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Tetraspanin CD151 Regulates Glycosylation of α3β1 Integrin*

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The tetraspanin CD151 forms a stoichiometric complex with integrin α3β1 and regulates its endocytosis. We observed that down-regulation of CD151 in various epithelial cell lines changed glycosylation of α3β1. In contrast, glycosylation of other transmembrane proteins, including those associated with CD151 (*e.g.* α6β1, CD82, CD63, and emmprin/CD147) was not affected. The detailed analysis has shown that depletion of CD151 resulted in the reduction of Fucα1–2Gal and bisecting GlcNAc-β(1→4) linkage on *N*-glycans of the α3 integrin subunit. The modulatory activity of CD151 toward α3β1 was specific, because stable knockdown of three other tetraspanins (*i.e.* CD9, CD63, and CD81) did not affect glycosylation of the integrin. Analysis of α3 glycosylation in CD151-depleted breast cancer cells with reconstituted expression of various CD151 mutants has shown that a direct contact with integrin is required but not sufficient for the modulatory activity of the tetraspanin toward α3β1. We also found that glycosylation of CD151 is also critical; Asn[159] → Gln mutation in the large extracellular loop did not affect interactions of CD151 with other tetraspanins or α3β1 but negated its modulatory function. Changes in the glycosylation pattern of α3β1 observed in CD151-depleted cells correlated with a dramatic decrease in cell migration toward laminin-332. Migration toward fibronectin or static adhesion of cells to extracellular matrix ligands was not affected. Importantly, reconstituted expression of the wild-type CD151 but not glycosylation-deficient mutant restored the migratory potential of the cells. These results demonstrate that CD151 plays an important role in post-translation modification of α3β1 integrin and strongly suggest that changes in integrin glycosylation are critical for the promigratory activity of this tetraspanin.

Transmembrane proteins from the tetraspanin superfamily are assembled in microdomains (referred to as tetraspanin-enriched microdomains (TERM)[2]) which also incorporate a number of tetraspanin-interacting receptors (*e.g.* integrins and receptor tyrosine kinases) (1). It has been shown that tetraspanins regulate the activity of the associated receptors via various mechanisms involving ligand binding, clustering, and trafficking (1–3).

N-Linked glycosylation is one of the most common and diverse modifications of transmembrane proteins. The role of glycosylation in regulation of protein stability, folding, and dimerization and trafficking to and from the plasma membrane as well as between intracellular organelles is documented in numerous reports (4–8). Furthermore, earlier studies have shown that glycosylation of a number of transmembrane proteins, which would be later identified as tetraspanin-associated partners, regulate their functions. Glycosylation of intercellular adhesion molecule 1 and components of major histocompatibility class I complex is important for interactions of the proteins with their respective receptors (9, 10). Trafficking of CD4 to the cell surface was impaired in cells treated with tunicamycin (11). More recently, it was found that glycosylation of H,K-ATPase β subunit, a partner for tetraspanin CD63, regulates internalization and subsequent degradation of the protein (12). A number of reports have described that surface expression, conformation, ligand binding, dimerization, and endocytosis of epidermal growth factor receptor are regulated by glycosylation (13–15). The *N*-linked glycan on ErbB3 prevents spontaneous heterodimerization and activation of the receptor (16).

There is a substantial body of evidence indicating that glycosylation of integrins plays a critical role in their function. Early experiments have shown that differential glycosylation of β2 integrin subunit is critical for its pairing with either αL or αM (17). Altered glycosylation of β1 integrins correlated with differences in attachment of cells to fibronectin and laminin (18). Similarly, binding of α5β1 integrin to its ligand was decreased in cells treated with 1-deoxymannojirimycin, a compound that inhibits conversion of the high mannose to hybrid and complex glycosylated species of the protein (19). Subsequent work from various laboratories has extended these observations to other integrins, including α6β1 (20), αv integrins (21), and α3β1 (22–25). In addition, it has been proposed that glycosylation-de-

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[2] The abbreviations used are: TERM, tetraspanin-enriched microdomain(s); DMEM, Dulbecco's modified Eagle's medium; Ab, antibody; mAb, monoclonal antibody.

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pendent interactions of integrins with their ligands involve gangliosides (26, 27).

Together, α3 and β1 integrin chains have 27 potential *N*-linked glycosylation sites: 14 on the α3 subunit and 13 on the β1 subunit. Detailed analyses of *N*-glycans by mass spectrometry have revealed significant diversity of oligosaccharides that decorate α3β1 integrin purified from various cell types (22, 23, 25, 28, 29). Importantly, specific changes in glycosylation of α3β1 observed in tumor cells correlated with their migratory and invasive potential (25, 29).

Recent studies have demonstrated that tetraspanin CD81 regulates glycosylation of its proximal partners CD19 and EWI-2 (30, 31). In the case of CD19, the region responsible for this activity was mapped to the predicted N-terminal cytoplasmic portion of the protein (30). In addition, modulation in the expression levels of CD82/KAI-1 affected maturation and surface expression of β1 integrin subunit in lung carcinoma cells (32).

α chains of canonical laminin-binding integrins (*i.e.* α3β1, α6β1/β4, and α7β1) are post-translationally cleaved in Golgi by proprotein convertases to form light and heavy chains that are held together by a single disulfide bond and β subunit (33). It is thought that cleavage of α subunits is required for "inside-out" activation of integrin heterodimers (34). There are two splice variants of the light chains described for α3 subunit, each having three potential *N*-linked glycosylation sites (35).

Tetraspanin CD151 forms stable and highly stoichiometric complexes with α3β1, α6β1/β4, and α7β1 integrin heterodimers (36, 37). Interactions with CD151 regulate ligand-binding and signaling properties of these integrins (38–47). Furthermore, CD151 regulates endocytosis of α3β1 integrin (45, 48, 49). In this report, we describe a previously unknown role for CD151 as a modulator of α3β1 glycosylation. We have also established that the direct interaction of CD151 with α3β1 is necessary but not sufficient for the modulatory activity of the tetraspanin. Finally, we provide strong evidence that changes in integrin glycosylation are important for the α3β1-dependent promigratory function of CD151.

EXPERIMENTAL PROCEDURES

Cells Lines and Antibodies

The MDA-MB-231 and HeLa cell lines were purchased from the Cancer Research UK. Cell lines were maintained in DMEM (Invitrogen) supplemented with 10% fetal calf serum (PAA Laboratories). The mouse anti-CD81 and anti-CD82 mAbs (M38 and M104, respectively) were kindly provided by Dr. O. Yoshie. The anti-CD63 (6H1) and anti-CD151 (5C11 and 11B1G4) mouse mAbs and rabbit anti-CD151 polyclonal Ab were described previously (50–53). The anti-CD9 mAb BU16 was from The Binding Site (Birmingham, UK). The anti-CD82 (TS82) mAbs were generously provided by Dr. E. Rubinstein (Villejuif, France). The anti-integrin mAbs used were A2-VIIC6 (anti-α2) (54), A3-IVA5 (anti-α3) (55), P1D6 (anti-α5) (56), A6-ELE (anti-α6) (57), TS2/16 (anti-β1) (58), G0H3 (anti-α6) (Chemicon International), and 3E1 (anti-β4) (Chemicon International). Rabbit polyclonal antibodies to α3 and α6 integrin subunits were gifts from Dr. F. Watt (Cambridge, UK) and Dr. A. Cress (Tucson, AZ). Laminin-332 was isolated from SCC25 cells as previously described (59). Biotinylated lectins were purchased from Vector Laboratories. To generate stable MDA-MB-231/CD151(−) and HeLa/CD151(−) cell lines, transfections were carried out using Fugene6 (Roche Applied Science) for HeLa or GeneJammer (Stratagene) for MDA-MB-231 cells. Transfected cells were selected and maintained in DMEM containing 0.5–1.0 μg/ml puromycin. CD151 knockdown was confirmed by flow cytometry analyses (COULTER Epics XL). CD151-positive and -negative populations were selected by cell sorting (BDFACSVantage SE) and confirmed by flow cytometry and Western blotting. MDA-MB-231/CD9(−), MDA-MB-231/CD63(−), and MDA-MB-231/CD81(−) cell lines were established using the same approach. To generate MDA-MB-231/rec series (MDA-MB-231/CD151(−) cells with the reconstituted expression of the CD151 wild-type or CD151 mutants) pZeoSV-based constructs were introduced into the cells using GeneJammer and selected in growth medium containing 100–300 μg/ml Zeocin. Various reconstituted CD151 cell lines were sorted to obtain a pool of cells expressing CD151 at levels similar to that of the control MDA-MB-231.

DNA Constructs

Plasmids Expressing Short Hairpin RNA—pSuperior-based constructs for specific targeting of tetraspanins were generated using a standard protocol. The constructs targeted the following sequences: CD151, 5′-AGTACCTGCTGTTTACCTACA (45); CD81, 5′-ATCTGGAGCTGGGAGACAA (60); CD63, 5′-GGTTTTTCAATTAAACGGA. pSuper-CD9 (kind gift from Dr. E. Rubinstein (Villejuif, France)) targets the following sequence: 5′-ACCTTCACCGTGAAGTCCT (61).

CD151 Constructs—The original CD151ΔC, CD151palm(−)/CD151Cys8, and SW6 mutants have been described earlier (38, 43, 62). CD151-QRD mutant was generated as described by others (39). CD151glyco(−) and 63-N-151 mutants were generated using a standard PCR approach; in CD151glyco(−) mutant, a predicted glycosylation site (Asn^{159}) was substituted to glutamine, and in 63-N-151 mutant, the N-terminal cytoplasmic sequence of CD151 was substituted to a corresponding region of CD63.

Flow Cytometry and Cell Sorting

Semiconfluent cells were detached using Cell Dissociation Buffer (Invitrogen), incubated with saturating concentrations of primary mouse mAb for 1 h on ice, washed twice in phosphate-buffered saline, and then labeled with fluorescein isothiocyanate-conjugated goat anti-mouse IgG for 1 h at 4 °C. Surface labeling was analyzed by flow cytometry using COULTER Epics XL. For sorting, cells were prepared as above except that all the solutions were sterilized by filtration.

Western Blot Analysis

Cells were lysed overnight at 4 °C in 1% Triton X-100/phosphate-buffered saline buffer containing inhibitors (2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10

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μg/ml leupeptin). The lysate was centrifuged at 12,000 × *g* for 10 min to remove insoluble material. Equal amounts of protein lysates were resolved by 12% SDS-PAGE under reducing and nonreducing conditions, transferred onto nitrocellulose membrane, and incubated with appropriate primary Ab. Protein bands were visualized after subsequent incubations with appropriate horseradish peroxidase-conjugated secondary Ab and Chemiluminescence Reagent Plus (PerkinElmer Life Sciences).

Immunoprecipitation

Cell lysates were prepared as above (except when the analysis of tetraspanin-tetraspanin association was performed) and precleared by incubation for 3 h at 4 °C with agarose beads conjugated with goat anti-mouse antibodies (mIgG-beads; Sigma). Immune complexes were collected using appropriate mAbs prebound to the mIgG-beads and washed four times with the immunoprecipitation buffer. The complexes were eluted from the beads with Laemmli sample buffer. Proteins were resolved by SDS-PAGE, transferred to the nitrocellulose membrane, and developed with the appropriate Ab. For the analysis of α3β1-tetraspanin (other than CD151) and tetraspanin-tetraspanin interactions, cell lysis and immunoprecipitation was carried out in 1% Brij 96 in the presence of aforementioned inhibitors.

Migration Assay

Migration was analyzed using a standard Boyden Chamber protocol. In brief, 1–2 × 10^5 cells were detached using Cell Dissociation Solution (Invitrogen) and suspended in 500 μl of serum-free DMEM. Cells were subsequently added into the inner compartment of Nunc's tissue culture inserts with polycarbonate membranes (8-μm pores), the bottom sides of which were coated with 2 μg/ml Laminin-332 or 10 μg/ml fibronectin (Sigma). Cells were allowed to migrate toward serum-free DMEM supplemented with 10 ng/ml epidermal growth factor for 8 h. Nonmigrated cells were removed, and nuclei of migrating cells were stained with 4′,6-diamidino-2-phenylindole. Membranes were mounted on glass slides and analyzed using a Nikon Eclipse E600 microscope. Up to seven random fields per membrane were photographed and scored using ImageJ nuclear/cell counter program. Each of the experiments was done in quadruplicates, and 2–3 independent experiments were carried out for each cell line.

Deglycosylation Treatment

Protein lysate (~100 μg) was boiled for 10 min in the presence of 0.5% SDS before deglycosylation. The denatured lysate or immunoprecipitated complexes eluted in 0.2× Laemmli buffer were deglycosylated overnight at 37 °C with either peptide:*N*-glycanase F in Buffer G7 or endoglycosidase H in Buffer G5 (New England Biolabs). Samples were then resolved by 12% SDS-PAGE, transferred onto nitrocellulose membrane, and detected with Abs for various proteins. Deglycosylation was performed in both reducing (in the presence of 40 mM dithiothreitol) and nonreducing conditions.

Cell Adhesion Assay

A standard static adhesion assay (30–35 min) was carried out as previously described (55); 2,7-bis-(2-carboxyethyl)-5-(and-6)carboxyfluorescein-labeled cells aliquoted into 96-well plates precoated overnight with various concentrations of laminin-332 or fibronectin.

RESULTS

CD151 Regulates Glycosylation of α3β1 Integrin—The tetraspanin CD151 forms stoichiometric complexes with various laminin-binding integrins, including α3β1, α6β1, α6β4, and α7β1. To investigate whether CD151 affects biosynthetic processing of integrins, we established two epithelial cell lines in which expression of the tetraspanin was decreased, using a specific short hairpin RNA construct (45); MDA-MB-231/CD151(−) and HeLa/CD151(−) lines express CD151 at levels of <5% of the corresponding parental cells (Fig. 1*A*). Flow cytometry and Western blotting have shown that down-regulation of CD151 did not affect surface and total levels of α3β1 and α6 integrins (α7β1 is not expressed in epithelial cells). Interestingly, we consistently observed that the protein band corresponding to the α3 integrin subunit runs slower in SDS-PAGE. This was particularly evident when we compared the positions of the α3 light chains. Light chains of α3 resolved as closely spaced three bands with the top, slower migrating band being more prominent in MDA-MB-231/CD151(−) than MDA-MB-231/CD151(+) cells (Fig. 1*B*). Conversely, the intensity of the lowest band was higher in the CD151-positive cells. The observed differences can be explained by one of the following: 1) differences in the furin-dependent cleavage; 2) differences in splicing; 3) differences in glycosylation. To distinguish between these possibilities, we compared the mobility of α3 light chains in SDS-PAGE after the treatment of protein lysates with peptide:*N*-glycanase. Since peptide:*N*-glycanase completely removes *N*-linked glycans from the protein backbone, this treatment would negate both qualitative and quantitative differences in glycosylation of the integrin in CD151-positive and CD151-negative cells. Indeed, we observed comparable patterns of the α3 light chains in the peptide:*N*-glycanase-treated lysates from the MDA-MB-231 and HeLa pairs (Fig. 2*A*, *lanes 3* and *4* and *lanes 9* and *10*). To examine differences in the integrin glycosylation in more detail, we analyzed mobility of the α3 light chains after treatment with endoglycosidase H, which specifically cleaves high mannose and hybrid forms of *N*-linked glycans. Although all three major glycoforms of the α3 light chain were sensitive to the endoglycosidase H treatment, the products of the digestion run slower in SDS-PAGE than completely deglycosylated species (Fig. 2*A*). These results indicated that the light chain of the α3 integrin subunit, which has three putative glycosylation sites, is modified by both complex and hybrid/high mannose oligosaccharides. Importantly, depletion of CD151 changed relative abundance of various endoglycosidase H-resistant glycoforms in MDA-MB-231 and HeLa cells (Fig. 2*A*, *lanes 5* and *6* and *lanes 7* and *8*). To gain further insight into the role of CD151 in matura-

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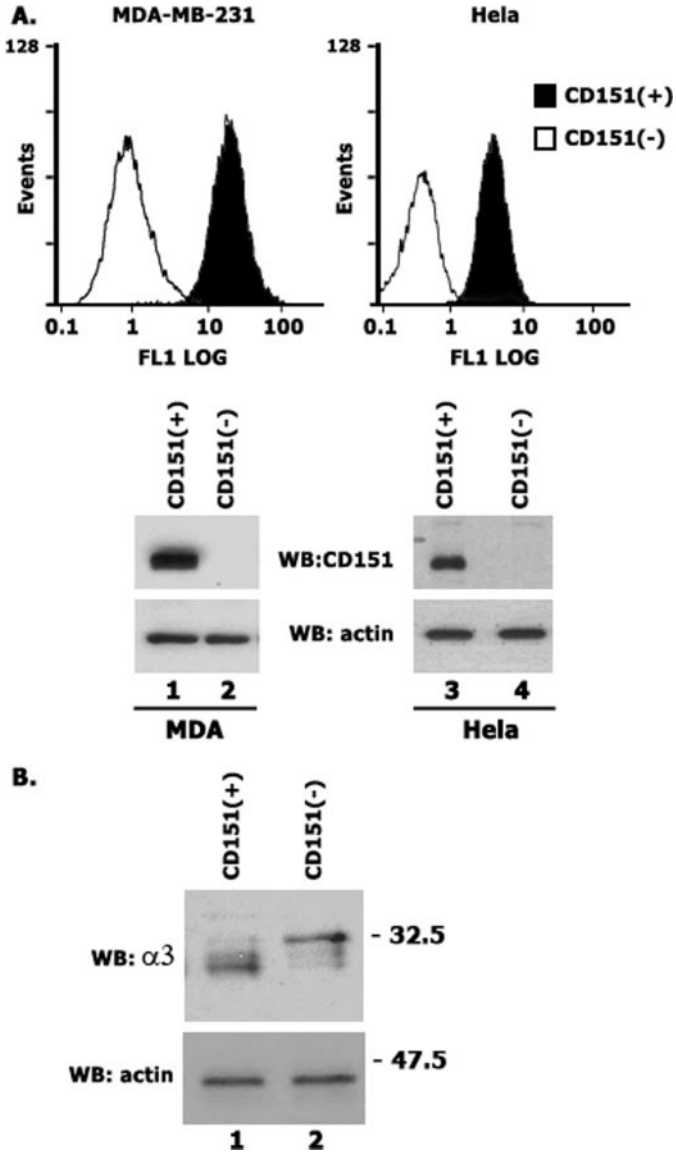

FIGURE 1. **Generation of stable CD151-depleted cells.** *A*, down-regulation of CD151 in MDA-MB-231 and HeLa cells was analyzed by flow cytometry (*top panels*) and by Western blotting (*WB*). Lysates prepared from MDA-MB-231, MDA-MB-231/CD151(−), HeLa, and HeLa/CD151(−) cells were resolved by 12% SDS-PAGE and analyzed by Western blotting with mouse mAbs to CD151 or β-actin. *B*, down-regulation of CD151 changes mobility of the light chain of α3 integrin subunit. Lysates prepared from MDA-MB-231 and MDA-MB-231/CD151(−) cells were resolved by 12% SDS-PAGE under reducing conditions and analyzed by Western blotting with rabbit Ab to α3 integrin subunit.

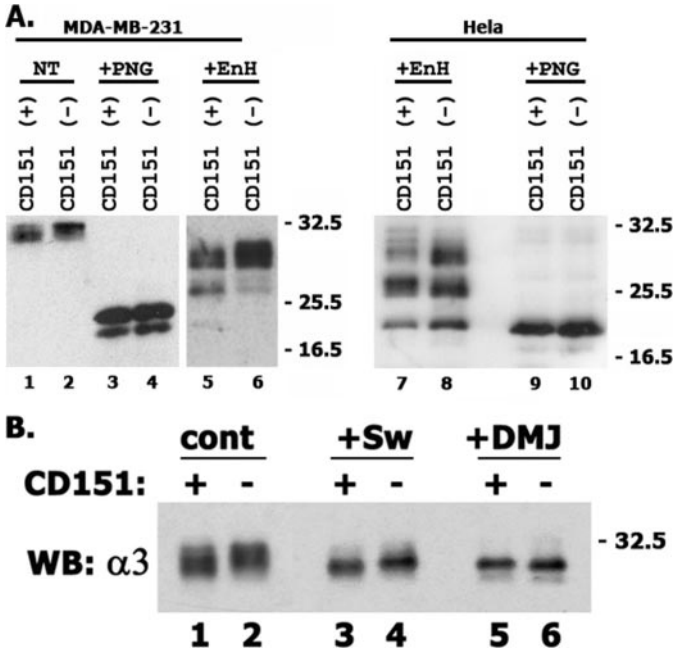

FIGURE 2. **Down-regulation of CD151 changes glycosylation of α3 integrin subunit.** *A*, cells were lysed in Triton X-100, and proteins were deglycosylated overnight with either peptide:*N*-glycanase F (*PNG*; *lanes 3*, *4*, *9*, and *10*) or endoglycosidase H (*EnH*; lanes 5–8). Samples reduced with dithiothreitol were resolved by 12% SDS-PAGE, transferred onto nitrocellulose membrane, and detected with rabbit Ab to α3 integrin subunit. *B*, cells were grown under standard conditions (*cont*) or in medium containing either 2 μg/ml swainsonine (*Sw*) or 2 mM 1-deoxymannojirimycin (*DMJ*) for 48 h. Glycosylation of the light chain of α3 integrin subunit was analyzed as described in *A*. *WB*, Western blot.

tion of α3β1, we analyzed glycosylation of the integrin in cells treated with swainsonine and deoxymannojirimycin. These chemicals block activities of trimming glycosidases and, thereby, inhibit processing of *N*-linked oligosaccharides to complex (swainsonine) and hybrid/complex forms (deoxymannojirimycin). Although less pronounced, differences between CD151(+) and CD151(−) cells were still visible after treatment with either swainsonine or deoxymannojirimycin (Fig. 2*B*). Taken together, these results demonstrated that CD151 influences glycosylation of α3β1 at the relatively early stages of the maturation process.

Depletion of CD151 Does Not Affect Glycosylation of Other Glycoproteins within TERM; Association of Tetraspanins with α3β1 Glycoforms—There are a large number of glycosylated proteins that are incorporated into TERM (63). To establish whether CD151 also targets other TERM-associated glycoproteins, we compared glycosylation patterns of CD63, CD82, and α6 integrin subunit. In addition, we analyzed glycosylation of EMMPRIN, a transmembrane protein that is known to associate with α3β1 (51). The results of these experiments showed that glycosylation of none of these proteins was affected by depletion of CD151 (Fig. 3*A*) (data not shown). It has been proposed that there exists a certain hierarchy of protein-protein interactions within TERM (64). Thus, we examined whether various α3β1 glycoforms preferentially associate with other tetraspanins. As shown in Fig. 3*B*, there was a subtle difference in the patterns of α3 glycoforms co-precipitated by the mAbs to CD9 and CD81; although one of the two lower glycoform species predominated over the other in the CD81 immunoprecipitate, relative abundance of these forms in the CD9 immunoprecipitate was comparable (*lanes 2* and *3*). These differences in glycosylation between the CD9- and CD81-associated α3 were observed in three independent experiments.

To examine whether these tetraspanins can modify glycosylation pattern of α3β1, we generated stable MDA-MB-231/CD9(−), MDA-MB-231/CD81(−) cell lines, in which expression of CD9 and CD81 was decreased by >95% (Fig. 3*C*). We also established a cell line (MDA-MB-231/CD63(−)) depleted of tetraspanin CD63 (Fig. 3*C*). Immunoprecipitation and Western blotting experiments have shown

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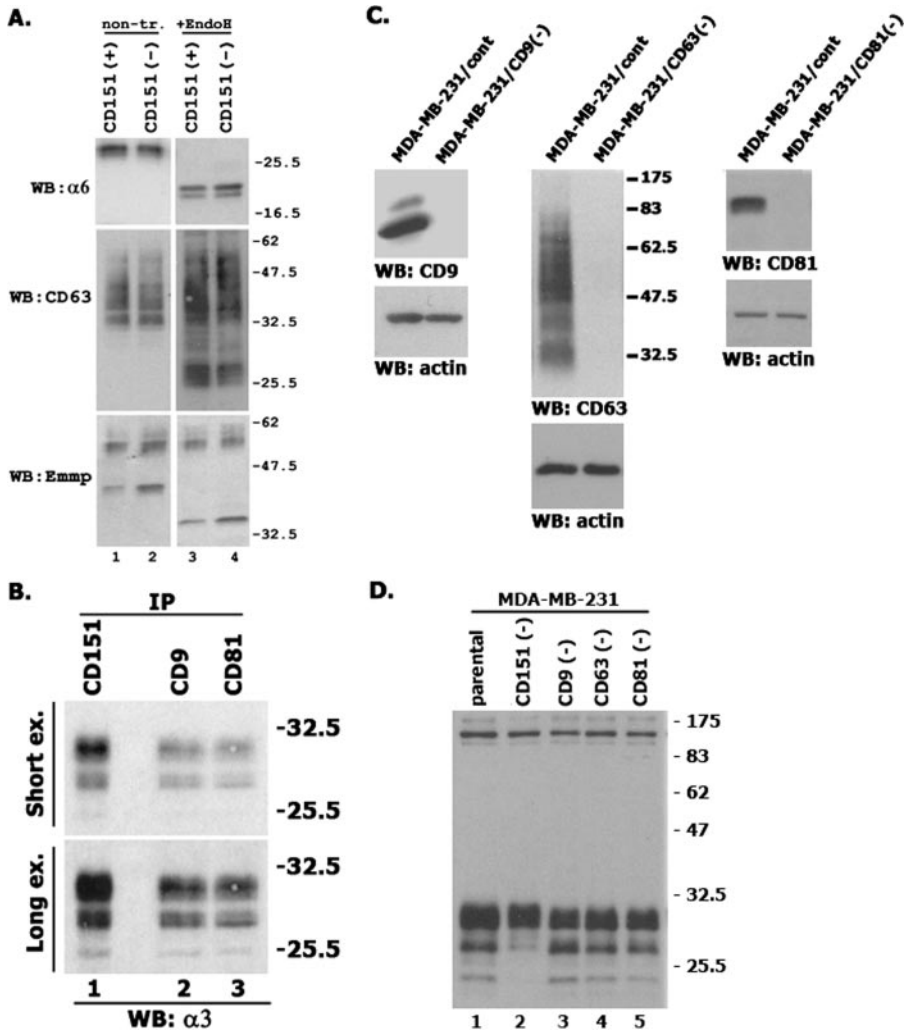

FIGURE 3. *A*, depletion of CD151 does not affect glycosylation of other transmembrane proteins. Cells were lysed in Triton X-100, and the lysates (or protein complexes immunoprecipitated with the mAb A6-ELE against α6 integrin subunit) were either left untreated or deglycosylated overnight with endoglycosidase H (*lanes 3* and *4*). Samples were resolved by 12% SDS-PAGE, transferred onto nitrocellulose membrane, and detected with rabbit Ab to α6 integrin subunit (*top*), mouse anti-CD63 mAb (1B5; *middle*), or mouse anti-emmprin mAb (8G6; *bottom*). *B*, association of CD9, CD81, and CD151 with glycoforms of α3β1. MDA-MB-231 cells were lysed in Brij98/Triton X-100, and tetraspanin complexes were immunoprecipitated (*IP*) with anti-CD151, anti-CD9, or anti-CD81 mAbs. Immunoprecipitated protein complexes were eluted and deglycosylated overnight with endoglycosidase H (*EndoH*). Samples reduced with dithiothreitol were resolved by 12% SDS-PAGE, transferred onto nitrocellulose membrane, and detected with rabbit Ab to the α3 integrin subunit. Note the differences between CD9- and CD81-associated α3 with regard to relative abundance of lower molecular mass glycoforms (indicated with *arrows*). Similar differences were observed in three experiments. *C*, characterization of MDA-MB-231/CD63(−), MDA-MB-231/CD81(−), and MDA-MB-231/CD9(−) cells. Lysates of the cells were resolved by 12% SDS-PAGE and analyzed by Western blotting (*WB*) with mAbs against tetraspanin or β-actin. *D*, depletion of CD63, CD9, or CD81 does not affect glycosylation of α3 integrin subunit by MDA-MB-231 cells. Cells were lysed in Triton X-100, and α3β1 integrin was immunoprecipitated with anti-α3 mAb A3-IVA5. Immunoprecipitated protein complexes were eluted and deglycosylated overnight with endoglycosidase H. Samples reduced with dithiothreitol were resolved by 12% SDS-PAGE, transferred onto nitrocellulose membrane, and detected with rabbit Ab to α3 integrin subunit.

that down-regulation of these tetraspanins in MDA-MB-231 cells did not change mobility of the α3 subunits in SDS-PAGE (Fig. 3*D*). Collectively, our data show that 1) the effect of CD151 on glycosylation of α3β1 integrin is specific, and 2) three other tetraspanins that are widely expressed in epithelial cells have no obvious role in glycosylation of this integrin.

The Role of Palmitoylation, Glycosylation, and the Large Extracellular Loop in the Activity of CD151 toward α3β1—A recent study has established that glycosylation of CD19 is controlled by the N-terminal cytoplasmic domain of the tetraspanin CD81 (30). To identify the region(s) required for the activity of CD151 toward α3β1, we used MDA-MB-231/CD151(−) cells to generate a panel of stable lines that reexpressed various mutants of CD151 (the MDA-MB-231/rec series) (Fig. 4*A*). The following mutants were used in these experiments. 1) CD151palm(−) (palmitoylation-deficient CD151) was used. Previously, we have shown that in rat fibroblasts, this mutant can form direct contact with α3β1, but recruitment of the complex into tetraspanin-enriched microdomains was impaired (38). 2) CD151glyco(−) (glycosylation-deficient CD151) was used. 3) CD151/QRD has been previously described by Kazarov *et al.* (39), who showed that mutation of QRD to INF (residues 194–196) within the large extracellular loop prevents direct interactions between CD151 and integrins when proteins are overexpressed in COS cells. Interactions of the CD151QRD mutant with other tetraspanins have not been affected. 4) CD151ΔC and SW6 mutants were described in our earlier studies. Both mutants retain their ability to associate with other tetraspanins and integrins (43, 62). 5) We used 63-N-151, a chimeric mutant in which the N-terminal cytoplasmic region of CD151 was substituted for a corresponding part of CD63. Where appropriate, an additional mutation was introduced into the mutant DNA constructs to make them resistant to short hairpin RNA-CD151. We also reconstituted the expression of the wild-type CD151 in these cells to establish MDA-MB-231/rec/wt line, which was used as a positive control in these experiments. Western blotting showed that the expression levels of all mutants and the wild-type CD151 were comparable in the reconstituted cells (Fig. 4*D*, *bottom*). Two of the CD151 mutants used in this study (*i.e.* CD151glyco(−) and 63-N-151) have not been characterized previously. Hence, we analyzed whether these mutations affected interactions of the CD151 with other tetraspanins and α3β1. As expected, both mutant proteins can be co-immunoprecipitated with CD9 and CD81 under the conditions that preserve tetraspanin-tetraspanin complexes (Fig. 4*B*). Furthermore, we found that α3β1-CD151glyco(−)

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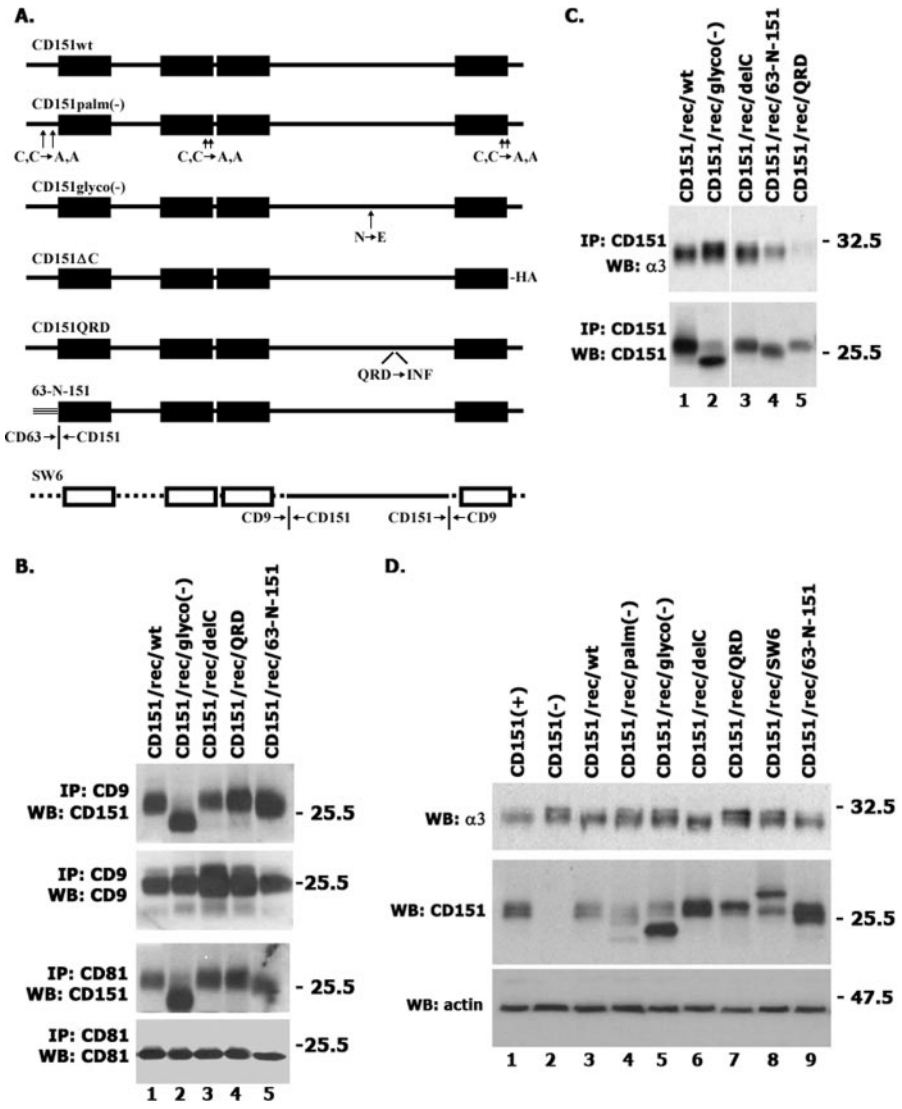

FIGURE 4. **The role of palmitoylation, glycosylation, and the large extracellular loop in the activity of CD151 toward α3β1.** *A*, schematic diagram of CD151 mutants used in this study. *B*, interactions of CD151glyco(−), CD151ΔC(delC), CD151/QRD, and 63-N-151 mutants with CD81 and CD9 in MDA-MB-231/rec cells. Cells were lysed in Brij98, and tetraspanin complexes were immunoprecipitated (*IP*) with anti-CD9 or anti-CD81 mAbs. Immunoprecipitated protein complexes were resolved by 12% SDS-PAGE, transferred onto nitrocellulose membrane, and detected with rabbit Ab to CD151 or mouse mAbs to tetraspanins. *C*, interactions of CD151glyco(−), CD151ΔC(delC), CD151/QRD, and 63-N-151 mutants with α3β1 in MDA-MB-231/rec cells. Cells were lysed in Triton X-100, and tetraspanin complexes were immunoprecipitated with anti-CD151 mAb. Immunoprecipitated protein complexes were resolved by 12% SDS-PAGE, transferred onto nitrocellulose membrane, and detected with rabbit Ab to CD151 or α3β1. *D*, Western blot analysis of α3 integrin light chain subunit (*top*) and CD151 (*bottom*) from MDA-MB-231/CD151(+), MDA-MB-231/CD151(−), and various MDA-MB-231/rec cell lines. *WB*, Western blot.

and α3β1·63-N-151 complexes were resistant to Triton X-100 (Fig. 4*C*), thus suggesting that both mutants are in direct contact with α3β1. Our experiments also confirmed that the Gln^{194}-Arg^{195}-Asp^{196} sequence (QRD mutant) plays a critical role in the interaction of CD151 with α3β1 in MDA-MB-231 cells (Fig. 4*C*, *lane 5*). We then analyzed the glycosylation pattern of α3 integrin subunit in MDA-MB-231/rec cells expressing CD151 mutants. As expected, we observed reconstitution of the glycosylation pattern for the α3 integrin subunit in MDA-MB-231/rec/wt cells (Fig. 4*D*, compare *lanes 1* and *3*). Of all of the mutants examined, only CD151ΔC and 63-N-151 were able to restore fully the SDS-PAGE mobility pattern of the α3 light chain (Fig. 4*D*, *lanes 6* and *9*). Based on these results, we have drawn the following conclusions. First, the presence of the intact CD151 large extracellular loop (and, therefore, direct contact between CD151 and α3β1) is not sufficient for the ability of the tetraspanin to modulate glycosylation of the integrin (results with MDA-MB-231/rec/SW6 and MDA-MB-231/rec/palm(−) cells; Fig. 4*D*, *lanes 4* and *8*). Second, neither N- nor C-terminal cytoplasmic regions are required for this purpose (Fig. 4*D*, *lanes 6* and *9*). Third, palmitoylation-dependent compartmentalization of CD151 to tetraspanin-enriched microdomains is essential for the activity of the protein toward α3β1 (results with MDA-MB-231/rec/palm(−) cells; Fig. 4*D*, *lane 4*). Finally, glycosylation of CD151 itself plays an important role in the modulatory activity of the protein (results with MDA-MB-231/rec/glyco(−) cells; Fig. 4*D*, *lane 5*).

Depletion of CD151 Results in the Reduction of Fucα1–2Gal and Bisecting GlcNAc-β(1→4) Linkage on N-glycans of α3β1—To examine the effect of CD151 depletion on glycosylation of the integrin in more detail, we probed α3β1 purified from MDA-MB-231/CD151(−) and MDA-MB-231/CD151(+) cells with various biotinylated lectins. As illustrated in Fig. 5, binding of biotinylated *Phaseolus vulgaris* erythroagglutinin and *Ulex europaeus* agglutinin I to α3 integrin subunit isolated from MDA-MB-231/CD151(−) cells was significantly reduced when compared with CD151-positive cells (*lanes 3* and *4* and *lanes 7* and *8*). These data indicated that depletion of CD151 resulted in a decrease in bisecting GlcNAc residues and Fucα1–2Gal *N*-glycans. By contrast, binding of *P. vulgaris* leucoagglutinin, which preferentially reacts with β1,6-branched GlcNAc, and wheat germ agglutinin, which binds to GlcNAc and sialic acid, was comparable in CD151-positive and CD151-depleted cells (Fig. 5, *lanes 5* and *6* and *lanes 9* and *10*).

Changes in Glycosylation in CD151-depleted Cells Correlate with Inhibition of α3β1-dependent Cell Migration—To investigate the functional consequences of CD151-dependent changes in glycosylation of α3β1 in MDA-MB-231 cells, we compared migration of CD151-positive and CD151-de-

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pleted cells toward laminin-332. The initial antibody-blocking experiments have shown that migration of MDA-MB-231 cells toward laminin-332 requires both α3β1 and α6 integrins. The inhibitory effect was more dramatic when cells were pretreated with the anti-α3 mAb (~90% of inhibition; Fig. 6*A*), compared with the inhibitory anti-α6 mAb, which reduced migration by ~40%. The major contribution of α3β1 integrin to migration of MDA-MB-231 cells toward laminin-332 was further confirmed when we analyzed motility of the α3-depleted cells (results are not shown). We then compared migration of MDA-MB-231/CD151(−) and MDA-MB-231/CD151(+) cells in Boyden chamber assays. As illustrated in Fig. 6*B*, depletion of CD151 dramatically reduced the migratory potential of MDA-MB-231 toward laminin-332. On the other hand, migration toward fibronectin was unaffected (Fig. 6*C*). Importantly, attachment of MDA-MB-231 to laminin-332 or fibronectin (as examined in static adhesion assays (30–40 min)) was not affected by the depletion of CD151 (Fig. 6*D*). These results provided further support for a specific functional link between CD151 and laminin-binding integrins during cell migration. We also analyzed migratory properties of MDA-MB-231/rec/glyco(−) cells. As shown above, this point mutant of CD151 displayed biochemical properties similar to those of the wild-type protein (*i.e.* the ability to associate with other tetraspanins and form tight complexes with integrins) but could not restore the glycosylation pattern of the α3 integrin subunit. Therefore, the CD151glyco(−) mutant was particularly useful to analyze the impact of the CD151-dependent changes in integrin glycosylation on its function. Notably, although reexpression of the wild-type CD151 restored migratory potential of the cells, migration of cells expressing the glycosylation-deficient mutant remained at the level of CD151-deficient MDA-MB-231 (Fig. 6*B*). Collectively, these data strongly suggest that CD151-dependent modulation of α3β1 glycosylation is functionally relevant and plays an important regulatory role in cell migration toward laminin-332.

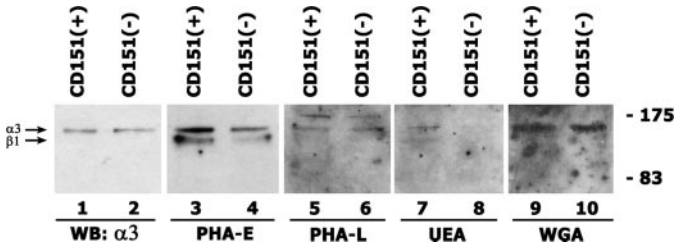

FIGURE 5. **Depletion of CD151 reduces Fucα1–2Gal and bisecting GlcNAc residues on the *N*-glycan of α3 integrin subunit.** Equal volumes of purified α3β1 were loaded five times (*i.e. lanes 1*, *3*, *5*, *7*, and *9* (for α3β1 purified from MDA-MB-231/CD151(+) cells) and *lanes 2*, *4*, *6*, *8*, and *10* (for α3β1 purified from MDA-MB-231/CD151(−) cells)) and resolved by 10% SDS-PAGE under nonreducing conditions. The proteins were transferred onto nitrocellulose membrane and detected with various biotinylated lectins (*lanes 3–10*) or polyconal Ab recognizing α3 integrin subunit (*lanes 1* and *2*). *Lanes 1* and *2* show that loaded volumes contain equal amounts of α3β1. *WB*, Western blot. WGA, wheat germ agglutinin. PHA-E, *P. vulgaris* erythroagglutinin; UEA, *U. europaeus* agglutinin I; PHA-L, *P. vulgaris* leucoagglutinin.

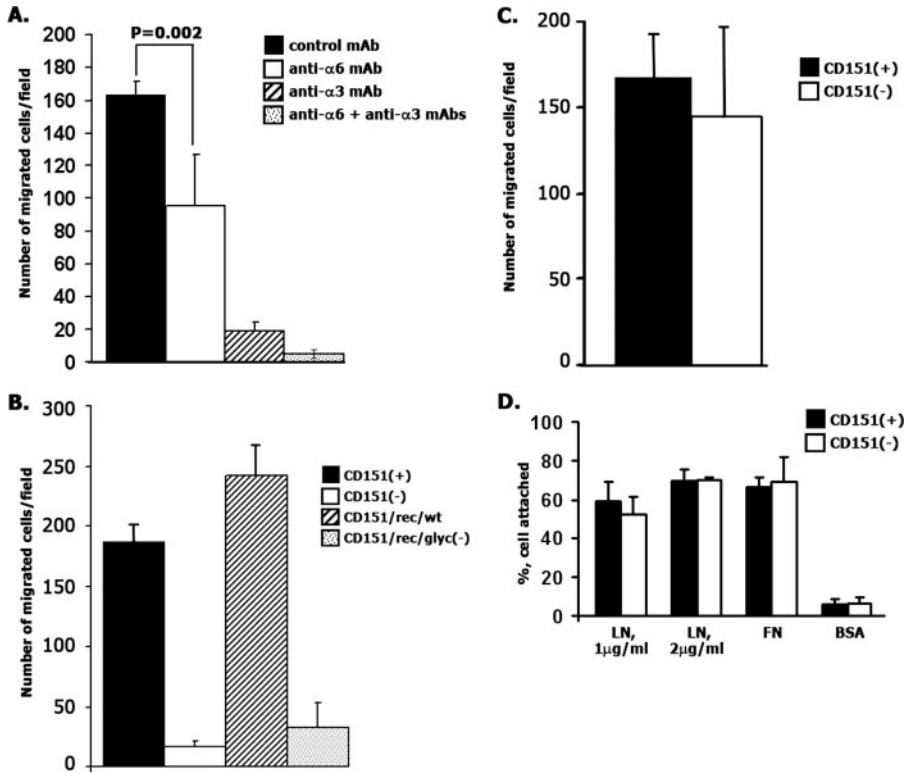

FIGURE 6. **Depletion of CD151 inhibits migration of MDA-MB-231 cells toward laminin-332.** The role for α3β1 glycosylation. *A–C*, cells were prepared for migration experiments as described under "Experimental Procedures." Cells were allowed to migrate toward laminin-332 (*LN*) (or fibronectin (*FN*)) for 8 h. Migration was quantified by counting cells in up to seven randomly chosen fields per membrane. In the antibody-blocking experiments (*A*), cells were preincubated with mAbs (10 μg/ml) for 30 min at 4 °C before the onset of the experiment. The following mAbs were used: A3-IVA5 (anti-α3 integrin subunit), G0H3 (anti-α6 integrin subunit), and isotype control IgG1. Data are presented as average number of cells per microscopic field (×10 objective). Results are shown as mean ± S.D. calculated from at least two separate experiments, each performed in quadruplicate. *p* values were calculated using a two-tailed *t* test. *D*, BCECF-AM-labeled cells were tested for adhesion to a 96-well microtiter plate coated with laminin-332 or fibronectin as described under "Experimental Procedures." *BSA*, bovine serum albumin. BCECF, AM (2,7-bis-(2-carboxyethyl)-5-(and-6)carboxyfluorescein), acetoxymethyl ester.

DISCUSSION

In this report, we identified tetraspanin CD151 as a modulator of glycosylation of α3β1 integrin. Importantly, glycosylation of other proteins that are associated with tetraspanin microdomains was not affected.

Although tetraspanin-dependent modulation of protein glycosylation is not a unique feature of CD151 (see Introduction), the underlying mechanisms are likely to be different. Indeed, although the N-terminal cytoplasmic end is critical for the modulatory activity of CD81 (30), a corresponding region of CD151 is not required. The differences in glycosylation of the β1 inte-

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grin subunit observed in cells overexpressing CD82 are likely to be due to a more general role of this tetraspanin in biosynthetic trafficking and/or delivering of integrins to the cell surface (32). In this regard, in agreement with previous data (45, 66), we found that CD151 does not affect surface levels of the associated integrins.[3]

Our results show that CD151 affects glycosylation of α3β1 integrin through multiple and interdependent mechanisms (Fig. 7). The most important factor seems to be the recruitment of α3β1 to the tetraspanin-enriched microdomains. This process requires direct interactions of CD151 with the integrin on one side and with other tetraspanins on the other. When either of these interactions was affected (as for the QRD and palmitoylation mutants), the modulatory activity of CD151 toward α3β1 was lost (Fig. 7). The modulatory deficiency of the CD151palm(−) mutant is particularly interesting. Previous data have shown that although early steps in the assembly of the α3β1-CD151 complex are not affected by the mutations, recruitment of the complex to TERM, which is thought to take place in Golgi, is impaired (38, 67). Thus, our data indicate that TERM can be used as a platform for diversification of the glycosylation process.

Although most tetraspanins possess one or more potential *N*-linked glycosylation sites, the role of glycosylation in various tetraspanin-associated activities remains largely unexplored. Studies involving CD82/KAI-1 have shown that glycosylation regulates interaction of the protein with integrins α5β1 and α3β1 and correlates with the antimigratory activity of the tetraspanin (68, 69). Differences in glycosylation of tetraspanin CD63 were observed during maturation of dendritic cells. This correlated with the changes in the morphology of the major histocompatibility complex class II compartments that are thought to be responsible for presentation of major histocompatibility complex II-peptide complexes on the cell surface (70). Finally, specific glycosylation of uroplakin Ia/TSPAN-20 is critical for FimH lectin-dependent binding of *Escherichia coli* to the surface of urothelial cells (71). Our data indicate for the first time that glycosylation of a tetraspanin protein modulates glycosylation of its specific partner. The CD151glyc(−) mutant could not restore the glycosylation pattern of α3β1 in MDA-MB-231/CD151(−) cells. Importantly, the interaction with α3β1 and recruitment of the complex to the tetraspanin-enriched microdomains was not affected by this mutation. Hence, direct contact with α3β1 and its compartmentalization to TERM are not the only factors that control the repertoire of oligosaccharides attached to the integrin complex. How do *N*-linked oligosaccharides on CD151 affect glycosylation of α3β1? One possibility is that oligosaccharides attached to CD151 cause subtle changes in the conformation of α3β1 complex that make integrin subunits a preferable substrate for particular glycosyltransferases. Alternatively, CD151 may function as a scaffolding platform for specific glycosyltransferases, which, while glycosylating the tetraspanin itself, may also act on the associated integrin complex (Fig. 7). In this regard, previous studies have shown the existence of a sequential glycosylation

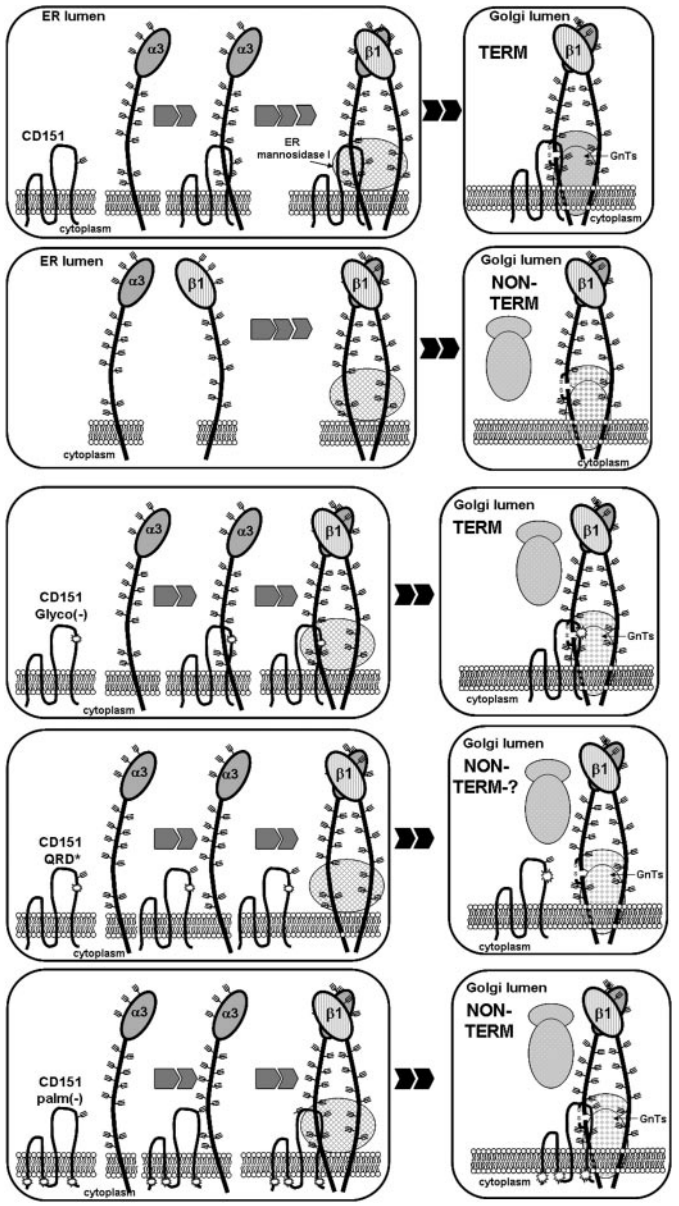

FIGURE 7. **Schematic diagram showing interrelationship between α3β1 and CD151 during integrin maturation.** The complex between CD151 and α3 integrin subunit is formed early during the biosynthesis. Association with CD151 is required for recruitment of α3β1-CD151 to tetraspanin-enriched microdomains. CD151 regulates glycosylation of α3β1 late in the endoplasmic reticulum (*ER*) and in Golgi. In the absence of CD151 (or when cells express palmitoylation-deficient CD151 and CD151 mutant, which lost direct contact with α3β1), the integrin is not recruited to TERM. Consequently, fucosylation (*i.e.* Fucα1–2Gal) of α3β1 and formation bisecting GlcNAc-β(1→4) linkage are decreased. This may be due to the differences in the abundance of various glycosyltransferase complexes in TERM and non-TERM compartments. Although glycosylation-deficient CD151 (CD151glyco(−)) retains a direct contact with α3β1 and targets the integrin to TERM, recruitment of specific GT complexes to the CD151-α3β1 complex may be affected, and this suppresses modifying activity of the tetraspanin.

mechanism whereby a preceding glycosyltransferase activity determines the following glycosyltransferase that will be recruited for glycosylation. Thus, the presence of CD151 may initiate the recruitment of a particular glycosyltransferase (or a multienzyme complex) to α3β1 and determine the order of subsequent glycosyltransferases that will come into contact with the integrin. Altered glycosylation of the SW6 mutant

[3] G. Baldwin and F. Berditchevski, unpublished results.

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(judged by the altered mobility in SDS-PAGE when compared with the wild-type protein) and, therefore, a distinct repertoire of recruited glycosyltransferases may explain inability of this mutant to restore the glycosylation pattern of α3β1.

Specific glycomodifications of the integrin within TERM may involve a more favorable presentation of α3β1 to (or shielding from) a TERM-associated glycosyltransferase. We found that depletion of CD151 specifically reduces Fucα1–2Gal and bisecting GlcNAc residues on the *N*-glycan of α3 integrin subunit (as revealed by the decreased binding of lectins). It is currently unknown how activities of fucosyltransferases I and II (FUT1 and FUT2) and *N*-acetylglucosaminyltransferase III (GnT-III), enzymes responsible for Fucα1–2Gal and bisecting GlcNAc modifications, respectively, are regulated by CD151. Sasai *et al.* (72) have shown that GnT-III is associated with caveolin-1. Furthermore, these authors proposed that this interaction regulates localization of the enzyme within Golgi and, ultimately, affects glycosylation pattern of cellular proteins. Although CD151 is not directly associated with caveolin (73), it is possible that, like some of the other tetraspanins (*e.g.* CD82 (74)), it may change compartmentalization of glycosyltransferases through a more general effect on lipid composition of cellular membranes.

We found that a decrease in bisecting GlcNAc structures on the α3 integrin subunit correlated with inhibition of tumor cell migration (see also below). By contrast, Zhao *et al.* (23) have recently reported that down-regulation of GnT-III and, consequently, decreased biosynthesis of bisecting GlcNAc residues resulted in an increased motility of neuroblastoma cells. There are at least two possible explanations for these seemingly contradictory results. First, Zhao *et al.* (23) found that in their cellular model, there was an inverse correlation between the amounts of bisecting GlcNAc and β1,6-GlcNAc-branched *N*-glycans on the α3 integrin subunit (23). Therefore, it is possible that in neuroblastoma cells, genuine suppression of cell motility associated with a decrease in bisecting *N*-glycans might have been negated by a strong promigratory activity of β1,6-GlcNAc. In this regard, we demonstrated that depletion of CD151 in MDA-MB-231 cells did not change the amount of β1,6-GlcNAc structures on the α3 subunit. Second, we found that the effect of CD151 on glycosylation of α3 integrin subunit in MDA-MB-231 cells is not limited to a decrease in bisecting GlcNAc structures. Indeed, as discussed above, depletion of CD151 also decreased Fucα1–2Gal residues on the α3 integrin subunit. Thus, it is feasible that this (or any other CD151-dependent changes in integrin glycosylation) could reverse the anti-migratory activity of bisecting GlcNAc structures.

Our results point to a new mode of regulation of integrin function by tetraspanins. We and others have previously suggested that CD151 controls cell motility by modulating integrin-dependent signaling and/or through regulation of integrin endocytosis and recycling (45, 49, 75). Here we established a strong correlation between the effect of CD151 on glycosylation of α3β1 and promigratory activity of this tetraspanin. Indeed, the CD151glyco(−) mutant with only a single change of amino acid within the large extracellular loop was unable to restore the glycosylation pattern of α3 integrin subunit in MDA-MB-231/CD151(−) cells and α3β1-dependent cell motility. Importantly, attachment of CD151-depleted MDA-MB-231 cells to laminin-332 in static adhesion experiments was not affected. Thus, the decrease in cell motility associated with CD151-dependent modifications in α3β1 glycosylation may be due to a more subtle effect on interactions of the integrin with laminin-332 (*e.g.* transient interactions at the leading edge). Alternatively, changes in glycosylation may influence the rate of integrin turnover in migrating cells. This may involve gangliosides (76), whose interactions with integrins require *N*-linked glycans (65). Further work will be necessary to distinguish between these possibilities.

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