An extracellular site on tetraspanin CD151 determines α3 and α6 integrin–dependent cellular morphology

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The α3β1 integrin shows strong, stoichiometric, direct lateral association with the tetraspanin CD151. As shown here, an extracellular CD151 site (QRD194–196) is required for strong (i.e., Triton X-100–resistant) α3β1 association and for maintenance of a key CD151 epitope (defined by monoclonal antibody TS151r) that is blocked upon α3 integrin association. Strong CD151 association with integrin α6β1 also required the QRD194–196 site and masked the TS151r epitope. For both α3 and α6 integrins, strong QRD/TS151r-dependent CD151 association occurred early in biosynthesis and involved α subunit precursor forms. In contrast, weaker associations of CD151 with itself, integrins, or other tetraspanins (Triton X-100–sensitive but Brij 96–resistant) were independent of the QRD/TS151r site, occurred late in biosynthesis, and involved mature integrin subunits. Presence of the CD151–QRD194–196→INF mutant disrupted α3 and α6 integrin–dependent formation of a network of cellular cables by Cos7 or NIH3T3 cells on basement membrane Matrigel and markedly altered cell spreading. These results provide definitive evidence that strong lateral CD151–integrin association is functionally important, identify CD151 as a key player during α3 and α6 integrin–dependent matrix remodeling and cell spreading, and support a model of CD151 as a transmembrane linker between extracellular integrin domains and intracellular cytoskeleton/signaling molecules.

Introduction

The integrins are a major family of cell surface receptors for extracellular matrix proteins, whereas laminins are key components within the extracellular matrix. The major laminin-binding integrins are α3β1, α6β1, α6β4, and α7β1 (Belkin and Stepp, 2000). Cell adhesion mediated through these integrins controls cell migration, differentiation, signaling, cytoskeletal organization, mechanical force generation, and many other functions (Wei et al., 1997; Burkin and Kaufman, 1999; Kreidberg, 2000; Mercurio et al., 2001). Consistent with the importance of the laminin-binding integrins, targeted deletion of the integrin α3 subunit led to lung, kidney, skin, and brain defects (Kreidberg et al., 1996; Dipersio et al., 1997; Anton et al., 1999), deletion of α6 caused severe blistering of skin and other epithelia (Georges-Labouesse et al., 1996), and absence of the α7 gene resulted in impaired function of myotendinous junctions (Mayer et al., 1997). Among the 24 known mammalian integrins, α3β1, α6β1, α6β4, and α7β1 not only are the best laminin-binding integrins but also form the strongest (i.e., most detergent-resistant) lateral associations with tetraspanin proteins (Hemler, 1998; Sterk et al., 2000, 2002; Boucheix and Rubinstein, 2001). Consistent with the specialized properties of these integrins, the α3, α6, and α7 subunits form a distinct subgroup among the 18 mammalian integrin α subunits based on protein sequence similarity (Hynes, 1992).

The tetraspanin family includes 28 or more mammalian proteins, with at least a few members abundantly expressed on nearly all cell and tissue types. Despite association with integrins, tetraspanins do not modulate integrin-dependent cell adhesion but rather are linked to cell migration, fusion, and signaling (Berditchevski, 2001; Boucheix and Rubinstein, 2001; Hemler, 2001; Yánez-Mó et al., 2001). A key function of tetraspanins may be to organize other transmembrane and membrane-associated proteins into specific complexes (Berditchevski, 2001; Boucheix and Rubinstein, 2001; Hemler, 2001). Thus, tetraspanins may act as transmembrane adapters, with extracellular domains linking to other transmembrane proteins, whereas cytoplasmic tails link to intracellular components. However, this model needs to be definitively tested.

Studies of tetraspanin protein complexes are complicated by the tendency of tetraspanins to associate with each other and to form large vesicular aggregates containing many diverse proteins. This is especially obvious when tetraspanins are
solvated in detergents that are less hydrophobic such as Brij 99 and CHAPS (Hemler, 1998; Bouchech and Rubinstein, 2001). However, membrane solubilization by detergents that are more hydrophobic (e.g., Brij 96, digitonin, NP-40, and Triton X-100) yields tetraspanin complexes of more limited complexity and more amenable to specific biochemical analysis (Indig et al., 1997; Hemler, 1998; Serru et al., 1999; Berditchevski, 2001; Bouchech and Rubinstein, 2001).

Compared with most other complexes involving either integrins or tetraspanins, the CD151–α3β1 complex shows a higher degree of stability (resistant to Triton X-100 and RIPA detergents), specificity, stoichiometry (nearly all α3 integrin bound to CD151), and proximity (as shown by direct covalent cross-linking) (Yauch et al., 1998, 2000; Berditchevski et al., 2001). Indeed, the α3β1 integrin has not yet been found in any cell or tissue in the absence of CD151 association (Yauch et al., 1998; Sterk et al., 2002). Sites required for strong association have been mapped to specific regions found in any cell or tissue in the absence of CD151 association, consistent with the CD151–integrin association properties, and (c) determine the functional relevance of strong CD151–integrin associations mediated through a specific extracellular CD151 site. To assess function, we analyzed integrin-dependent cell spreading and also used a Matrigel cell cable formation assay that is dependent on CD151 and associated integrin (Zhang et al., 2002). This latter assay is an excellent readout for cellular exertion of tractional forces and extracellular matrix remodeling. Cells dispersed on the surface of Matrigel, a model basement membrane, exert mechanical force onto the Matrigel and subsequently migrate along lines of mechanical tension to assemble over the next 8–12 h into a pattern of intersecting cellular cables (Davies and Camarillo, 1995; Vernon and Sage, 1995). Thus, in addition to evaluating the relevance of strong CD151–integrin association we could test the hypothesis that CD151 provides a critical link between integrin-mediated adhesion and mechanical force generation.

Results

Identification of a minimal CD151 site needed for strong α3 integrin association

Previous studies using CD151–NAG-2 and CD151–CD9 tetraspanin chimeras showed that CD151 large extracellular loop residues 186–216 (Yauch et al., 2000) or 195–205 (Berditchevski et al., 2001) are required for strong α3 integrin association. A problem with CD151–CD9 chimeras is that CD9 lacks two critical cysteines and cannot readily be aligned with CD151 in the 194–207 region. We prefer the corresponding region in CD63), and this minimal human integrins in CD63), and this minimal human CD151–INF-2 chimeras were prepared (Fig. 1) and tested for α3 integrin association in HT1080 cells. As indicated, CD151 association with α3 integrin under stringent conditions (Triton X-100) was lost when CD151 residues 194–216 or 194–207 were replaced with the corresponding regions from NAG-2. In contrast, replacement of CD151 residues 209–216 did not abolish α3 integrin association.

Within the 194–207 region, we next focused attention on CD151194–196 because that region is quite different from the corresponding region in NAG-2 (Table I). The CD151 QRD194–196 residues were replaced with "INF" (from the corresponding region in CD63), and this minimal human CD151–INF-194–196 mutant was expressed in Cos7 cells together with the human α3 integrin subunit. Upon lysis in Triton X-100 and immunoprecipitation of CD151, no associated α3 was detected by immunoblotting (Fig. 2 A, top). In the same experiment, wild-type CD151 showed association...
with abundant α3 integrin (Fig. 2 A, top). Control α3 immunoblotting experiments indicate comparable α3 expression in Cos7 cells transfected with vector alone, wild-type CD151, or CD151–INF194–196. Also, anti-HA blotting indicates comparable expression of HA-tagged wild-type CD151 and CD151–INF194–196. In a separate experiment, wild-type CD151 and CD151–INF194–196 were immunoprecipitated from Triton X-100 lysates of 35S metabolically labeled HT1080 cells. Again, α3 integrin was immunoblotted

with wild-type CD151 but not with CD151–INF194–196 (unpublished data). In addition, metabolic labeling demonstrated that wild-type CD151 and CD151–INF194–196 are synthesized and maintained almost exactly in parallel at 30-min, 1-h, and 36-h time points (unpublished data). These results provide assurance that despite loss of α3 integrin association CD151–INF194–196 maintained its overall integrity.

As shown previously (Serru et al., 1999) and again below (see Fig. 4 A), α3 integrin association specifically conceals a CD151 epitope recognized by monoclonal antibody (mAb)* TS151r. Accordingly, replacement of CD151 residues 185–216 abolished the TS151r epitope in parallel with loss of α3 association (Yauch et al., 2000). As indicated by flow cytometry (Fig. 3, bottom), the CD151–INF194–196 mutant also completely lost the TS151r epitope, whereas that epitope was retained by wild-type CD151. In contrast, the epitope defined by mAb 5C11 is not concealed by α3 association

Table I. Comparison of tetraspanin sequences in the CD151 QRD region

| Tetraspanin   | Sequence† |
|---------------|-----------|
| Nag2/Tspan4   | CGL-HAPGTWKN--------APC 177–190 |
| CD151-hum     | CGQRDHASNLYKVE------GCG 192–208 |
| CD151-mnk     | CGQRDHASNLYKVE------GCG 192–208 |
| CD151-mou     | CGQRDHASNLYKVE------GCG 192–208 |
| CD151-rat     | CGQRDHASNLYKVE------GCG 192–208 |
| BAB22942      | CGQRAHPSNYKVE------GCG 192–208 |
| TM4-B         | CTPQRDADKXVNN------EGC 183–197 |
| A15/Talla-1   | CNPQDLHNLTAATKXVNG--KGC 181–201 |
| TM4S6e        | CTPQRDADKXVNN------EGC 183–197 |
| Net1/Tspan1   | CTRQKADQKV--------EGC 187–200 |
| CD63f         | CSINPMKAIHK------EGC 177–191 |
| CD82f         | CEAPFRKTQSNHPFDPVXQEUGC 193–216 |
| CD37f         | CAVFAESHITYR--------EGC 217–230 |
| Co029         | CGSYNGKYKX--------ETC 181–193 |
| UP1a          | CRLGHDYDLF--------TXGC 205–218 |
| UP1b          | CKLGVPGFYHN--------QGC 205–218 |

An additional seven tetraspanins (BAB5318, Net5, Net4/Tspan5, MGC11352, Net7, Tspan3, Net2) contain no residues similar or identical to the corresponding CD151 QRD motif.

These tetraspanins (CD53, CD9, CD81, Tspan2, SAS, Net2, TSSc6) lack a cysteine comparable to the QRD proximal cysteine in CD151, thus hindering alignment in this region.

Residues similar or identical to “QRD” are bolded. TM4S6e contains a “QRD” (underlined), but it is offset by one residue. CD63 “INF” residues chosen to replace “QRD” in CD151 are also underlined.

These tetraspanins do not show strong (Triton X-100-resistant) associations with α3 or α6 integrins (Berditchevski et al., 1995, 1996; Wu et al., 1993; Tachibana et al., 1997; Serru et al., 1999).

These tetraspanins have not yet been tested for associations with integrins.
(Yauch et al., 2000) and is well expressed by both CD151–INF194–196 and wild-type CD151 (Fig. 3, top). During preparation of the previously described CD151(216)–N chimera (Yauch et al., 2000), a random mutation of Cys192 (Cys→Tyr) occurred. That C192Y point mutation also caused a loss of both H9251 integrin association and the TS151r epitope. Indeed, for all 10 tetraspanin structures summarized in Fig. 1, H9251 integrin association (in Triton X-100 conditions) was retained or lost exactly in parallel with the TS151r epitope. Together, these biochemical and immunochemical results establish that the QRD/TS151r region of CD151 is required for strong H9251 integrin association.

Do α3 and α6 integrins show similar CD151 association properties?

In some studies, CD151 associated similarly with α3 and α6 integrins (Serru et al., 1999; Sincock et al., 1999), whereas in other studies α3 association seemed substantially stronger (Sterk et al., 2000; Stipp and Hemler, 2000; Yauch et al., 2000). Here we compared α3β1 and α6β1 integrins with respect to (a) dependence on the CD151 QRD motif, (b) concealment of the TS151r epitope, and (c) timing of CD151 association during biosynthesis. Similar to the α3 integrin (Fig. 2 A), α6 integrin also completely lost association with the CD151–INF194–196 mutant under Triton X-100 conditions (Fig. 2 B). By comparison, abundant α6 was recovered in association with wild-type CD151. Control experiments (Fig. 2 B, bottom) showed comparable levels of α6 integrin in the various transfectants and comparable levels of HA-tagged CD151–INF194–196 and wild-type CD151.

Fig. 4 A confirms that the α3 integrin suppresses the TS151r epitope. The ratio of TS151r to 5C11 anti-CD151 mAb staining was >1.0 for endogenous CD151 in mock-transfected K562 cells (Fig. 4 A, middle). In contrast, in K562-α3 transfectants the TS151r to 5C11 ratio was markedly diminished (down to ~0.2) (Fig. 4 A, right). The left panel confirms that α3 was indeed present in K562–α3 cells. Expression of α6 in K652–α6 cells was too low for definitive study of TS151r epitope masking. However, in Cos7 cells a strong and selective diminution of TS151r antibody binding was observed upon coexpression of human CD151 with human α6, but not with α2, or vector control (Fig. 4 B, right column). In contrast, binding of anti-CD151 mAb 5C11 was minimally altered by integrin α subunits (Fig. 4 A, middle), indicating that the 5C11 epitope is insensitive to integrin association and that there are comparable levels of human CD151 in each cell.

To address integrin–CD151 association during biosynthesis, 293 cells were transiently transfected with CD151 together with integrin α3 or α6 or vector control. After a 1-h pulse of 35S metabolic labeling, followed by a 0- or 1-h chase with unlabeled cysteine and methionine, CD151 immuno-
precipitation yielded nonreduced ~140-kD precursor forms of both α3 and α6 integrins (Fig. 5). After a 10- or 20-h chase time, α3 and α6 precursor forms were converted to ~150-kD mature forms that were more diffuse and less obvious (especially α6). In 293 cells transfected with CD151 but no integrin subunit, little or no association with endogenous integrin was apparent (Fig. 5, left lanes). Together, the results in Figs. 2, 4, and 5 indicate that CD151 association with α3 and with α6 integrins can occur by highly similar mechanisms.

**CD151 associations independent of the QRD site and TS151r epitope**

Association of the CD151–INF194–196 mutant with α3 and α6 integrins in Cos7 lysates was completely lost in Triton X-100 and greatly diminished in 1% Brij 96 conditions (Fig. 2). Nonetheless, the residual α3 (Fig. 2 A) and α6 (Fig. 2 B) remaining associated with CD151–INF194–196 indicates that in Brij96 conditions a QRD-independent alternative mechanism must exist. As established in Figs. 3 and 4, the TS151r antibody is a useful tool for exploring QRD-dependent (or -independent) integrin associations. The TS151r mAb failed to immunoprecipitate any integrin-like material from 1% Triton X-100 lysates of [35S] metabolically labeled HT1080 (Fig. 6 C), consistent with the TS151r epitope being blocked by tightly associated α3 or α6 integrins. However, from 1% Brij96 lysate the TS151r antibody did immunoprecipitate a mixture of α3 and α6 integrins (Fig. 6 D). Thus, we have additional evidence for a TS151r/QRD-independent association mechanism that is maintained in 1% Brij96 but disrupted in 1% Triton X-100. Importantly, TS151r/QRD-independent integrin associations with CD151 observed in Brij96 were only seen at later metabolic labeling time points (25 and 56 h) and involved only the ~110-kD reduced mature forms of the integrins (Fig. 6 D). In contrast, Triton X-100–resistant CD151–α3 and CD151–α6 associations (the type that are QRD/TS151r dependent) were observed at early time points (2.5 and 5 h) and involved uncleaved ~140-kD integrin α chain precursor forms (Fig. 6 B). Similar results were obtained when α3 integrin was directly immunoprecipitated with anti-α3 antibody (Fig. 6 A).

What is the mechanism for QRD/TS151r-independent linkage of CD151 to α3 and α6 integrins? Since tetraspanin–tetaspanin interactions are maintained in Brij96 detergent conditions (Berditchevski et al., 1996; Boucheix and Rubinstein, 2001), we suspected that the CD151–INF194–196 mutant may link to endogenous wild-type CD151, thereby allowing the CD151–INF194–196 to associate indirectly with integrins in a QRD/TS151r-independent manner. To probe for endogenous CD151 associated with CD151–INF194–196, we used anti-CD151 mAb 1A5, which blots wild-type CD151 (Fig. 7 A, middle lanes) but not CD151–INF194–196 itself (Fig. 7 A, right lanes) in either Triton X-100 or Brij96 conditions. As expected, anti-HA immunoprecipitation of wild-type CD151 from human HT1080 cells yielded abundant CD151 (transfected plus endogenous) as detected by mAb 1A5 (Fig. 7 B, top panel, middle lane) and abundant associated α3 integrin as blotted by anti-α3 serum D23 (Fig. 7 B, middle panel, middle lane). In contrast, anti-HA immunoprecipitation of CD151–INF194–196 yielded a low level of form to fragments of ~120 and 30 kD (not depicted). Labeled β1 subunit (110 kD), appearing at later time points, closely comigrates with 120 kD α subunit fragments. As seen elsewhere, CD81 associated bands of ~120 kD from HT1080 cells (such as in panel e) have been clearly identified as corresponding to α3 and α6, with α3 being ~2–3-fold more abundant than α6 in HT1080 cells (Berditchevski et al., 1996, 1997; Stipp et al., 2001).
Brij96-resistant links to QRD mutation and therefore could account for indirect, association was seen in Triton X-100 lysate (unpublished less of whether QRD mutant or wild-type CD151 was im-
amounts of associated GFP–CD151 were obtained regard-
precipitation yielded an abundance of GFP–CD151. Similar
separately tagged HA–CD151 and GFP–CD151 were coex-
CD151–INF194–196 were present in HT1080 cells at compa-
polyclonal (D23), and anti-HA mAb in reducing conditions.
Having established that CD151 associates strongly with
CD151–INF194–196 via endogenous wild-type CD151.
QRD-independent CD151 associations.

Figure 7. QRD-independent CD151 associations. (A) Cos7 cells
were transiently transfected with the indicated human CD151
constructs, lysed in the presence of 1% Triton X-100 or 1% Brij 96,
and immunoprecipitated with anti-CD151 5C11 mAb. Resolved
proteins were then immunoblotted using anti-CD151 mAb 1A5 in
nonreducing conditions. Note that mAb 1A5 does not blot monkey
CD151. (B) HT1080 cells were transiently transfected with the
indicated HA-tagged CD151 constructs and immunoprecipitated
using anti-HA mAb in a buffer containing 1% Brij 96. Immune
complexes were resolved by SDS-PAGE and immunoblotted with
anti-CD151 mAb 1A5 in nonreducing conditions, or anti-α3 polyclonal (D23), and anti-HA mAb in reducing conditions.
endogenous human wild-type CD151 (Fig. 7 B, top panel,
right lane) and a low level of α3 integrin subunit (Fig. 7 B,
middle panel, right lane) that presumably is linked indirectly
to CD151–INF194–196 via endogenous wild-type CD151.
Control experiments indicate that HA-tagged CD151 and
CD151–INF194–196 were present in HT1080 cells at compara-
table levels (Fig. 7 B, bottom). To extend results in Fig. 7,
separately tagged HA–CD151 and GFP–CD151 were coex-
pressed in Cos7 cells. From Brij96 lysate, anti-HA immuno-
precipitation yielded an abundance of GFP–CD151. Similar
amounts of associated GFP–CD151 were obtained regardless
of whether QRD mutant or wild-type CD151 was immu-
noprecipitated (unpublished data). No CD151–CD151
association was seen in Triton X-100 lysate (unpublished
data). These latter results confirm that in Brij96 lysate,
CD151–CD151 association occurs and is unaffected by the
QRD mutation and therefore could account for indirect,
Brij96-resistant links to α3 integrins. In 1% Brij96, other
tetraspanins could also link indirectly to integrins. In sup-
port of this, antibody to CD81 coimmunoprecipitated only
the mature forms of α3 and α6 integrin and only at the later
time points (Fig. 6 E). As expected (Berditchevski et al.,
1996), CD81–integrin associations were not seen in Triton
X-100 detergent (unpublished data).

Functional consequences of CD151–INF194–196 mutagenesis
Having established that CD151 associates strongly with
both α3 and α6 integrins by a similar QRD/TS151r-depen-
dent mechanism, we then examined the functional conse-
quences of disrupting association. For this we used a Matrigel
cellular cable formation assay shown previously to involve
CD151–α6 integrin complexes (Zhang et al., 2002). Mock-
 or human CD151–transfected Cos7 cells, grown on Matrigel for 18 h showed a similar pattern of cellular cable
formation (Fig. 8 a, A and B). In marked contrast, expres-
sion of CD151–INF194–196 almost completely abolished cel-
lar cable formation (Fig. 8 a, C). Confirming the func-
tional role of CD151, anti–human CD151 mAb 5C11 had
a pronounced inhibitory effect when human wild-type
CD151 was present (Fig. 8 a, D) but had no effect on Cos7
cells lacking human CD151 (unpublished data). Treatment
of CD151–INF194–196–transfected cells with 5C11 mAb re-
sulted in perhaps a slight additional inhibition of cable for-
mation that was already largely abolished due to the QRD
mutation (unpublished data).

To determine which integrins are involved, antibody in-
hibition experiments were performed (Fig. 8 b). As indi-
cated, anti–α6 mAb GOH3 (binding to monkey α6) substan-
tially inhibited cable formation by mock- and
CD151–transfected Cos7 cells (Fig. 8 b, A and C). When
GOH3 was added to Cos7–INF transfectants, even the resi-
dual cellular aggregates were now abolished (Fig. 8 a, C,
comparing with 8 b, E). Although slightly less potent than
the anti-α6 antibody, an anti–α3 mAb (A3-IIF5) also had
a strong inhibitory effect on mock- and CD151–transfected
Cos7 cells (Fig. 8 b, B and D). The minimal cable forma-
tion by Cos7–INF cells was not further inhibited by mAb
A3-IIF5 (Fig. 8 b, F). As shown previously (Zhang et al.,
2002), antibodies to other highly expressed cell surface pro-
teins (e.g., CD44 and CD9) had no inhibitory effects on
cellular cable formation. In agreement with previous results
(Zhang et al., 2002), NIH3T3–CD151 transfectants also
formed a robust network of CD151–integrin cellular cables
when plated on Matrigel for 20 h (Fig. 8 c, left). CD151–
INF194–196 transfection again almost completely abolished
this network (Fig. 8 c, right). Compared with endogenous
CD151, the CD151–INF194–196 mutant was expressed at a
level ~1–3-fold higher (in Cos7) and 2–4-fold higher (in
NIH3T3 cells). In both cases, this was sufficient to override
functional contributions of endogenous CD151, consistent
with CD151–INF194–196 exerting a dominant negative effect.
As seen previously (Zhang et al., 2002), cell adhesion was
essentially unaltered by transfection with either wild-type or
mutant CD151. Also, α3 and α6 integrins were maintained
at comparable levels in Cos7 cells (see Fig. 8 legend).

Although cell adhesion to a thin coating of Matrigel (con-
taining laminin-1) was not different between wild-type and
mutant CD151, we did observe pronounced differences in
cell spreading on Matrigel. Compared with Cos7–CD151
wild-type cells, Cos7–CD151–INF cells showed markedly
reduced spreading as indicated in photos of spread cells (Fig.
9) and by quantitation of the percentage of spread cells (Fig.
10). Spreading differences were especially obvious at early
time points (Fig. 9, 20 min, and Fig. 10, 0–15 min). Even
even after the majority of the CD151–INF cells had spread (Fig.
9, 40 min), they were typically spread over a smaller area
than the CD151 wild-type cells. In contrast to spreading on
Matrigel, the rate of cell spreading on fibronectin was essen-
tially identical (Fig. 10, bottom).
Discussion

Mapping α3 integrin association to the QRD region

To have a tool for definitive functional studies, we first needed to define a minimal CD151 mutation that would eliminate strong (Triton X-100–resistant) integrin association. Initially we used CD151–NAG-2 chimeras to define progressively smaller CD151 regions required for strong \(\alpha_3\beta_1\) association, thus focusing attention on CD151 residues 194–207. Subsequent point mutations indicated that C\(^{192}\) and QRD\(^{194–196}\) are each essential for strong CD151 association with \(\alpha_3\beta_1\). Because the cysteine mutation is potentially more disruptive to CD151 structure (due to disulfide bond elimination), we chose the QRD mutant for subsequent experiments. Our results are consistent with previous studies indicating a requirement for CD151 residues 186–216 (Yauch et al., 2000) or 195–205 (Berditchevski et al., 2001). Several experiments suggest that our QRD→INF mutation does not appreciably disrupt CD151 structure. Wild-type CD151 and the QRD mutant were both expressed at comparable levels on the surface of Cos7 cells as determined by flow cytometry (not depicted). (b) Mock- or CD151-transfected cells (A–C) were plated on the surface of a thick layer of Matrigel in 5% FBS-DME at \(5 \times 10^5\) cells per well in a 24-well plate, analyzed using a ZEISS Axiovert 135 microscope, and photographed after 18 h as described previously (Zhang et al., 2002). In one case (D), anti-CD151 mAb 5C11 was added (at 7.5 \(\mu\)g/ml) at the beginning of the experiment. mAb 5C11 does not bind to endogenous monkey CD151. (b) Cos7 cell stable transfectants were treated as in part a, except that mAbs to integrin \(\alpha_6\) (GoH3) or \(\alpha_3\) (A3IIIF5) were added (at 7.5 \(\mu\)g/ml) at the beginning of the experiment. The GoH3 and A3IIIF5 mAbs do recognize monkey \(\alpha_6\) and \(\alpha_3\) integrins. Note that compared with \(\alpha_3\) levels in mock-transfected Cos7 cells (MFI = 222), \(\alpha_3\) levels in mutant or wild-type CD151-transfected cells varied by \(\leq 1.2\)-fold. Compared with \(\alpha_6\) levels in mock-transfected Cos7 cells (MFI = 55), \(\alpha_6\) levels in mutant or wild-type CD151-transfected cells varied by \(\leq 1.9\)-fold. Previously we learned that twofold differences in \(\alpha_6\) levels had essentially no effect on static cell adhesion or on cell cable formation on Matrigel (Zhang et al., 2002). (c) Stable NIH3T3 cell transfectants were grown in 5% FBS-DME at \(10^5\) cells per well (24 well plate) on the surface of Matrigel for 20 h before photographs were taken.
complement and independently validate CD151 mutation results. Accordingly, the CD151 site for strong α3 integrin association is here called the QRD/TS151r site. The TS151r epitope on CD151 is also masked in many tissues (Sterk et al., 2002). Thus CD151–integrin complexes occur not only in lysates and in cell lines but also in vivo.

Within the QRD sequence, it is not yet clear which individual residues are essential. Conservation of R195 in CD151 from four different animal species (Table I) suggests that this residue may be particularly important. Conservative substitutions of Q194 and D196 by Lys and Glu appear in rodent CD151 (Table I), indicating some flexibility at those positions. So far, CD151 is the only tetraspanin that strongly associates with α3/β1. None of other 26 tetraspanins mentioned in Table I (see also Table I legend) contain a fully aligned Q/K-R-D/E motif. The BAB22942, TM4-B, and TM4SF6 tetraspanins have some similarity in this region but have not yet been tested for integrin association. Although the QRD region is clearly necessary, it is not sufficient for strong integrin association. Indeed, transfer of aa 158–216 from CD151 into NAG-2 was still not sufficient to confer strong α3 integrin association (unpublished data). However, transfer of CD151 aa 149–213 into the backbone of another tetraspanin did confer strong α3 association (Berditchevski et al., 2001). In conclusion, although additional elements are required to fully reconstitute a strong α3 integrin association site, we nonetheless have achieved the goal of defining a minimal CD151 mutation that wholly eliminates strong integrin association.

Comparisons between α3 and α6 integrins
Our results indicate major similarities between α3 and α6 integrins in terms of CD151 association properties. Both integrins can form Triton X-100–resistant CD151 complexes that require the QRD site, conceal the TS151r epitope, and occur early in biosynthesis. Association of CD151 with α3 and α6 precursor forms, including α3 that has not yet been proteolytically processed, is consistent with CD151 complex formation occurring in the ER. Covalent cross-linking results indicate that CD151 directly contacts the integrin α3 subunit and not β1 (unpublished data). We predict that CD151 should also directly contact the integrin α6 subunit. Furthermore, although we have limited our studies here to the α6β1 heterodimer, our conclusions should also apply to CD151–α6β4 complexes (Sterk et al., 2000). The similarity between α3 and α6 integrins in terms of CD151 association is likely related to their overall protein sequence similarity. In this regard, the integrin α7 subunit is also structurally similar to the α3 and α6 subunits and also may associate
strongly with CD151 (Sterk et al., 2002). Thus, α7β1 association should also require the QRD region of CD151, but this has not yet been tested.

Given the major similarities between α3 and α6 integrins in terms of CD151 association, then why are pronounced differences sometimes observed? For example, in K562 and NT2 cells, CD151 associates strongly with α3 but not with α6β1 or α6β4 integrins (Sterk et al., 2000; Stipp and Hemler, 2000; Yauch et al., 2000). Also, compared with α3 integrin, α6 expression in K562 cells did not bring as much CD151 to the cell surface (Yauch et al., 2000) and not as much α6 associated with CD151 in 293 cells (Fig. 5). We suspect that in some cells (especially if CD151 is limiting), an abundance of weakly associating other tetraspanins could associate with α6 integrins and thereby block access to CD151, or if α3 is in excess, it could compete more favorably for CD151.

Multiple levels of association

Strong CD151–integrin association is Triton X-100 resistant, utilizes the QRD/TS151r site, occurs early in biosynthesis, and involves both precursor and mature α3 and α6 subunits. In contrast, a second level of CD151–integrin association is maintained in Brij 96 but not Triton X-100, does not use the QRD/TS151r site, occurs late in biosynthesis, and involves mature integrin α chains. Our results reinforce the idea that strong QRD/TS151r-dependent integrin association is unique for CD151, whereas the QRD/TS151r site is not needed for weaker CD151 associations with itself, other tetraspanins (such as CD81) or α3 and α6 integrins in Brij 96 lysates. In this regard, we confirm and extend previous studies showing that the large extracellular loop of CD151 is needed for strong CD151–α3 associations but not for second level CD151 associations. In fact, replacement of the entire large extracellular loop of CD151 with irrelevant protein did not prevent secondary interactions with itself, α3 integrin, or other tetraspanins (Berditchevski et al., 2001). The biochemical basis for these second level CD151 interactions is not yet entirely clear, although a role for tetraspanin palmitoylation has been established (Yang et al., 2002).

Functional studies

In a previous study involving neurite outgrowth, α3β1 integrin collaborated equally well with strongly associated CD151 and with more weakly associated CD81 (Stipp and Hemler, 2000). Such results suggested that strong, direct tetraspanin–integrin association was functionally indistinguishable from second level associations. The CD151–INF194–196 mutant now provides a tool to disrupt strong integrin association without affecting secondary CD151 engagement with CD81 and other components in the multicomponent tetraspanin web. Indeed, we demonstrate here that CD151–INF194–196 mutants failed to support Matrigel cellular cable formation by either Cos7 or NIH3T3 cells. Sensitivity of the assay to both anti-α3 and -α6 integrin antibodies indicates that strong CD151 associations with both α3 and α6 integrins are functionally relevant. Importantly, weak second level associations of CD151–INF194–196 that were retained as seen in Brij 96 conditions were not sufficient to overcome the QRD deficit.

Our anti-α3 inhibition results with Cos7 cells are in contrast to previous results with human α3–NIH3T3 transfectants, in which mAb anti–human α3 failed to inhibit cell cable formation (Zhang et al., 2002). Possibly in the previous case, the inhibitory effects of anti–human α3 antibody on α3-transfected NIH3T3 cells were diminished due to an unknown contribution from murine α3. Furthermore, in the α3–NIH3T3 transfectants used previously, the ratio of α3 to α6 recognized by inhibitory antibodies was ∼2:1, whereas in Cos7 cells described here, the ratio of α3 to α6 is between ∼4:1 and 7:1. Because α3β1 is a poorer receptor for laminin-1 (Delwel et al., 1994; Eble et al., 1998), a higher ratio of α3 to α6 may be needed for the contribution of α3 integrin to become evident.

When plated on the surface of a malleable Matrigel thick layer, multiple cell types can exert tensile forces on the basement membrane and then migrate along these “matrix guidance pathways” until they are assembled into a network of cellular cables (Vernon et al., 1992; Davis and Camarillo, 1995; Vernon and Sage, 1995). In collagen gels, newly sprouted endothelial capillary cells organize into a very similar cellular network pattern, with cells aligning in the direction of tensional forces (Korff and Augustin, 1999). The generation of matrix tensional forces in model systems not only directs cell morphology but also reorganizes the matrix, thus providing insights into tissue morphogenesis and wound healing (Bell et al., 1979; Harris et al., 1981). On a very thin, nonmalleable layer of Matrigel, mechanical force transduction results in cell spreading rather than cable formation and matrix remodeling. Based on our cable formation and cell spreading results, CD151 potentially could play a critical role in mechanical force transduction wherever laminin-binding integrins are involved. For example, CD151 could affect mechanical force transmission by carcinoma and endothelial cells using α6β4 and α6β1 (Davis and Camarillo, 1995; Rabinovitz et al., 2001), and endothelial cell CD151 has already been shown to play a role during α6β1-dependent Matrigel cable network formation (Zhang et al., 2002). Interestingly, CD151 is colocalized with α7β1 in skeletal and cardiac muscle (Sterk et al., 2002), but a role in force generation remains to be investigated. Likewise, it remains to be seen how strong and specific association with CD151 may affect the many other functions of α3β1, α6β1, α6β4, and α7β1 integrins on both normal and transformed cells (Weitzman et al., 1996; Wei et al., 1997; Burkin and Kaufman, 1999; Kreidberg, 2000; Mercurio et al., 2001). Conversely, the role of CD151 as a regulator of cell migration (Yánez-Mó et al., 1998; Yauch et al., 1998), tumor cell metastasis (Testa et al., 1999; Kohno et al., 2002), neurite outgrowth (Stipp and Hemler, 2000), and other functions could be largely due to strong and specific association with laminin-binding integrins.

How does strong QRD/TS151r-dependent association of CD151 affect integrin function? Here we define an extracellular site (QRD194–196) that is essential for strong association with integrins, optimal cell spreading, and cell cable formation on Matrigel. Previously, we showed that the short (8 aa) COOH-terminal cytoplasmic tail of CD151 is also essential for integrin-dependent cell spreading and cable formation on Matrigel. Together these results support a transmembrane
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CAGCCGCCCTCCAC, internal antisense to amplify the 5’ region on CD151 template; and CAGCTGGGCCCGCGCCCTCCCA, internal antisense to amplify the 3’ region on CD151 template.

Although antisense oligonucleotides and mutant tetraspanins can dramatically alter the consequences of integrin-mediated cell adhesion, these reagents consistently have little effect on integrin-mediated cell adhesion itself (Hemler et al., 1996; Yauch et al., 1998; Zhang et al., 2002). Thus, tetraspanins are selectively influencing “outside-in” integrin signaling. Previous studies of outside-in signaling have largely focused on integrin extracellular ligand-binding sites, and integrin cytoplasmic domains. Indeed, with respect to mechanical force transduction, specific integrin cytoplasmic domains do play a key role (Chan et al., 1992). However, our results now emphasize that a specific, membrane proximal CD151–integrin lateral association site is also playing a key role. Such specific lateral interactions (Woods and Couchman, 2000), mediated through novel sites, provide an important new dimension to our understanding of integrin signaling.

Materials and methods

Cell lines, transfectants, and antibodies

Erythroleukemic K562 cells were maintained in RPMI 1640 supplemented with 10% FBS and antibiotics. Cos7, 293 cells, NIH3T3, and HT1080 cells were grown in DME with 10% FBS supplemented with sodium pyruvate and antibiotics. For transient transfection, 10 × 10^6 K562 cells were electroporated with 50 μg of plasmid DNA at 960 microfarads and 320 V (Mc-Caffrey et al., 1997) and analyzed within 36–48 h. Transfection efficiency was 45–63% as estimated using GFP-containing expression vector. Cos7 and HT1080 cells were transiently transfected using FuGene6 reagent (Roche Molecular Biochemicals) according to manufacturer’s instructions. 293 cells were transiently transfected using calcium phosphate, with 40–65% as estimated using GFP-containing expression vector. Cos7 and NIH3T3 cells using FuGene6 reagent, cells were selected in media containing Zeocin (0.2 mg/ml) for 2 wk, and pooled cells were further sorted by flow cytometry using mAb SC11.

The following antiintegrin mAbs were used: anti-a2, A2-HE10 (Bergelson et al., 1994); anti-a3, A3-III5 and A3-X8 (Weitzman et al., 1993); and anti-a6, Ab-ELN (Lee et al., 1995). Other mAbs were anti-CD151, SC11 (Yauch et al., 1998); TS151r (Serru et al., 1999), 1A5 (Testa et al., 1999); and anti-CB81, JS64 (Pesando et al., 1986). Polyclonal rabbit antisera D23 recognizes aα cytoplasmic domain (Dpersio et al., 1995), and 6843 (gift from Dr. V. Quanara) recognizes the a6 cytoplasmic domain. Anti-α-HA mAb 3F10 was from Roche Molecular Biochemicals. HRP-conjugated goat anti-mouse, goat anti-rabbit and HRP-conjugated streptavidin were from Sigma-Aldrich.

Construction of HA-tagged CD151 mutants

Construction of HA-tagged wild-type CD151 and NAG-2 proteins and HA-tagged chimeric proteins, with exception of the last four proteins in Fig. 1, was done by recombinant PCR as described (Yauch et al., 2000). To construct new mutants, we used the same recombinant PCR approach and existing HA-tagged templates: for mutant C(193)–N–C(208), AAAGGGCGGCTGCA-CACAAGTTG, internal sense to amplify the 3’ region on CD151 template; and CTTGGTGATGCAGCCGCTTCCTCA, internal antisense to amplify the 5’ region on C(193)–N–C(217) template; for CD151–INF194–196, TGTTGAATATTTCTGCTGCTAC, internal sense to amplify 5’ region on CD151 template, and CGCAAGAAATAAATCCACAAACACC, internal antisense to amplify the 5’ region on CD151 template. As the external primers, in each case we used either T3 or SP6 primers encoded by expression plasmid pZeoSV (Invitrogen). Final recombinant PCR was performed using purified PCR products and T3 and SP6 primers. Products were ligated into Spel and EcoR1 restriction sites of pZeoSV and confirmed by sequencing.

Cell labeling, immunoprecipitation, and immunoblotting

To determine association of CD151 with integrins during biosynthesis, 293 and HT1080 cells were labeled with 1-μCi [35S]methionine/cysteine mixture (NEN Life Science Products). Cells were washed twice in PBS, starved in methionine- and cysteine-free medium for 1 h, and then labeled using 0.5 μCi/ml of [35S]methionine/cysteine in methionine/cysteine-free medium supplemented with 5% dialyzed FBS. Subsequently, cells were collected (time 0 after labeling) or chased for various times by replacing labeling medium with chasing medium (5% dialyzed FBS and 25% excess of unlabeled methionine and i-cysteine). Labeled cells were washed in PBS several times and processed for immunoprecipitation.

For immunoprecipitation, cells were lysed for 1 h at 4°C in RIPA buffer (25 mM Tri-HCl, pH 7.2, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS) or in PBS (8.0, 5 mM MgCl2, 150 mM NaCl, with 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml apro- tinin, 10 μg/ml leupeptin, and detergent (1% Brij 96 [Sigma-Aldrich]), or 1% Triton X-100 (Roche Molecular Biochemicals)). Insoluble material was removed by centrifugation, and lysates were immunoprecipitated with mAbs prebound to protein G–Sepharose (Amersham Biosciences) at 4°C overnight. Immune complexes were washed three to four times with the same buffer then resolved on acrylamide SDS-PAGE gel, transferred to nitrocellulose (Schleicher & Schuell) and blotted with primary antibody and HRP-conjugated secondary antibody, and then visualized with chemiluminescence reagent (NEN Life Science Products).

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References

Anton, E.S., J.A. Kredberg, and P. Rakic. 1999. Distinct functions of alpha3 and alpha6 integrin receptors in neuronal migration and laminar organization of the cerebral cortex. Neuros. 22:277–289.
Belkin, A.M., and M.A. Stepp. 2000. Integrins as receptors for laminins. Micro. Res. Tech. 51:280–301.
Bell, E., B. Iverson, and C. Merrill. 1979. Production of a tissue-like structure by skin fibroblasts. J. Biol. Chem. 254:1205–1211.
Berditchevski, F. 2001. Complexes of tetraspanins with integrins: more than meets the eye. J. Cell Sci. 114:4143–4151.
Berditchevski, F., G. Bazzoni, and M.E. Hemler. 1995. Specific association of CD63 with the VLA-3 and VLA-6 integrins. J. Biol. Chem. 270:17784–17790.
Berditchevski, F., M.M. Zutter, and M.E. Hemler. 1996. Characterization of novel complexes on the cell surface between integrins and proteins with 4 transmembrane domains (TM4 proteins). Mol. Biol. Cell. 7:193–207.
Berditchevski, F., S. Chang, J. Bodor, and M.E. Hemler. 1997. Generation of monoclonal antibodies to integrin-associated proteins: evidence that αβ1 complexes with EMMMPRI/haigin/67/M6. J. Biol. Chem. 272:29174–29180.
Berditchevski, F., E. Gilbert, M.R. Griffiths, S. Fitter, L. Ashman, and S.J. Jenner. 1996. Clonal antibodies to integrin-associated proteins: evidence that αβ1 complexes with EMMPRI/haigin/67/M6. J. Biol. Chem. 272:29174–29180.
Bergelson, J.M., N.F. St. John, S. Kawaguchi, R. Pasqualini, F. Berditchevski, M.E. Hemler, and R.W. Finberg. 1994. The 1 domain is essential for echovirus 1 potential in vitro. Proc. Natl. Acad. Sci. USA. 76:1274–1278.
Bouchard, C., and E. Rubinstein. 2001. Tetraspanins. Cell. Mol. Life Sci. 58:1189–1205.
Burkin, D.J., and S.J. Kaufman. 1999. The alpha3beta1 integrin in muscle devel-

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opment and disease. Cell Tissue Res. 296:183–190.
Chan, B.M.C., P.D. Kassner, J.A. Schro, H.R. Byers, T.S. Kupper, and M.E. Hemler. 1992. Distinct cellular functions mediated by different VLA integrins in subunit cytoplasmic domains. Cell 68:1051–1060.
Davis, G.E., and C.W. Camarillo. 1995. Regulation of endothelial cell morphogenesis by integrins, mechanical forces, and matrix guidance pathways. Exp. Cell Res. 216:113–125.
Delwel, G.O., A.A. de Melker, F. Hogervorst, L.H. Jaspars, D.L.A. Fleis, I. Kuikman, A. Lindblom, M. Paulsson, R. Timpl, and A. Sonnenberg. 1994. Distinct and overlapping ligand specificities of the alpha 3beta1 and alpha 6beta1 integrins: recognition of laminin isoforms. Mol. Biol. Cell. 5:203–221.
Dipersio, J.M., P. Hoffman, and T. Conrad. 1986. Malignant human B cells express raspan interactions. J. Cell Biol. 114:1126–1135.
Poinney, C., C.A. Buendia, P. Dols, M. Billard, D.O. Azorsa, F. Lanza, C. Boucheix, and E. Ruhrmoser. 1999. Selective tetraspan-integrin complexes (CD81/alpha beta 1, CD151/alpha beta 1, CD151/alpha beta 4) under conditions disrupting tetraspan interactions. Biochem. J. 340:103–111.