Tetraspan CD151 Promotes Cell Migration by Regulating Integrin Trafficking*

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Regulation of cell migration is an important feature of tetraspan CD151. Although it is well established that CD151 physically associates with integrins, the mechanism by which CD151 regulates integrin-dependent cell migration is basically unknown. Given the fact that CD151 is localized in both the plasma membrane and intracellular vesicles, we found that CD151 and its associated integrins undergo endocytosis and accumulate in the same intracellular vesicular compartments. CD151 contains a YXXφ type of endocytosis/sorting motif, in its C-terminal cytoplasmic domain. Mutation of this motif markedly attenuated CD151 internalization. The loss of CD151 trafficking completely abrogated CD151-promoted cell migration on extracellular matrices such as laminin and diminished the internalization of its associated integrins, indicating a critical role for integrin trafficking in regulating cell motility. In conclusion, the YXXφ motif-mediated internalization of CD151 promotes integrin-dependent cell migration by modulating the endocytosis and/or vesicular trafficking of its associated integrins.

Tetraspan CD151 is functionally linked to cancer metastasis, hemidesmosome formation, neurite outgrowth, and vascular morphogenesis (1–5). Although the mechanism remains obscure, cell movement appears to be the common theme for many of these events. The regulatory role of CD151 in cell migration is supported by the knock-out studies in mice (6, 7) and knockdown experiments in vitro (8). Also, the association of CD151 with integrins, especially the laminin (LN)β3-binding integrins, stands out as a prominent feature (3, 9–11). CD151 is expressed in a variety of tissues and is especially abundant in epithelia, endothelia, cardiac muscle, smooth muscle, and megakaryocytes (12). Notably, the tissue distribution of CD151 is similar to the distribution of LN-binding integrins such as α3β1, α6β1, α6β4, and α7β1 (13), which is consistent with its ability to associate with these integrins and with a likely concomitant function during development (14, 15). At the cellular level, CD151 is present on plasma membrane and in intracellular vesicles (1). On the cell surface, CD151 is localized in the basolateral membrane in endothelial and epithelial cells (1, 16, 17), whereas intracellularly, CD151 resides in endosomal/lysosomal vesicles (1).

Because CD151 directly associates with LN-binding integrins, it is not surprising that CD151 regulates cell-matrix adhesion. CD151 is needed for cell adhesion strengthening mediated by LN-binding integrin α6β1 (18). CD151 also potentiates the ligand binding activity of integrin α3β1 by stabilizing the activated conformation of this integrin (19). Furthermore, CD151 is a component of hemidesmosomes that anchor epithelial cells to basal lamina (3). Finally, CD151 was reported to up-regulate integrin α5β1-dependent static adhesion to fibronectin (FN) (20). Interestingly, CD151 also plays a role in cell-cell adhesion (10). For example, CD151 promotes the formation of epithelial cell-cell contacts in a protein kinase C (PKC) and Cdc42-dependent manner (17). CD151 recruits integrin α3β1 to cell-cell contacts to form a multimolecular complex that includes PKC, phosphotyrosine phosphatase μ, E-cadherin, and β-catenin (21).

CD151 contains two extracellular loops and two short cytoplasmic tails. The large extracellular loop is required for the association with α3 and α6 integrins (22, 23). The CD151 C-terminal cytoplasmic domain plays a key role in signaling. The deletion or exchange of the CD151 C-terminal cytoplasmic tail markedly impaired integrin α6β1-dependent cell spreading and cellular morphogenesis (5, 18). In addition, the C-terminal cytoplasmic tail of CD151 negatively regulates Ras activity (24). Moreover, the C-terminal tail determines integrin α6β1-mediated adhesion strengthening to LN-1 (18). Despite several lines of evidence indicating an essential role of the C-terminal tail of CD151, it is still unclear how only eight amino acids at the C-terminal tail regulate these CD151-mediated activities.

The C-terminal cytoplasmic domain of CD151 contains a YXXφ sequence that meets the criteria of the YXXφ-sorting motif, in which Y is tyrosine and X indicates any amino acid, and φ represents the amino acid residue with a bulky hydrophobic side chain (25, 26). The YXXφ motifs in the cytoplasmic domain of transmembrane proteins are recognized by adaptor protein-2 complex, a core component of clathrin endocytic machinery (26, 27). This led us to hypothesize that the YXXφ motif drives CD151 endocytosis, is responsible for the func-
tional importance of CD151 C-terminal cytoplasmic domain, and ultimately regulates CD151 activities such as integrin-dependent cell movement.

MATERIALS AND METHODS

Cells and Transfectants—NIH3T3 and Du145 cell lines were obtained from the ATCC and cultured at 37 °C in 5% CO₂ in DMEM (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Human dermal microvascular endothelial cells (HMEC-1) were obtained from the Centers for Disease Control and Prevention and cultured in ECBM-2 media (Cambrex).

CD151 wild type and the YRSL → ARSA mutant (termed the Yala mutant) were constructed into a eukaryotic expression vector pZeoSV2 (Invitrogen) as described in our earlier study (5). Briefly, the CD151 Yala mutant was generated by simultaneously replacing the Tyr and Leu residues in the YRSL sequence of wild type CD151 C-terminal cytoplasmic domain with alanine residues through PCR. The full-length cDNAs encoding human wild type and Yala mutant CD151 were constructed into pZeoSV2 vector, respectively, through the XbaI and HindIII sites. The CD151 coding information was confirmed by DNA sequencing. NIH3T3 cells were transfected with plasmid DNA using Lipofectamine2000 (Invitrogen) and selected with Zeocin (Invitrogen) at a concentration of 0.2 mg/ml. Hundreds of Zeocin-resistant clones were pooled, and CD151-positive clones were collected by flow cytometric cell sorting. The pooled and sorted CD151-positive clones were the CD151 wild type and Yala stable transfectants.

Antibodies and Reagents—The monoclonal antibodies (mAb) used in this study were human CD151 mAbs 5C11 (9) and 8C3 (19; kindly provided by Drs. K. Sekiguchi and M. Yamada of Osaka University) and mouse CD9 mAb KM114 (BD Biosciences), mouse CD81 mAb 2F7 (SouthernBiotech, Birmingham, AL), mouse CD44 mAb KM114 (BD Biosciences), mouse integrin α2 mAb HMα2 (BD Biosciences), and a negative control mAb mouse IgG2b (clone MOPC 141) (Sigma). The polyclonal antibodies (pAb) include integrin α3 cytoplasmic domain pAb D23 (28) and integrin α6 cytoplasmic domain pAb (5). Alexa 488- or Alexa 594-conjugated human CD151 mAb 5C11 was prepared using the Alexa protein labeling kit (Molecular Probe). FITC-conjugated human integrin α5 mAb CBL497F and integrin α6 mAb GoH3 were purchased from BD Biosciences, and all second antibodies were from Invitrogen. Alexa 488-conjugated transferrin was from Molecular Probes. Extracellular matrix proteins used in the study include human plasma fibronectin and mouse laminin 1 (Invitrogen).

Flow Cytometry—NIH3T3 transfectant cells were detached with 2 mM EDTA/PBS, washed once with PBS, treated with DMEM supplemented with 5% goat serum at 4 °C for 10–15 min, and then incubated with primary mAb at 4 °C for 1 h. After washing, cells were further labeled with FITC-conjugated secondary antibody at 4 °C for 1 h and then analyzed on FACSCalibur flow cytometer.

Immunofluorescence and Confocal Microscopy—Cells were fixed with 3% paraformaldehyde at room temperature for 10 min, permeabilized with 0.1% Brij 98 at room temperature for 2–5 min, blocked with 20% goat serum at room temperature for 1 h or at 4 °C overnight, and then incubated with primary mAbs at room temperature for 30 min, followed by staining with a secondary antibody at room temperature for 30 min. After each antibody incubation, the cells were washed with PBS five times; each wash lasted 15 min. For double staining, the cells were blocked with murine IgG and then further stained with Alexa 488- or Alexa 594-conjugated human CD151 mAb 5C11. For immunofluorescence analysis, the cells were examined with an Axioptofluorescent microscope (Carl Zeiss), and images were captured using an Optronics digital camera. For confocal microscopic analysis, the coverslips were examined using LSM 510 laser scanning microscope (Carl Zeiss) equipped with 63×/1.4 Plan-APoCHROMAT oil immersion objectives. Green fluorescent protein, FITC, or Alexa 488 and rhodamine or Texas Red were excited by the 488- and 543-nm lines of Kr-Ar lasers, respectively, and individual channels were scanned in a series to prevent cross-channel bleed through. Each image represents a stack of ~1.0 µm “Z” optical sections of the cells.

Immunoprecipitation—Immunoprecipitations were carried out as described previously (29). Briefly, cells were lysed with 1% Brij 97 and/or 1% Nonidet P-40 lysis buffer at 4 °C for 1 h. In some experiments, the cell surface was labeled with 0.5 mg/ml EZLink sulfo-NHS-LC biotin (Pierce) before cell lysis. After removing the insoluble material by 14,000 × g centrifugation, the precleared lysates were incubated with primary mAb preabsorbed protein A- and G-Sepharose beads (Amersham Biosciences) from 3 h to overnight at 4 °C. The precipitates were washed with the lysis buffer three times, dissolved in Laemlli sample buffer, heated at 95 °C for 5 min, separated by SDS-PAGE, and then electrically transferred to nitrocellulose membranes (Bio-Rad). For immunoblots experiments, the membranes were blotted sequentially with primary Ab and horseradish peroxidase-conjugated secondary Ab (Sigma), followed by chemiluminescence (PerkinElmer Life Sciences). For biotinylation experiments, the membranes were blotted with horseradish peroxidase-conjugated extravidin (Sigma), followed by chemiluminescence.

Internalization Assay—The internalization of CD151 and integrins was examined with antibody uptake assay. Cells were incubated with fluorescein-isothiocyanate-conjugated CD151 mAb and/or integrin mAb at 4 °C for 1 h. The unbound antibodies were removed by three washes with serum-free medium. The cells were then incubated at 37 °C for internalization for typically 30 or 60 min or for various periods of times as specified in the particular experiments. The antibodies that bound to the cell surface and were not internalized were removed by acid washes with the 0.1 M glycine, 0.1 M NaCl buffer (pH 3.0). The acid wash typically removed ~80–90% of the cell surface-bound antibodies. The cells were then fixed and examined with immunofluorescent or confocal microscopy.

The kinetics of integrin and CD151 internalization were determined following the established protocol (29–33). Cells were incubated with reducible sulfo-NHS-SS-biotin at the saturated concentration (0.5 mg/ml) at 4 °C to label the cell surface proteins. The labeling lasted for 1 h to allow maximal biotinylation of the cell surface proteins. The free biotin was removed by three washes with ice-cold PBS. The labeled cells were incubated at 37 °C for various times for the internalization and then
treated with a reducing solution for 20 min on ice to remove cell surface-bound biotin. For the internalization of CD151 and its associated integrin, the reducing solution contained 42 mm GSH, 1 mm EDTA, 75 mm NaOH, and 1% bovine serum albumin. After washing with PBS, the cells were lysed in 1% Nonidet P-40, 1% Brij 97 lysis buffer at 4°C for 1 h, and CD151 was immunoprecipitated as aforementioned. After SDS-PAGE and electric transferring, the biotinylated integrin or CD151 was detected by peroxidase-conjugated extravidin and visualized by chemiluminescence. The integrin and CD151 bands were quantitated by densitometric analysis as described previously (29). Integrin internalization at an indicated time point was calculated with the following formula: (the densitometry of internalized integrin at that time point – the densitometry of internalized integrin at time 0)/the total surface integrin × 100%.

Cell Migration Assay—Transwell cell migration assay was performed as described previously (34). Briefly, cells were detached from culture plates with EDTA, washed once with PBS, and replated onto the inserts of 8-μm pore size Transwell chambers (Costar). The undersides of the inserts were pre-coated with either FN (10 μg/ml) or LN 1 (10 μg/ml) at 4°C overnight and blocked with 0.1% heat-inactivated bovine serum albumin at 37°C for 45 min. The cell number was 8 × 10³ per insert filter. The media include 500 μl of DMEM containing 1% fetal calf serum in the lower well and 300 μl DMEM containing 0.1% heat-inactivated bovine serum albumin in the insert. After incubating at 37°C for 3 h, cells that had not migrated through inserts were removed, and cells that had migrated to the undersides of the inserts were fixed and stained with Diff-Quick (Baxter Merz & Dade). The cells that had migrated through the insert were counted. Data from independent experiments were pooled and analyzed using two-tailed Student’s t test.

For wound healing assay, NIH transfectant cells were cultured in Lab-Tek chamber slides (Nunc, Rochester, NY). After the monolayers were formed, wounds were generated by scraping the monolayers with sterile pipette tips. The detached cells were rinsed away with PBS, and the wounded monolayers were replenished with the complete medium. After overnight culture at 37°C, the monolayers were fixed and photographed under inverted light microscope (Olympus, Center Valley, PA).

RESULTS

The YRSL Sequence in CD151 Cytoplasmic Domain Determines Its Trafficking—To determine whether the YRSL sequence of CD151 (Fig. 1A) is involved in regulating its intracellular trafficking and function, we mutated this motif in human CD151 molecule. The Tyr and Leu residues in CD151 YRSL motif were replaced with alanine residues simultaneously, and the resulting YRSL → ARSA mutant was designated as the Yala mutant. We transfected human CD151 WT and Yala mutant cDNA into NIH3T3 mouse fibroblast cells and established stable transfectants by pooling and sorting hundreds of CD151-expressing clones by flow cytometry. As shown in Fig. 1B, CD151 WT and the Yala mutant were stably expressed at comparable levels on the surface of NIH3T3 cells when cells reached confluence in culture. When cells were less confluent in culture, the cell surface level of CD151 on the Yala mutant was higher than that on the wild type. For example, when cells were at the 60–70% confluent stage, the mean fluorescent intensity of CD151 was 134 in wild type and 189 in the Yala mutant. The expression levels of other cell surface proteins such as CD44 (Fig. 1B, top panel) were equivalent on all transfectants and not altered upon CD151 expression. The total cellular levels of CD151 proteins were equivalent between CD151 wild type and the Yala transfectants, with a slightly higher level in the wild type (densitometry of CD151 band in the wild type: that in the Yala mutant was 1.088) (Fig. 1B, bottom panel). The total CD151 proteins remained stable at different stages during cell culture.

The effect of the Yala mutation on CD151 subcellular localization was analyzed at the steady state (Fig. 1C). Typically, CD151 proteins are localized on the cell surface and in intracellular endosomes and lysosomes (1). CD151 Yala mutant was distributed mainly at the cell surface and largely lost the vesicular staining observed with CD151 WT, suggesting that the YRSL motif is important for regulating CD151 localization. In NIH3T3-CD151 wild type transfectant cells, we confirmed that some of CD151-positive intracellular vesicles were EEA1- or Rab5-positive early endosomes (Fig. 1C, bottom panel).

We further analyzed the internalization of CD151 in the WT and Yala transfectants using an antibody uptake assay. After a 30-min endocytosis, a substantial fraction of WT CD151 was internalized and accumulated in intracellular vesicles, whereas CD151 Yala mutant was barely internalized in this time frame (Fig. 1D, top panel). The quantitative analyses showed the significant diminution of CD151 endocytosis in the Yala mutant (Fig. 1D, bottom panel). A small fraction of the CD151 Yala mutant proteins were internalized after prolonged incubation at 37°C, such as 2 h, and the internalized CD151 Yala mutant proteins remained at the cell peripheral area (data not shown), suggesting a deficiency in the trafficking following endocytosis. Thus, the YRSL motif is required for CD151 endocytic processes.

CD151 Trafficking Regulates Integrin-dependent Cell Migration—Regulation of cell motility is a prominent feature of CD151. We tested whether CD151 internalization is important for cell migration. We measured the directional motility of NIH3T3-CD151 transfectants on the substratum coated with FN or LN1, a ligand of α5 or α6 integrins, respectively. Because the α6 integrin expressed in NIH3T3 cells is the α6β1 integrin (5), cell migration on LN1 is mediated primarily by integrin α6β1. As shown in Fig. 2A, the expression of WT CD151 in NIH3T3 significantly enhanced cell migration on both FN and LN1, consistent with previous results (1, 2, 9, 16). The ability of CD151 Yala mutant cells to migrate toward FN and LN1 was markedly reduced compared with that of CD151 WT and Mock transfectant cells. The diminished cell migration of the Yala mutant indicates that YRSL internalization signal is not only required for CD151 pro-migratory activity but also affects constitutive motility of NIH3T3 cells. We also used wound healing assay to assess the effect of the Yala mutation on cell migration. Consistent with the results obtained from the transwell cell

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migration assay, CD151 WT promoted wound healing, whereas CD151 Yala mutant exhibited delayed wound healing compared with the CD151 WT transfectant (Fig. 2B). Thus, CD151 endocytosis plays an essential role in regulating integrin-dependent cell migration.

Deficient Endocytosis of CD151 Does Not Alter the Integrin-CD151 Association—To determine why the loss of CD151 internalization affects integrin-mediated cell migration, we first assessed if CD151 Yala mutant altered the CD151-integrin complex. We compared the CD151-integrin associations between NIH3T3-CD151 WT and Yala transfectants. CD151 immunoprecipitation profiles of surface-biotinylated NIH3T3 cells revealed that integrins coprecipitated equally well with CD151 WT and the Yala mutant under either a stringent lysis condition, i.e. 1% Nonidet P-40 (Fig. 3A), or a mild lysis condition, i.e. 1% Brij 99 (data not shown). Immunoblotting analysis indicated that the coprecipitated integrin under the stringent lysis condition was endogenous murine α3, and the ones under the mild conditions were α5 and α6 (Fig. 3B). The transmembrane protein CD44 (Fig. 3A) and housekeeping protein tubulin (Fig. 3B) were included to indicate the equal loadings of the cell lysate. The blotting of CD151 immunoprecipitates with CD151 mAb ensured that the biotinylated ~30-kDa band in CD151 immunoprecipitate is indeed CD151 (Fig. 3A, bottom panel). Together, these results indicate that the Yala mutation did not alter the association of CD151 with cell surface integrins and that CD151 endocytosis is not required for CD151-integrin complex formation. In addition, the association of CD151 with endogenous murine tetraspanins such as CD9 was not altered by the Yala mutation (Fig. 3C). Thus, CD151 endocytosis does not appear to be critical for the partitioning of CD151 into tetraspanin webs (34) or tetraspanin-enriched microdomains (TEM) (36).
CD151 Endocytosis Regulates Cell Migration

Integrin Is Internalized into CD151-containing Vesicular Compartments—Based on the fact that CD151 physically associates with integrins, we investigated whether CD151-associated integrins could also be internalized. To address this issue, we used human dermal microvascular endothelial cells (HMECs) that express CD151 and α3β1, α5β1, and α6β1 integrins. As shown in Fig. 4, α3β1, α5β1, and α6β1 integrins can be detected in the intracellular vesicles of HMECs after internalization. Moreover, a significant fraction of the internalized α3β1, α5β1, and α6β1 integrins accumulated in intracellular vesicles that also contained internalized CD151 (Fig. 4). These results suggest that CD151 was cointernalized with its associated integrins, and CD151-integrin complexes traffic through, at least in part, the same endocytic compartments. In 84% cells examined, the endocytic vesicles positive for both CD151 and integrin α3 were detected. These vesicles were distributed throughout the cytoplasm with a higher density in the perinuclear area. Approximately 33.3% of the intracellular vesicles positive for internalized CD151 also contained the internalized integrin α3 (Table 1, ImageJ analysis) (Image, National Institutes of Health, Bethesda) (38). The staining of internalized α3 or CD151 in cointernalization experiments was less than the staining when the internalization was performed individually for α3 or CD151. This is probably caused by the steric hindering effect of the α3 mAb-α3 binding on the CD151 mAb-CD151 binding and/or vice versa because of the tight association of α3 and CD151. Thus, the colocalization of internalized α3 or CD151 could be substantially underestimated in this cointernalization experiment. Endocytic vesicles that contained both internalized CD151 and internalized integrin α5 were found in 49% cells and were mainly located in the perinuclear area (Fig. 4). In intracellular vesicles, the internalized integrin α5 and internalized CD151 are partially colocalized with each other (Table 1). The endocytic vesicles that contain both internalized CD151 and internalized integrin α6 were found in 80% of cells and were also primarily perinuclear (Fig. 4). In the majority of cells, the internalized integrin α6 and internalized CD151 were partially colocalized (Table 1, ImageJ analysis), whereas in ~10% of the cells integrin α6 and CD151 were fully colocalized after internalization. These integrin- and CD151-positive vesicles belong to an unidentified vesicular compartment. The colocalization of internalized CD151 with internalized integrins was quantitated using both visual counting and ImageJ software (Table 1), and the results obtained from both measurements were consistent with each other. The extent of colocalization of CD151 with the internalized α3, α6, or α5 integrin also correlated with the stoichiometries and stability of the physical associations of CD151 with these integrins. We then analyzed the colocalization of internalized CD151 and internalization. Moreover, a significant fraction of the internalized α3β1, α5β1, and α6β1 integrins accumulated in intracellular vesicles that also contained internalized CD151 (Fig. 4). These results suggest that CD151 was cointernalized with its associated integrins, and CD151-integrin complexes traffic through, at least in part, the same endocytic compartments. In 84% cells examined, the endocytic vesicles positive for both CD151 and integrin α3 were detected. These vesicles were distributed throughout the cytoplasm with a higher density in the perinuclear area. 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Figure 3. The effect of Yala mutation on CD151-integrin association. A, NIH3T3-Mock, -CD151 WT, and -CD151 Yala transfectant cells were biotinylated and subsequently lysed in 1% Nonidet P-40 lysis buffer. Cell lysates were immunoprecipitated (IP) with CD151 mAb. The precipitated proteins were resolved by nonreducing SDS-PAGE. Following transfer to nitrocellulose, the membrane was blotted with extravidin, and the proteins visualized by chemiluminescence. The CD44 immunoprecipitates were included as protein loading controls (middle panel). The CD151 immunoprecipitates were blotted with CD151 mAb 8C3 to confirm the presence of CD151 in precipitates (bottom panel). B, CD151 immunoprecipitates were blotted with anti-human/murine integrin α3 cytoplasmic tail, a pAb against human/murine integrin α5, and a pAb against human/murine integrin α6 cytoplasmic tail, respectively. For integrin α3 coprecipitation, cells were lysed in 1% Nonidet P-40; whereas for integrins α5 and α6 coprecipitations, cells were lysed in 1% Brij 98. The cell lysates were blotted for tubulin as a loading control. C, NIH3T3 transfectant cells were lysed in 1% Brij 98 lysis buffer, and the cell lysates were immunoprecipitated with human CD151 mAb SC11. The precipitated proteins were resolved by nonreducing SDS-PAGE. Following transfer to nitrocellulose, the membrane was blotted with murine CD9 mAb KM08, and the CD9 proteins were visualized by chemiluminescence. The cell lysates were blotted for tubulin to demonstrate the equal loading.

Figure 4. Integrins are internalized into CD151-positive vesicles. For the cointernalization of integrin α3, α5, or α6 and CD151, HMECs grown on glass coverslips overnight were incubated with FITC-conjugated α3, α5, or α6 mAb and Alexa 594-conjugated CD151 mAb SC11 at 4 °C for 1 h. The FITC-conjugated mAbs against human integrins were integrin α3 mAb X8, integrin α5 mAb CBL497F, and integrin α6 mAb GoH3. For the control, cells were incubated with Alexa 488-conjugated transferrin and Alexa 594-conjugated CD151 mAb. After the unbound mAbs and transferrin were removed by washes with cold PBS, the cells were then incubated at 37 °C for 1 h for internalization. The cells were treated with two consecutive acid washes to remove the cell surface uninternalized mAbs or transferrin. The confocal images were captured at the magnification ×63.

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Because CD151 and its associated integrins can be internalized into the same intracellular vesicular compartment and CD151 Yala mutant is still able to associate with integrins, the attenuation in CD151 internalization by the Yala mutation may result in the impaired endocytosis of its associated integrins. We thus investigated whether the endocytic defect of the CD151 Yala mutant affected the endocytosis of its associated integrins.

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CD151 Regulates the Endocytosis of Its Associated Integrin—Because CD151 and its associated integrins can be internalized into the same intracellular vesicular compartment and CD151 Yala mutant is still able to associate with integrins, the attenuation in CD151 internalization by the Yala mutation may result in the impaired endocytosis of its associated integrins. We thus investigated whether the endocytic defect of the CD151 Yala mutant affected the endocytosis of its associated integrins.

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biotin, we determined the internalized integrin based on the inaccessibility of integrin to biotin release upon reduction. As shown in Fig. 5C, the internalization of CD151-associated integrin in the Yala mutant was indeed attenuated, compared with that in the WT transfectant. The internalization rate of the integrin that physically associates with CD151 Yala mutant was significantly lower than the rate of the integrin that physically associates with CD151 WT. The actual quantities of the CD151-associated, internalized integrins at each of the time points were also less in the Yala than in the WT transfectant, and the differences were particularly marked between 15 and 45 min. It has been demonstrated in earlier studies that, at 1% Nonidet P-40 lysis condition, the biotinylated α3 integrin is the only CD151-associated protein that is detectable in the biotin-avidin blot (9, 22). Because NIH3T3 transfectant cells were lysed with 1% Nonidet P-40 detergent in the internalization experiments, we predict that CD151-associated integrin was murine α3 integrin. Using a pAb that was raised with human α3 cytoplasmic domain but that cross-reacts with murine α3 cytoplasmic domain, we confirmed that the CD151-associated integrins were indeed α3 integrin by immunoblotting (Fig. 5C). CD151-associated total α3 integrin and the level of tubulin in cell lysates were included to ensure equal loading at each time point.

Finally, we analyzed the internalization kinetics of CD151-associated α6 and α5 integrins in NIH3T3-CD151 wild type and -CD151 Yala transfectants. CD151-associated α6 and α5 integrins were re-immunoprecipitated using their specific mAbs, respectively, from the CD151 immunoprecipitates that were performed under 1% Brij 98 cell lysis condition. Similar to α3 integrin, we found that the internalization of CD151-associated α6 and α5 integrins was diminished in the Yala mutant, especially evident between 15 and 45 min (Fig. 5D), indicating that the Yala mutation also inhibits the endocytosis of CD151-associated α6 and α5 integrins.

Besides interacting with integrins, CD151 also physically associates with tetraspanins such as CD9 and CD81 (35, 39). To investigate if the defect in CD151 endocytosis affects the internalizations of tetraspanins, we analyzed the endocytosis of endogenous CD9 and CD81 in NIH3T3-CD151 transfectants. For CD9, we found that no CD9 was internalized in 60-min endocytosis experiments in ~70% WT and Yala cells. In ~30% cells, the internalized CD9 was detected in intracellular vesicles (Fig. 6A). In these cells, CD9 was internalized at similar degrees between CD151 WT and the Yala mutant cells (Fig. 6B), suggesting that CD9 endocytosis is not altered by the CD151 Yala mutation. The expression level of CD81 in NIH3T3 cells appears to be negligible because we barely detected any CD81 protein expression using a workable murine CD81 mAb (40). Thus, the effect of the CD151 Yala mutation on CD81 endocytosis cannot be assessed in NIH3T3 cells.

**DISCUSSION**

The YXXφ Motif-mediated Endocytosis and Trafficking of CD151—As predicted, the YXXφ motif is a crucial intrinsic determinant governing CD151 internalization. Mutation of the CD151 YXXφ motif resulted in diminished internalization and subsequent endocytic trafficking of CD151 and in increased

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**TABLE 1**

| Internalized proteins | Internalized CD151 that colocalizes with integrin or CD71* |
|------------------------|--------------------------------------------------------|
| α3                     | 84.0                                                  |
| α5                     | 49.0                                                  |
| α6                     | 80.0                                                  |
| CD71                   | 18.5                                                  |

*The results are the average of three individual experiments.

*The number of cells that contain the intracellular vesicles positive for both CD151 and integrin or CD71 are presented as the percentage of total cells. Approximately 50 cells were quantified in each measurement.

*The integrin- or CD71-colocalized CD151 staining is presented as the weighted colocalization coefficient for CD151. Thirty cells containing the internalized CD151 were quantitated in each measurement. The p values are <0.01 between CD71 and any integrin group.
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A

Total Integrin α5 Endocytosis

Total Integrin α6 Endocytosis

B

Laminin 1  Fibronectin

Integrin α5/CD151

Integrin α6/CD151

C

CD151 Wt  CD151 Yala

Surf 0 15 30 45 60 90

CD151-associated

Internalized

Total

α-tubulin

CD151-associated

Integrin α3

Endocytosis

D

CD151 Wt  CD151 Yala

L.P.: CD151

Re-L.P.: α5 integrin

L.P.: CD151

Re-L.P.: α6 integrin

Biot: α-tubulin

CD151-associated

Integrin α5

Endocytosis

CD151-associated

Integrin α6

Endocytosis
CD151 Endocytosis Regulates Cell Migration

A, NIH3T3-CD151 transfectant cells cultured on coverslips overnight were incubated with murine CD9 mAb at 4 °C for 1 h. After the unbound Abs were removed by PBS washes, the cells were placed at 37 °C for 1 h for internalization, followed with the acid washes to remove uninternalized Abs. The cells were then fixed, permeabilized, and stained with secondary Ab. The images were acquired under confocal microscopy as described above. B, CD9 endocytoses were quantitated by assessing either the percentage of the transfectant cells having the internalized CD9 (left panel) or the number of intracellular vesicles containing CD9 per cell (right panel). The histograms represent the mean of three experiments ± S.D. (left panel) or S.E. (right panel). In each experiment, 100 cells (left panel) or 30 cells containing internalized CD9 (right panel) were quantitated from each transfectant, respectively. There were no statistical significances (p > 0.05) in CD9 endocytoses between CD151-wild type and the Yala transfectants.

FIGURE 6. The internalization of CD9 is not affected by the Yala Y56A mutation. A, NIH3T3-CD151 transfectant cells cultured on coverslips overnight were incubated with murine CD9 mAb at 4 °C for 1 h. After the unbound Abs were removed by PBS washes, the cells were placed at 37 °C for 1 h for internalization, followed with the acid washes to remove uninternalized Abs. The cells were then fixed, permeabilized, and stained with secondary Ab. The images were acquired under confocal microscopy as described above. B, CD9 endocytoses were quantitated by assessing either the percentage of the transfectant cells having the internalized CD9 (left panel) or the number of intracellular vesicles containing CD9 per cell (right panel). The histograms represent the mean of three experiments ± S.D. (left panel) or S.E. (right panel). In each experiment, 100 cells (left panel) or 30 cells containing internalized CD9 (right panel) were quantitated from each transfectant, respectively. There were no statistical significances (p > 0.05) in CD9 endocytoses between CD151-wild type and the Yala transfectants.

distribution of CD151 at the cell surface and/or periphery, indicating that the YXXΦ motif is indeed a functional endocytosis or trafficking motif. Similar observations have been made for other tetraspanins such as CD63 (41), A15,4 and CD82 when the YXXΦ motifs in the C-terminal cytoplasmic tail were mutated. Thus, using the YXXΦ motifs to traffic between different cellular compartments appears to be a common mechanism for the tetraspanins that contain this motif. For the tetraspanins that do not contain the YXXΦ motifs, such as CD9 and CD81, their endocytosis may depend on unidentified intrinsic sorting signals or on their directly associated tetraspanins that contain this motif, such as CD63, CD82, and/or CD151. For example, we found in this study that CD9 underwent endocytosis, but its endocytosis was not markedly altered by the CD151 Yala mutation. In this case, CD9 endocytosis may be determined by its own endocytosis signal that remains to be identified. In terms of the mechanism that governs CD151 endocytosis, we have demonstrated elsewhere that CD151 is internalized via a dynamin- and Arf6-independent but phosphatidylinositol 3-kinase- and actin-dependent endocytosis pathway.3

Although the defect in endocytosis is obvious in the CD151 Yala mutant, the Yala effect was more profound when endocytosis was measured using antibody uptake than using biotinylation (data not shown). It is possible that the antibody causes the cross-linking of the cell surface CD151 or that the pH conditions during the cell surface stripping are different in these two kinds of endocytosis assays. The clustering of cell surface CD151, however, likely occurs physiologically, because the engagement of its associated integrins with multivalent ECM ligands or the reorganization of TEM can induce CD151 clustering. Also, if the ligand or counter-rec-
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tector of CD151 is present, the antibody may imitate the CD151 ligand or counter-receptor to elicit CD151 signaling and trigger its internalization. Thus, the CD151 internalization measured by either antibody uptake or biotinylation could reflect its endocytosis under different physiological settings.

Tetraspanin and integrin have been shown previously to colocalize in the vesicle-like intracellular structure in an earlier study (42). Upon PKC inhibition, integrin β1 can actually be trapped in an unidentified recycling compartment that contains tetraspanin CD81 (43). However, it is not known whether they are internalized via the same endocytic pathway and traffic through the same endocytic compartment. Our study for the first time demonstrates that tetraspanin and integrin are possibly delivered to the same vesicular compartment following endocytosis, although this remains to be confirmed by immunoelectron microscopy in the future studies. The colocalization of CD151 and its associated integrins in endocytic vesicles implies that they are either internalized through the same endocytosis pathway or trafficked to the same intracellular vesicular compartment after differential internalizations. Because CD151 physically associates with integrin, it is more likely that they share the same endocytosis pathway or internalization route. Thus, the cotrafficking of tetraspanin with integrin could be an immediate functional consequence of the well documented tetraspanin-integrin physical association.

CD151 Trafficking Regulates Cell Migration—CD151 regulates cell migration, but the mechanism remains elusive. Notably in this study, when the YXXφ motif was mutated, the WT CD151-promoted cell migration on FN and LN1 was completely inhibited. This reduction in cell migration correlated with the marked reduction in the internalization of the CD151 Yala mutant. These results strongly suggest that CD151 endocytosis and/or trafficking is needed for integrin-mediated cell migration. Meanwhile, our study is the first to demonstrate that the trafficking of tetraspanin can play a key role in regulating cell migration. Endocytosis of other transmembrane proteins such as MT1-MMP has also been found to regulate cell migration (44). For motility-related transmembrane proteins, it appears that their endocytosis takes part in the motility regulation. A role for vesicular trafficking in regulating cell migration had been postulated long ago (45). On the one hand, disrupted endocytosis is accompanied by a deficiency in cell migration (46). On the other hand, polarized exocytosis occurs at the leading edge of motile cells and may assist cells migrating forward (47). Regulating cell motility is a common feature of most tetraspanins, and tetraspanins such as CD63, CD82, A15, and CD151 are present in intracellular vesicles (39, 48). Vesicular trafficking could therefore be a common point that tetraspanins act on for regulating cell motility.

How does CD151 internalization regulate cell migration? We postulated that the association of CD151 with tetraspanin webs (35) or glycosynapse (49) or TEM (36), which are mainly formed by tetraspanins, cell adhesion molecules, and glycosphingolipids, holds the key. CD151 internalization possibly induces endocytosis of its associated tetraspanin web or glycosynapse or TEM, and the latter in turn facilitates the recycling and reutilization of TEM components. Indeed, we demonstrated in this study that trafficking of integrin, a TEM component, was significantly affected by CD151 (see following). Likely, CD151 also regulates the trafficking of other TEM components. If it is true, the directionality of CD151 trafficking in migrating cells, the cotrafficking of TEM components with CD151, and the identity of CD151-containing vesicles become key issues to be addressed in future studies.

The Role of CD151 Trafficking in Integrin Function—Although the traction force generated from integrin-ECM engagement is crucial for propelling cells to move, integrin disengagement from ECM during cell migration is equally important as its engagement in maintaining motility (50). The ECM-engaged integrins in the rear of migrating cells could be diffused laterally across the cell surface through the plasma membrane and/or internalized and redelivered to the cell surface (51). The observations of integrin endocytosis and recycling of integrin-containing vesicles at the cell rear or in circulation (30, 52) led to the notion that integrins are internalized from plasma membrane at the rear of migrating cells and likely recycled back to plasma membrane at the leading edge to engage with ECM again (53). The vesicle trafficking of integrins therefore is postulated to regulate cell migration (54). Indeed, integrin distribution is polarized in migrating cells and maintained by the cycles of endocytosis from the rear and recycling to the front (55). Also during cell migration, integrin traffics via endocytic and recycling vesicles from the rear portion of a cell through the perinuclear region to the base of the leading lamellipodia (56–58). The structural elements in integrin responsible for its trafficking in cell migration have been elucidated in recent studies. For example, the YXXφ motif in the integrin β2 subunit is essential for recycling of the internalized β2 integrins, and a mutation of this motif disrupted integrin β2-dependent cell migration (33, 59). The trafficking pathways of integrin are also becoming better understood (37, 43, 59–65). For example, the PKC-dependent internalization of integrin β1 in MCF-7 breast cancer cells requires dynamin activity, and the dominant-negative dynamin inhibits the PKC-promoted, integrin-mediated cell motility on collagen (60).

Regulation of cell migration via CD151 trafficking raised a question whether integrin trafficking is involved in this regulatory process. The direct association of CD151 with LN-binding integrins such as α3β1, α6β1, α6β4, and α7β1 strongly suggests that CD151 directly regulates the trafficking of these integrins. Although CD151 may not directly bind to FN receptor α5β1 integrin (36), this integrin could be part of a tetraspanin web or glycosynapse or TEM and indirectly associates with CD151 (1, 20). Therefore, the trafficking of α5β1 integrin could also be affected by CD151 when CD151 is expressed in fibroblast cells such as NIH3T3 in which α5β1 is one of the major integrins. Indeed, the endocytosis (and/or the trafficking immediately following the endocytosis) of CD151-associated integrins such as α3β1, α6β1, and α5β1 is markedly attenuated by the CD151 Yala mutation. Consistently, the endocytosis of integrin α3β1 was impaired when CD151 was silenced, correlating with the diminished cell migration (8). Although recent studies have shown that tetraspanins regulate integrin endocytosis (8, 29), our study is the first to identify the structural element in tetraspanin that determines the trafficking of its associated inte-
grins and to provide a mechanism for how CD151 regulates integrin trafficking. The fact that CD151 alters only the trafficking of its associated integrins, which are the integrins in the activated conformation (19) or the integrins actively involved in cell migration (this study), strongly suggests that CD151 links integrin activation to integrin trafficking in migrating cells. Based on these observations, we extrapolate that activated integrins participate in cell migration through vesicle trafficking.

Besides the highly stoichiometric association of CD151 with integrin α3β1, the associations of CD151 with other integrins have relatively low stoichiometries (36). Especially in NIH3T3 transfectants, a portion of integrins is preoccupied by endogenous murine CD151, resulting in even lower stoichiometry of human CD151-murine integrin associations. Thus, it is not surprising that the global trafficking of α5β1 and α6β1 integrins was not substantially altered by human CD151 in NIH3T3 transfectant cells. Although we could not determine the effect of the CD151 Yala mutation on the global internalization of murine integrin α3 in NIH3T3 transfectants, we predict that the total α3 integrin trafficking will be significantly attenuated by the highly stoichiometric association of CD151 with integrin α3β1, which is different from α5β1 and α6β1 integrins.

Because the cytoplasmic domains of many integrin β subunits contain NPXY endocytosis signals, the trafficking of integrins could also be regulated by their own sorting signals. By associating with tetraspanin web or glycosynapse or TEM, the trafficking pathways and/or dynamics of integrins could be modified by the intrinsic trafficking signals of their associated tetraspanins such as the YRSL motif of CD151. Thus, the trafficking of integrins and other TEM components are guided by their intrinsic trafficking signals, whereas tetraspanin adds another layer of complexity and a new dimension to the trafficking of its associated TEM components such as integrin. It remains to be tested in a future study whether CD151 provides or alters the trafficking directionality of integrins in migrating cells. Given the fact that CD151 expression is frequently up-regulated in invasive and metastatic cancers (2), this notion actually points to a novel and potential mechanism that the altered trafficking pattern of integrins in cancer cells, because of the increased level of CD151, may facilitate cell movement and consequently promote the invasiveness and metastatic potential of cancer cells. Also, given the fact that pathological angiogenesis becomes deficient in CD151-null mice (7), the role of CD151 in regulating the trafficking of integrins in endothelial cell movements needed for blood vessel formation and remodeling is an intriguing issue remaining to be tested.

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