H]palmitate was detected. The α3 subunits include α3X3/3C5 (α3 tail and transmembrane regions are replaced by those regions from α5; Yauch et al., 1998) and α3C106/5 (palmitoylation site point mutant).

![Image 277x53 to 361x196](image)

Figure 4. Comparable expression of wild-type and mutant β4 in MDA-MB-435 cells. (A) MDA-MB-435 cells stably expressing vector or wild-type (Wt) or 7C/S β4 were analyzed using anti-β4 (mAb AA3), anti-CD151 (mAb 5C11), anti-α6 (mAb A6-ELE), and anti-α3 (mAb A3-X8). Histograms represent Wt β4 cells (thick line), 7C/S β4 cells (dashed line), background autofluorescence (dotted line), and vector control cells (thin line). Data are representative of three independent experiments. [B] MDA-MB-435 cells stably expressing vector or Wt or 7C/S β4 were surface labeled with biotin and lysed in 1% Brij 96, and then the α6β4–CD151 complex was immunoprecipitated (using anti-β4 mAb AA3) and analyzed by blotting for biotin-labeled proteins (avidin blotting; top), total β4 (rabbit pAb; middle), or CD151 (mAb 1A5; bottom).
had increased by which confirmed that we could readily distinguish cells in which the area was validated by quantitation of cell area using Scion Image software, that were no longer round and phase-bright. The accuracy of this method (B) Cell spreading was estimated by determining the percentage of cells stably expressing vector or wild-type (WT) or 7C/S in each experiment, with at least 50 cells/field. (C) MDA-MB-435 cells were suspended at 37°C overnight with either laminin-5 (LN; subpanels A–C) or vitronectin (VN; subpanels D–E), and photographed after 45 min. Cell spreading was monitored using a microscope (Axiovert 135; Carl Zeiss Micro Imaging, Inc.) as described previously (Stipp and Hemler, 2000). Bar, 50 μm. (B) Cell spreading was estimated by determining the percentage of cells that were no longer round and phase-bright. The accuracy of this method was validated by quantitation of cell area using Scion Image software, which confirmed that we could readily distinguish cells in which the area had increased by ≥1.2-fold. Results (mean ± SD) are derived from at least two separate experiments, counting at least two representative fields in each experiment, with at least 50 cells/field. (C) MDA-MB-435 cells were suspended at 37°C for 30 min, and then either retained in suspension or plated on vitronectin (VN), laminin-1, or laminin-5 for 45 min. Some samples (lanes 8–10) were preincubated with 1.0 μg/ml PP2 in DMSO for 20 min before plating. Cells were collected and lysed in RIPA, and p130Cas was immunoprecipitated. After SDS-PAGE, samples were blotted with antiphosphotyrosine mAb 4G10. To assess protein loading, the blot was stripped and reblotted with p130Cas-specific antibody. Similar results were seen in multiple experiments.

and wild-type β4 associated similarly with their core partner, CD151 (Fig. 4 B, bottom), and brought similarly elevated amounts of CD151 and α6 to the cell surface (Fig. 4 A). Levels of cell surface α3 (Fig. 4 A) and β1 (not depicted) also remained very similar when either wild-type or mutant β4 was expressed.

The membrane-proximal regions of integrin α3, α6, and α7 cytoplasmic tails contain a single cysteine residue (Fig. 3 A) that is highly conserved in diverse animal species, but not in most other integrin α chains. Upon replacement of this key residue in α3 with serine (C1067S mutation), or upon replacement of the entire α3 cytoplasmic tail and transmembrane with the tail and transmembrane of α5 (X3TC5), palmitoylation was completely lost (Fig. 3 C, lanes 4 and 5). In the same experiment, palmitoylation of wild-type human α3A and α3B was readily observed (Fig. 3 C, lanes 2 and 3). Despite loss of palmitoylation in the C1067S and X3TC5 mutants, these integrins retained association with palmitoylated tetraspanin CD151 (Fig. 3 C, lanes 4 and 5).

Effects of integrin β4 palmitoylation on cell functions

We observed comparable hemidesmosome-like staining for GFP-tagged wild-type and mutant β4 in A431 cells (unpublished data). Because a high level of endogenous β4 precluded further functional studies in A431 cells, we switched to MDA-MB-435 cells, with minimal endogenous β4, for studies of stably expressed wild-type and 7C/S β4. On laminin-5, spreading of 7C/S cells was markedly impaired compared with that of cells with control vector or wild-type β4 (Fig. 5 A, top). All cells spread equally well on vitronectin (Fig. 5 A, bottom). Quantitation of multiple experiments confirmed deficient 7C/S β4 cell spreading on laminin-5 but not vitronectin substrate (Fig. 5 B). A marked defect in tyrosine phosphorylation of p130Cas was also observed for 7C/S β4 cells (Fig. 5 C, lanes 7 and 12) compared with wild-type β4 cells (Fig. 5 C, lanes 6 and 11), when plated on laminin-1 or laminin-5. No defect was seen on control ligand (vitronectin; Fig. 5 C, lanes 3 and 4), and minimal p130Cas phosphorylation was seen for cells in suspension (Fig. 5 C, lanes 1 and 2). In contrast to results with p130Cas, mutant and wild-type β4 showed little difference in phosphorylation of FAK (unpublished data). In concert with cell spreading, p130Cas is typically phosphorylated by a mechanism dependent on Src family kinases (O’Neill et al., 2000). Consistent with this, the Src family kinase inhibitor PP2 (Hanke et al., 1996) abolished both p130Cas phosphorylation (see Fig. 7 A, lanes 8–10) and wild-type β4–MDA-MB-435 cell spreading on laminin-5 (not depicted). Similar spreading and signaling defects were also seen for 7C/S β4–transfected SK-MEL-5 melanoma cells (unpublished data).

Figure 6. β1-dependent cell adhesion is unaffected by β4 mutation. (A) To assess cell adhesion, MDA-MB-435 cells were plated on specific substrates for 30 min, and then nonadherent cells were removed by washing and adherent cells were stained using Wright-Giemsa and then quantitated at OD 590 using an ELISA 96-well plate reader. Starting with 70,000 cells/well, ~70% cell adhesion corresponds to OD 590 = 0.3. (B) Cells were incubated with ~10 μg/ml of the indicated mAbs at RT for 15 min before being plated on laminin-5. Adhesion was determined as in A. (C) Effects of indicated antibodies on adhesion of wild-type (WT) β4 cells was determined as in A and B. Results shown are the means of triplicate determinations ± SD.
Lipid rafts, growth factor receptors, and Src kinases

Palmitoylation diminishes the solubility of some lipid raft–type proteins, leading to increased appearance in “light membrane” fractions of sucrose gradients and decreased extractability in nonionic detergents (Dunphy and Linder, 1998; Resh, 1999). However, CD151 palmitoylation neither decreased density in sucrose gradients nor decreased detergent solubility (Yang et al., 2002). Similarly, in A431 cells, palmitoylation of β4 (compared with 7C/S β4) did not yield decreased density in sucrose gradients in either stringent (Triton X-100) or mild (1% Brij 96) detergent conditions (Fig. 7 A). Furthermore, neither endogenous [3H]palmitate-labeled β4 nor endogenous β4 cell surface–labeled with biotin appeared in the low density fractions of a sucrose gradient, even when mild Brij 96 detergent conditions were used (Fig. 7 B). In control experiments, abundant endogenous caveolin-1 appeared in low density fractions (Fig. 7 B, Fract 1–4) and transferrin receptor (CD71) marked the dense fractions (Fig. 7 B, Fract 8–12). Furthermore, in MDA-MB-435 cells, palmitoylated β4 integrin (compared with the 7C/S mutant) showed increased solubility in nonionic detergent. Upon extraction of cells with 1% Brij 96 for 10 or 20 min, the solubility of wild-type β4 was 69% and 82%, respectively, relative to the total extractable β4 (in RIPA). In marked contrast, the 7C/S mutant remained at 40% solubility at the 10- and 20-min time points, although by 30 min, both mutant and wild-type β4 were completely extracted. In conclusion, palmitoylation did not endow β4 with lipid raft–like properties in either A431 or MDA-MB-435 cells.

As mentioned in the Introduction, integrin β4 can associate with activated EGFR-type growth factor receptors and Src family kinases. However, in EGFR-stimulated A431 cells, we observed no difference in levels of tyrosine-phosphorylated EGFR associating with GFP-tagged 7C/S and wild-type β4 (Fig. 8 A, top, lanes 2 and 4). In control experiments, deletion of portions of the β4 tail did diminish association with tyrosine-phosphorylated EGFR (Fig. 8 A, top, lanes 6 and 8) and EGFR protein (Fig. 8 A, middle, lanes 5–8). Tyrosine-phosphorylated EGFR was not detected in the absence of EGF stimulation (Fig. 8 A, top, lanes 1, 3, 5, and 7) or when β4 was not immunoprecipitated using anti-GFP pAb. Samples were blotted for phosphoryrosine (mAb 4G10) and β4 (mAb anti-GFP). Del1, β4 deleted after the third fibronectin repeat (aa 1582); Del2, β4 deleted after the second fibronectin repeat (aa 1412). (B) Samples were prepared as in A, and then EGFR and fyn were immunoprecipitated and blotted for phosphoryrosine, using mAb 4G10. (C) A431 transfecents were stimulated and lysed as in A; α2, α6, and β4 immunoprecipitations were performed; and samples were blotted for fyn (using pAb). W, wild-type β4; 7, 7C/S β4. (D) MDA-MB-435 transfecents were treated with or without 100 ng/ml hergulin for 10 min and lysed in RIPA, and then α6β4 was immunoprecipitated using mAb GoH3. ErbB3 was blotted for phosphoryrosine (top) and β4 was blotted using anti-β4 pAb (bottom). (E) MDA-MB-435 cell lysate was prepared in D, and ErbB3 was immunoprecipitated. Samples were blotted for phosphoryrosine, total ErbB3, and p85 subunit of PI 3-K.
tated (Fig. 8 A, lane 9). Comparable amounts of β4 were recovered in each lane (Fig. 8 A, bottom, lanes 1–8). As expected (Mariotti et al., 2001), an abundance of tyrosine-phosphorylated EGFR was recovered in association with the Src family kinase fyn (Fig. 8 B), but no fyn was recovered in association with either mutant or wild-type β4 that had been immunoprecipitated with antibodies to either α6 or β4 (Fig. 8 C). In addition, there were no consistent differences in tyrosine phosphorylation of 7C/S and wild-type β4 (unpublished data). Integrin α6β4 also associates with ErbB2 (Hintermann et al., 2001), which in MDA-MB-435 cells forms dimers with ErbB3 (Adelsman et al., 1999). However, after heregulin treatment to induce ErbB phosphorylation, we observed no association of either wild-type or mutant β4 with tyrosine-phosphorylated ErbB3 (Fig. 8 D) or ErbB2 (not depicted). Also, for mutant β4 in MDA-MB-435 cells, we saw no diminution in ErbB3 tyrosine phosphorylation or ErbB3 association with the p85 subunit of PI 3-K (Fig. 8 E).

**Diminished association with tetraspanins**

Removal of CD151 palmitoylation sites did not affect core association with α3β1 or α6β4, but did alter secondary associations with other tetraspanins (Berditchevski et al., 2002; Yang et al., 2002). Likewise, removal of β4 palmitoylation sites did not affect core association with CD151 (Fig. 4 B), but did markedly alter secondary associations with other tetraspanins (Fig. 9 A). As indicated by immunoprecipitations of α6 or CD151, association of CD9, cell surface CD9 (sCD9), CD81, and CD63 was impaired in 7C/S–MDA-MB-435 cells, in five different experiments. In control experiments, the levels of wild-type and 7C/S β4 associated with α6 were unaltered, as were the total levels of CD151, CD9, CD81, and CD63 (Fig. 9 B). Similar levels of CD63 were seen by flow cytometry (Fig. 9 C, top).

Flow cytometry showed that surface levels of tetraspanin CD9 were very similar, as indicated by mAb ALB6 staining (Fig. 9 C, middle), but different as indicated by mAb C9BB staining (Fig. 9 C, bottom). Indeed, the C9BB/ALB6 staining ratio (0.59 in wild-type cells) decreased to 0.34 in 7C/S cells. After β4-transfected MDA-MB-435 cells had been cultured for a few more months, there was an even larger difference in C9BB/ALB6 ratios (0.56 in wild-type vs. 0.07 in 7C/S cells), whereas mutant and wild-type β4 levels remained equal (unpublished data). Wild-type β4–GFP and 7C/S β4–GFP were expressed in another cell line (SK-MEL-5), and again there was similar staining of CD9 by mAb ALB6, but selectively diminished staining of the mutant cells by mAb C9BB, such that the C9BB/ALB6 ratio decreased by ~40% (unpublished data). These results indicate that cell surface tetraspanin organization is affected by β4 palmitoylation.

**Functional link between CD9 and β4**

In cells where CD9 associates with α6β4, anti-CD9 antibodies can alter α6β4-dependent cell motility (Jones et al., 1996; Baudoux et al., 2000). Here, we confirm a functional connection between CD9 and laminin-binding integrins in MDA-MB-435 cells. Addition of an anti-CD9 antibody (ALB6) stimulated cell spreading in 7C/S cells, and to an even greater extent in cells expressing wild-type β4 (Fig. 10). Background spreading was observed in the presence of control anti-α2 and anti-CD81 antibodies. As expected from the results shown in Fig. 5, spreading on laminin-5 was elevated for wild-type β4 cells. The anti-CD9 mAb ALB6 did not promote spreading of either wild-type (Fig. 10) or 7C/S (not depicted) cells on vitronectin, and had no effect on static cell adhesion to laminin-1 or laminin-5.
**Discussion**

**CD151 and β4 palmitoylation similarities**

There are several striking similarities between β4 and tetraspanin CD151, with respect to palmitoylation. First, multiple membrane-proximal cysteines are used. Although six cysteines may contribute to CD151 palmitoylation (Berditchevski et al., 2002; Yang et al., 2002), removal of seven cysteines eliminated all detectable [3H]palmitate incorporation into β4. Removal of only one, two, three, or five membrane-proximal cysteines yielded incomplete results (Gagnoux-Palacios et al., 2003). The seven mutated cysteines are exactly conserved in β4 from multiple species (e.g., humans, rats, and mice), but do not appear in any other integrin β subunits. Second, palmitoylation of both CD151 (Berditchevski et al., 2002; Yang et al., 2002) and β4 occurs on newly synthesized protein and remains stably attached, with minimal turnover on the mature proteins. Third, removal of palmitoylation sites from CD151 (Berditchevski et al., 2002; Yang et al., 2002) or β4 increased neither protein density nor detergent solubility/extractability (see TEMs and lipid raft localization section). Fourth, removal of palmitoylation sites from CD151 (Berditchevski et al., 2002; Yang et al., 2002) or β4 did not alter static cell adhesion, but did alter α6β4-dependent cell signaling and morphology. Fifth, immunoprecipitation of either CD151 or α6β4 yielded strikingly similar patterns of palmitoylated proteins. Sixth, loss of palmitoylation from either molecule did not disrupt the core CD151–α6β4 complex. However, seventh, loss of palmitoylation from either CD151 (Berditchevski et al., 2002; Yang et al., 2002) or β4 did markedly alter secondary associations with other tetraspanins. These observations reinforce the concept that the CD151–α6β4 core complex is similarly affected by palmitoylation on either CD151 or β4.

**Tetraspanin-enriched microdomains (TEMs)**

It is well established that tetraspanin proteins (including CD151 and CD9) and their partners associate with each other in extended complexes known as the tetraspanin web (Trusolino et al., 2001), or TEMs (Berditchevski et al., 2002; Hemler, 2003). TEMs contain primary complexes (e.g., CD151–integrin [Serru et al., 1999; Yauch et al., 2000; Kazarrov et al., 2002; Sterk et al., 2002] and tetraspanin homodimers [Kovalenko et al., 2004]) occurring through direct, protein–protein interactions. These are then brought together into extended secondary complexes, with tetraspanin palmitoylation playing a key role (Berditchevski et al., 2002; Charrin et al., 2002; Yang et al., 2002; Hemler, 2003). Given the abundance of evidence regarding CD151 palmitoylation, and the strong similarities between CD151 and β4 (see previous section), it is not surprising that β4 palmitoylation would also contribute to localization into TEMs. The evidence for this is as follows. First, in multiple cell lines, palmitoylated β4 associates with other palmitoylated proteins (CD9, CD81, CD63, and others) in a pattern that is strikingly similar whether immunoprecipitated with antibodies to CD151, to CD9, or to integrins (α6β4 or α3β1). The specificity of interaction among these particular components is emphasized by the exclusion of other abundant cell surface molecules (e.g., integrin α2β1 and tetraspanin CD82). Consistent with our results, others have also shown that CD9 can associate with α6β4 (Jones et al., 1996; Baudoux et al., 2000). Second, loss of β4 palmitoylation diminished secondary associations with other tetraspanins (CD9, CD81, and CD63), just as seen previously upon removal of CD151 and CD9 palmitoylation sites (Berditchevski et al., 2002; Charrin et al., 2002; Yang et al., 2002). Third, not only was CD9 substantially dissociated from the palmitoylation-deficient α6β4–CD151 complex, it also showed reorganization on the cell surface, which is consistent with reduced clustering. Pairs of monoclonal antibodies, diagnostic for cell surface clustering, have been described for other molecules, including CD147 (Koch et al., 1999) and CDw78 (Drbal et al., 1999). In each case, a lower affinity antibody (such as anti-CD9 mAb C9BB) showed more dependence on bivalent binding, and hence more sensitivity to cell surface clustering, compared with a higher affinity antibody (such as anti-CD9 mAb ALB6).

It is likely not a coincidence that the integrin α subunits (α3, α6, and α7) best able to interact with tetraspanins also contain palmitoylation sites. The integrin α3, α6, and α7 subunits contain single, membrane-proximal cysteine palmitoylation sites, conserved in orthologous subunits across a wide range of animal species. For α3 and α6, the key cysteine is present in both major (α3A and α6A) and minor (α3B and α6B) alternatively spliced forms. In contrast to the integrin α3, α6, and α7 subunits, the other 13 integrin α subunits (except for αE and α8) don't contain membrane-proximal cysteines.

**Functional consequences**

Removal of CD151 palmitoylation did not affect static cell adhesion but did alter α6β4-dependent kidney epithelial cell morphology (Yang et al., 2002) and integrin-dependent signaling in fibroblasts (Berditchevski et al., 2002). Similarly, removal of β4 palmitoylation did not alter cell surface expression levels, association with α6 or CD151, or cell adhesion to laminins, but did alter α6β4-dependent spreading and associated signaling through p130Cas in MDA-MB-435 cells. Although β4 has not previously been linked to p130Cas signaling, p130Cas is preferentially activated when cells are plated on laminin, compared with fibronectin (Gu et al., 2001). Inhibition by the Src family kinase inhibitor PP2 is consistent with cell spreading depending on Src family kinase phosphorylation of p130Cas (Njoima et al., 1996; Panetti, 2002). Presently, we cannot determine whether impaired cell spreading, due to the absence of β4 palmitoylation, is a consequence or a cause of diminished Src kinase → p130Cas signaling. Notably, Gagnoux-Palacios et al. (2003) have also reported altered Src family kinase–dependent signaling upon removal of β4 palmitoylation sites. Upon removal of palmitoylation from CD151 (Berditchevski et al., 2002) or β4, signaling through FAK was unaltered, consistent with cell adhesion being unaltered. Our results are in accord with previous evidence that FAK and p130Cas can sometimes be differentially regulated (Gu et al., 2001).

For several reasons, we suggest that β4 palmitoylation–dependent recruitment of CD151–α6β4 into TEMs plays a ma-
ajor role in determining cell spreading and signaling. First, dimin-
ished spreading and signaling occurred in parallel with re-
duced secondary associations of α6β4 with tetraspanins (CD9,
CD81, and CD63) and reorganization of cell surface CD9. Sec-
ond, CD9 is functionally connected to α6β4, consistent with its
physical association. As seen elsewhere, anti-CD9 antibodies
inhibited the motility of cultured keratinocytes under conditions
where motility is at least partially α6β4-dependent (Jones et al.,
1996; Baudoux et al., 2000). Consistent with this, we used an
anti-CD9 antibody to stimulate MDA-MB-435 cell spreading,
which is partly α6β4-dependent, as evidenced by the negative
effects of β4 mutation. It was a little surprising that anti-CD9
antibody also stimulated spreading of MDA-MB-435 cells
bearing mutant β4. However, we note that removal of β4 pal-
mitoylation does not completely dissociate CD151–α6β4 from
CD9 and other tetraspanins. There remain six palmitoylation
sites in CD151, and one site in α6, that likely contribute to con-
tinued CD9 association. Third, an association with TEMs pro-
vides a mechanism to link CD151–α6β4 with several other
molecules known to regulate cell morphology and signaling.
These include other CD9-associated proteins such as EWI-2
(Stipp et al., 2003a; Kolesnikova et al., 2004), PKC (Zhang et
al., 2001), and phosphatidylinositol 4-kinase (Yauch and Hem-
ler, 2000). In this regard, CD9 has also been linked to signaling
of c-kit and EGFR tyrosine kinases (Higashiyama et al., 1995;
Shi et al., 2000; Anzai et al., 2002). It was suggested previously
that CD9 regulation of α6β4 might only be relevant when cells
are migrating and/or when the α6β4 is associated with the actin
cytoskeleton rather than stably interacting with intermediate fil-
aments within hemidesmosome-like structures (Baudoux et al.,
2000). Indeed, tetraspanins CD9 and CD81 are not associated
with the CD151–α6β4 complex in hemidesmosomes (Sterk et
al., 2000), and mutation of β4 palmitoylation sites did not af-
flect hemidesmosome localization, as observed here and by
Gagnoux-Palacios et al. (2003).

Our β4 mutation had minimal effect on cell adhesion to laminin-1 or laminin-5. This result is consistent with previous
results in which β4 in MDA-MB-435 cells modulated down-
stream signaling but not initial cell adhesion to laminin-1
(Shaw et al., 1997). Furthermore, our β4 mutant was deficient in
spreading and signaling on both laminin-5 and laminin-1,
even though only the former is a very good ligand for α6β4.
Hence, a mechanism is needed to explain how α6β4 can exert
substrate-dependent effects on cell morphology, signaling, and/
or motility, even when it is not primarily responsible for cell
adhesion. In this regard, we have demonstrated that multiple
laminin-binding integrins can be linked together within over-
lapping complexes. Not only were the profiles of palmitoylated
proteins associated with α6β4 and α3β1 strikingly similar, but
also we observed α6β4 and α3β1 coimmunoprecipitations
from multiple cell lines. Hence, we propose that α6β4 can
physically associate with α3β1 (and likely also α6β1), in the
context of TEMs. This provides a mechanism for the close co-
ordination of initial adhesion, mediated by α3β1 (or α6β1),
with subsequent α6β4-dependent spreading, signaling, or mi-
gration. Though novel, these results are not unexpected. It was
previously established that α6β4, α6β1, and α3β1 all can asso-
ciate with CD151 (Berditchevski, 2001), and that CD151 may
appear predominantly as a homodimer (Kovalenko et al., 2004),
thus having the potential to link distinct integrins. Furthermore,
all of these integrins were known to associate with additional
tetraspanins (CD9, CD81, CD63, etc.), which can associate
with each other (Boucheix and Rubinstein, 2001; Berditchevski
et al., 2002; Hemler, 2003).

TEMs and lipid raft localization

Protein palmitoylation can lead to localization into lipid rafts
and decreased solubility in nonionic detergents (Dunphy and
Linder, 1998; Resh, 1999). Indeed, it was suggested else-
where that β4 palmitoylation promotes lipid raft localization,
thus providing a mechanism for bringing β4 into functionally
important complexes with Src family kinases such as fyn and
yes (Mariotti et al., 2001; Gagnoux-Palacios et al., 2003).
However, we found no evidence for palmitoylation promot-
ing either integrin or tetraspanin association with lipid rafts.
First, in β4-transfected A431 cells, the presence of β4 palmi-
toylation was not associated with decreased density in either
Brij 96 or Triton X-100 lysates. Second, neither the palmi-
toylated subset nor the cell surface–labeled subset of endoge-
nous β4 in A431 cells appeared in low density sucrose gradi-
ent fractions, even in mild Brij 96 detergent. Third, β4 palmi-
toylation did not decrease detergent solubility in MDA-
MB-435 cells. In fact, in a short-term detergent (Brij 96) sol-
ubility assay, β4 palmitoylation increased solubility. Fourth,
our β4 results are in complete agreement with prior results re-
garding tetraspan palmitoylation. Protein density was not
affected by removal of palmitoylation from either CD151 or
CD9 (Berditchevski et al., 2002; Charrin et al., 2002; Yang et
al., 2002), and CD151 palmitoylation did not decrease deter-
gent solubility (Yang et al., 2002). Fifth, we did not see changes in association of mutant β4 with signaling proteins
(EGFR, ErbB3/2, and fyn) that can be found in lipid rafts.
Despite the potential of tetraspanins to associate with gangli-
osides and cholesterol, TEMs consistently remain distinct
from lipid rafts. In contrast to lipid rafts, TEMs are resistant
to cholesterol depletion, typically appear in the soluble phase
upon extraction by nonionic detergents, and are not disrupted
at 37°C (Hemler, 2003). An extensive proteomics analysis of
lipid rafts did not reveal any tetraspanins or palmitoylated in-
tegrins (Foster et al., 2003). Conversely, we have identified,
by mass spectrometry, numerous potential partners for tet-
raspanins and palmitoylated integrins, but this list does not
contain typical raft-type proteins such as caveolins and GPI-
linked proteins (unpublished data).

In conclusion, palmitoylation of β4 integrin, as seen pre-
viously for CD151, plays a key role in integrin-dependent cell
spreading and signaling, and in the maintenance of secondary
interactions with additional tetraspanin proteins. We suggest
that CD151–α6β4, CD151–α3β1, and CD151–α6β1 assemble
with other tetraspanins into novel signaling platforms (TEMs).
These provide a framework for the regulation of integrin-de-
pendent postadhesion functions (spreading, signaling, and mo-
tility) and coordination of the functions and distributions of
laminin-binding integrins at the cell–substrate interface.
Materials and methods

Reagents and integrins

mAbs to integrins α2 (A2-III-E10), α3 (A3-XIII B and A3 IV A), α6 (A6-ELE; to tetratraspanins CD9 (9C8B and DU-ALL1), CD63 (6H1), CD81 (M38 and JS64), CD82 (M104), and CD151 (SC11 and 1A5); and to CD147 (8G6) were referenced previously (Kazazov et al., 2002; Yang et al., 2002). Other mAbs used were anti-integrin α9 (GoH3; BD Biosciences), anti-β4 (AA3; provided by V. Quaranta, Vanderbilt University, Nashville, TN) anti-CD9 (Ab; CHEMICON International), and anti-CD71 (OKT9; American Type Culture Collection). Also used were rabbit polyclonal antibodies to β4 and β1 integrins (CHEMICON International) and to the cytoplasmic domains of integrins α3A (Dipersio et al., 1995) and α6A (Ab 6843; a gift from V. Quaranta). mAbs to phosphotyrosine (4G10) and PI 3-kinase were purchased from Upstate Biotechnology, and antibodies to EGFR, ErbB2, and ErbB3 were purchased from Transduction Laboratories. Antibodies to FAK, Fn, Src, and p130Cas were obtained from Santa Cruz Biotechnology, Inc., and anti-GFP was obtained from CLONTech Laboratories, Inc.


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