Potentiation of the ligand-binding activity of integrin α3β1 via association with tetraspanin CD151

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CD151, one of the tetraspanins, forms a stable complex with integrin α3β1, the major laminin receptor on the cell surface. We found that 8C3, an anti-CD151 mAb obtained by screening for reactivity with integrin α3β1–CD151 complexes, was capable of dissociating CD151 from integrin α3β1, thereby allowing us to deplete CD151 from purified integrin α3β1–CD151 complexes. The CD151-free integrin α3β1 thus obtained showed a significant reduction in its ability to bind to laminin-10/11, a high-affinity ligand for integrin α3β1, with a concomitant reduction in its reactivity with mAb AG89, which recognizes activated β1-containing integrins. These results raised the possibility that the association of integrin α3β1 with CD151 potentiates the ligand-binding activity of the integrin through sustaining its activated conformation. In support of this possibility, the ligand-binding activity was restored when CD151-free integrin α3β1 was reassociated with purified CD151. 8C3-induced dissociation of CD151 from integrin α3β1 was also demonstrated on the surface of living cells by fluorescent resonance energy transfer imaging, accompanied by a concomitant reduction in the cell adhesion to laminin-10/11-coated substrates. CD151 knock-down by RNA interference also resulted in a reduction in the adhesive activity of the cells. Taken together, these results indicate that CD151 association modulates the ligand-binding activity of integrin α3β1 through stabilizing its activated conformation not only with purified proteins but also in a physiological context.

cell adhesion | laminin

Integrins are heterodimeric adhesion receptors for extracellular matrix proteins, consisting of an α-subunit and a β-subunit. In mammals, 18 α-subunits and 8 β-subunits have been identified, combinations of which yield >20 different integrins with distinct ligand-binding specificities (1). Among these, integrins α3β1, α6β1, α7β1, and α6β4 specifically bind to laminins, the major cell-adhesive proteins in basement membranes. Unlike other integrins, the laminin-binding integrins form complexes with tetraspanins (2). Tetraspanins are widely expressed membrane proteins characterized by four transmembrane segments and well conserved cysteine residues in a large extracellular loop (2). More than 30 tetraspanin proteins have been identified in mammals. Among these tetraspanins, CD151 forms a stable complex with integrin α3β1, the major laminin-binding integrin ubiquitously expressed in many cell types (3–5). Tetraspanins also bind to each other, yielding distinct membrane microdomains, referred to as the "tetraspanin web," in which integrins and other membrane proteins such as Ig superfamily proteins and growth factor receptors are incorporated (6).

The association of CD151 with integrin α3β1 occurs early in their biosynthesis and has been implicated in sorting of integrin α3β1 to the cell surface (2). CD151 also interacts with phosphatidylinositol 4-kinase and protein kinase C, thereby linking integrin α3β1 to these signaling molecules (3, 7). Several lines of evidence have indicated that CD151 is involved in integrin

α3β1-mediated adhesive events, including cell spreading, migration and cellular cable formation (8, 9), and anti-CD151 antibodies have been shown to inhibit the α3β1-dependent motility of neutrophils and endothelial cells (3, 10) as well as neurite outgrowth (11). Furthermore, a CD151 mutant defective in association with integrins, due to substitution of the Gln-Arg-Asp sequence in the large extracellular loop with Ile-Asn-Phe, blocks cell spreading on Matrigel-coated substrata and cellular cable formation on thick Matrigel layers (8), although it has minimal effects on integrin-mediated cell adhesion per se (2, 8). However, it remains to be defined whether the association of CD151 with laminin-binding integrins is involved in modulation of their ligand-binding activities or the conformations relevant to their activities, because the mutant CD151 did not prevent the association of endogenous CD151 with integrin α3β1.

In the present study, we obtained an anti-human CD151 mAb that was capable of dissociating CD151 from integrin α3β1, and succeeded in isolating CD151-free integrin α3β1 after incubation of purified integrin α3β1–CD151 complexes with this mAb immobilized on agarose beads. The binding activity of CD151-free integrin α3β1 to laminin-10/11, the highest affinity ligand for integrin α3β1 (12), was significantly reduced, and could be restored to the level of integrin α3β1–CD151 complexes upon reassociation with purified CD151. We also provide evidence that association with CD151 modulates the conformation of integrin α3β1 in an activation-dependent manner.

Materials and Methods

Cell Lines, Reagents, and Antibodies. A549 human lung adenocarcinoma cells, U251 human glioma cells, and Cos7 monkey kidney cells were cultured in DMEM containing 10% FBS. Laminin-10/11 was purified from conditioned medium of A549 cells (13). Human fibronectin was purchased from Sigma–Aldrich. mAbs against human integrin α3 (3G8) and CD151 (8C3) were produced as described (14). Other antibodies used were as follows: anti-human integrin α3 (P1B5) from Chemicon; anti-human CD151 (11G5) from Serotec; control mouse IgG and goat anti-human integrin α3 (I-19) from Santa Cruz Biotechnology; an activating anti-human integrin β1 mAb (TS2/16) from American Type Culture Collection (ATCC); anti-human CD151 (SFA1.2B4), anti-human integrin β1 (AG89) recognizing activated β1 integrins, FITC-conjugated AG89, and FITC-conjugated TS2/16 were produced as described (15, 16).

Purification of Integrin α3β1–CD151 Complexes and CD151. Integrin α3β1–CD151 complexes were purified from human placenta (12). Integrin-free CD151 was purified from a detergent extract
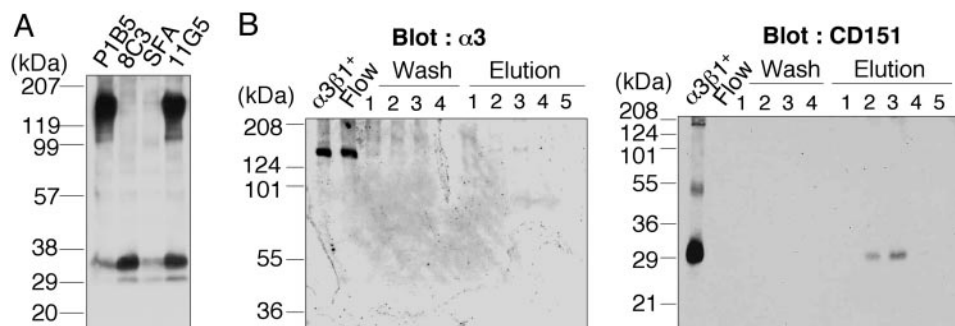
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Fig. 1. Dissociation of CD151 from integrin $\alpha3\beta1$ by mAb 8C3. (*A*) U251 cells surface-labeled by biotinylation were lysed and immunoprecipitated with mAbs 8C3, SFA1.2B4, 11G5 (anti-CD151), or P1B5 (anti-$\alpha3$). Precipitates were resolved by 4–20% SDS/PAGE under nonreducing conditions, followed by detection of the labeled proteins with streptavidin-horseradish peroxidase. (*B*) Integrin $\alpha3\beta1$–CD151 complexes purified from placenta ($\alpha3\beta1^+$) were incubated with mAb 8C3-conjugated agarose beads overnight, followed by chromatographic separation of the unbound and bound proteins. The flow-through (Flow), wash (Wash), and 0.1 M triethylamine-eluted (Elution) fractions were collected separately and analyzed by immunoblotting with anti-$\alpha3$ (I-19; *Left*) and anti-CD151 (8C3; *Right*) antibodies. The minor band in $\alpha3\beta1^+$ migrating at $\approx$55 kDa was identified as the CD151 dimer by N-terminal amino acid sequencing.

of human placenta by immunoaffinity chromatography by using the anti-CD151 mAb 8C3. The column was washed with TBS (+) (20 mM Tris·HCl, pH 7.5/0.13 M NaCl/1 mM CaCl$_2$/1 mM MgCl$_2$) containing 1 mM PMSF and 50 mM *n*-octyl-β-D-glucoside, and the bound CD151 was eluted with 0.1 M triethylamine containing 50 mM *n*-octyl-β-D-glucoside and neutralized with 2 M NaHPO$_4$.

Immunoprecipitation. Surface biotinylation of U251 cells and subsequent immunoprecipitation were performed as described (17).

Depletion of CD151 from Integrin $\alpha3\beta1$–CD151 Complexes. The integrin $\alpha3\beta1$–CD151 complexes purified from placenta were incubated with 8C3-conjugated agarose beads overnight at 4°C on a rotary shaker. The slurry was poured into an empty column, and CD151-free integrin $\alpha3\beta1$ was collected in the flow-through fractions. The bound proteins were eluted with 0.1 M triethylamine containing 50 mM *n*-octyl-β-D-glucoside and neutralized with 2 M NaHPO$_4$. The CD151-free integrin $\alpha3\beta1$ and purified CD151 were stored at 4°C.

Integrin Binding Assays. Solid-phase binding assays using purified integrins (10–20 μg/ml) reconstituted into ^{3}H-labeled liposomes were performed as described (12), except that the assays were performed in the presence of 1 mM MgCl$_2$ and 1 mM CaCl$_2$.

Fluorescent Resonance Energy Transfer (FRET) Imaging. Expression vectors for cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) were constructed as described (18). The CFP and YFP expression vectors were cleaved by *Nhe*I/*Kpn*I and *Hin*dIII/*Kpn*I, respectively, to insert the coding sequences for human integrin $\alpha3$ chain and CD151, yielding the expression vectors for CFP-tagged integrin $\alpha3$ and YFP-tagged CD151. A 6-aa spacer (Gly-Gly-Thr-Val-Pro-Arg) was introduced before the fluorescent protein tags. Cos7 cells were cotransfected with these expression vectors by using Lipofectamine 2000 (Invitrogen) and replated after 48 h on glass-bottom dishes coated with 50 μg/ml fibronectin in phenol red-free DMEM containing 5% FBS. Twelve hours after replating, the cells were imaged at 1-min intervals by using an Olympus IX71 microscope equipped with a cooled charge-coupled device camera (CoolSNAP HQ, Roper Scientific, Trenton, NJ) controlled by METAMORPH software (Universal Imaging, West Chester, PA) as described (18). The medium was replaced with medium containing control IgG (20 μg/ml) and then with medium containing one of the anti-CD151 mAbs (20 μg/ml each) at 5 and 15 min after the onset of recording, respectively. After background subtraction, a YFP/

CFP ratio image was produced by METAMORPH software and used to represent the FRET efficiency.

Cell Adhesion Assays. Cell adhesion assays were performed as described (14). A549 cells were preincubated with the indicated mAbs (20 μg/ml) for 20 min at room temperature and plated on 24-well plates coated with 1 nM laminin-10/11. The cells were incubated for 20 min at 37°C, washed, fixed with 3.7% formaldehyde, and stained with 0.5% toluidine blue. The attached cells were counted in three independent fields per well.

CD151 Knock-Down by RNA Interference (RNAi). Double-stranded small interfering RNAs (siRNAs) were purchased from Dharmacon (Lafayette, CO), and used to knock-down CD151 expression. The target sequence of the most effective siRNA was 5′-ccagacagcugcugcaag-3′. Nonspecific Control Duplex VII RNA (Dharmacon) was used as a control. The siRNAs were transfected into A549 cells by using Lipofectamine 2000, and the cells were used for cell adhesion assays at 96 h after transfection. RNAi-mediated knock-down of the CD151 expression was verified by flow cytometry by using mAb 8C3.

Results

Production of an Anti-CD151 mAb That Dissociates CD151 from Integrin $\alpha3\beta1$. Previously, we produced a panel of mAbs against integrin $\alpha3\beta1$ based on their reactivity to integrin $\alpha3\beta1$ purified from human placenta (14). One of these mAbs, named 8C3, immunoprecipitated a 30-kDa protein from surface-labeled U251 human glioma cells, instead of the 150-kDa/125-kDa integrin $\alpha3\beta1$ (Fig. 1*A*). Because integrin $\alpha3\beta1$ has been shown to associate with tetraspanin family proteins (3, 4), we also performed immunoprecipitation with a panel of anti-tetraspanin mAbs. Among these mAbs, anti-CD151 mAbs SFA1.2B4 and 11G5 precipitated a 30-kDa protein migrating to the same position as the protein precipitated with 8C3 (Fig. 1*A*), indicating that the 30-kDa protein recognized by 8C3 is CD151. The identity of the 30-kDa protein as CD151 was verified by determination of the N-terminal amino acid sequence of the protein.

The tetraspanin CD151 has been shown to form a stable complex with integrin $\alpha3\beta1$ (3, 4). Indeed, the purified integrin $\alpha3\beta1$ used in our screening for anti-integrin $\alpha3\beta1$ mAbs contained a significant amount of CD151 (data not shown), thus resulting in the isolation of mAb 8C3 recognizing CD151. Interestingly, many anti-CD151 mAbs have been shown to coimmunoprecipitate integrin $\alpha3\beta1$ and CD151 as a complex, as was the case with mAb 11G5 (Fig. 1*A*). Given the stability of integrin $\alpha3\beta1$–CD151 complexes under stringent detergent con-

ditions (i.e., 1% Triton X-100), the sole precipitation of CD151 with mAb 8C3 raised the possibility that this mAb was capable of dissociating CD151 from integrin $\alpha3\beta1$. To explore this possibility, we incubated integrin $\alpha3\beta1$–CD151 complexes purified from placenta with 8C3-conjugated agarose beads, followed by chromatographic separation into unbound and bound proteins (Fig. 1*B*). Western blot analyses showed that the flow-through fractions contained only integrin $\alpha3\beta1$, whereas the bound fractions contained only CD151, supporting the possibility that 8C3 is capable of dissociating CD151 from integrin $\alpha3\beta1$. Although the precise mechanism by which 8C3 dissociates CD151 from integrin $\alpha3\beta1$ remains to be defined, 8C3 can be used to deplete CD151 from integrin $\alpha3\beta1$–CD151 complexes, thereby allowing us to examine the role of CD151 association in integrin $\alpha3\beta1$ functions.

CD151 Potentiates the Ligand-Binding Activity of Integrin $\alpha3\beta1$. The ligand-binding activity of CD151-free integrin $\alpha3\beta1$ (designated $\alpha3\beta1^-$) was compared with that of CD151-bound integrin $\alpha3\beta1$ (designated $\alpha3\beta1^+$) by integrin-liposome binding assays by using laminin-10/11 as an adhesive ligand. We reconstituted $\alpha3\beta1^+$ and $\alpha3\beta1^-$ in ^{3}H-labeled phosphatidylcholine liposomes, and examined their binding to surface-adsorbed laminin-10/11 (Fig. 2*A*). Binding of integrin liposomes to a surface-adsorbed anti-$\alpha3$ mAb (P1B5) was used as a reference to normalize the amount of bound integrin liposomes. The validity of P1B5 as a reference was confirmed by comparable binding of the integrin liposomes to surface-adsorbed mAb TS2/16, an anti-integrin $\beta1$ mAb. Notably, significantly less $\alpha3\beta1^-$ bound to laminin-10/11 than $\alpha3\beta1^+$, indicating that depletion of CD151 decreases the ligand-binding activity of integrin $\alpha3\beta1$. The ligand-binding activity of integrins has been shown to be dependent on their conformation (19, 20). To probe the activation-dependent conformations of $\alpha3\beta1^+$ and $\alpha3\beta1^-$, we examined their reactivity toward mAb AG89, an mAb that selectively recognizes activated $\beta1$-containing integrins (16, 21). Consistent with the reduced laminin-10/11 binding, $\alpha3\beta1^-$ showed significantly reduced binding to mAb AG89 compared with $\alpha3\beta1^+$ (Fig. 2*B*). These results indicate that CD151 stabilizes the activated conformation of integrin $\alpha3\beta1$, thereby potentiating its binding to laminin-10/11.

To confirm that CD151 association potentiates the ligand-binding activity of integrin $\alpha3\beta1$, we purified CD151 from human placenta by 8C3-immunoaffinity chromatography, and examined whether reassociation with CD151 could restore CD151-free $\alpha3\beta1$ to the full ligand-binding activity. Incubation of $\alpha3\beta1^-$ with the purified CD151, followed by immunoprecipitation with an anti-integrin $\alpha3$ mAb, demonstrated that $\alpha3\beta1^-$ was capable of reassociating with purified CD151 (Fig. 2*C*). No CD151 was precipitated by the anti-$\alpha3$ mAb in the absence of $\alpha3\beta1^-$, confirming the specificity of the immunoprecipitation assays. When reconstituted in ^{3}H-labeled liposomes and examined for its ligand-binding activity, $\alpha3\beta1^-$ reassociated with CD151 was significantly more potent than $\alpha3\beta1^-$ in binding to laminin-10/11, and exhibited comparable activity to that of $\alpha3\beta1^+$ (Fig. 2*D*). Liposomes reconstituted with the purified CD151 alone did not show any significant binding to laminin-10/11 (Fig. 6, which is published as supporting information on the PNAS web site), excluding the possibility that CD151 *per se* could bind to laminin-10/11 and thereby exert an additive effect on the ligand-binding activity of $\alpha3\beta1^-$ upon reassociation with CD151.

MAb 8C3-Induced Dissociation of CD151 from Integrin $\alpha3\beta1$ on the Cell Surface. To examine whether mAb 8C3-induced dissociation of CD151 from integrin $\alpha3\beta1$ could occur on the cell surface, we used FRET imaging assays using Cos7 cells cotransfected with CFP-tagged integrin $\alpha3$ chain and YFP-tagged CD151. The
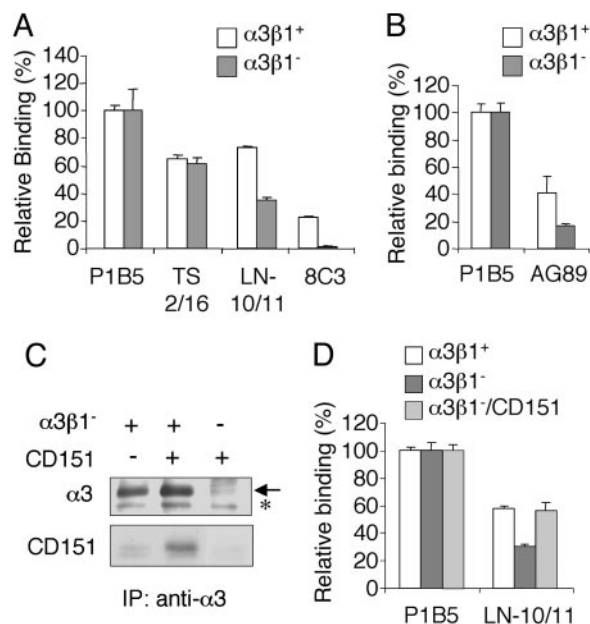

Fig. 2. Modulation of the ligand-binding activity of integrin $\alpha3\beta1$ by CD151 association. (*A*) Integrin $\alpha3\beta1$–CD151 complexes ($\alpha3\beta1^+$; open bars) and CD151-free integrin $\alpha3\beta1$ ($\alpha3\beta1^-$; filled bars) were reconstituted in ^{3}H-labeled liposomes and incubated with laminin-10/11 (LN-10/11) or mAbs against integrin $\alpha3$ (P1B5), $\beta1$ (TS2/16), or CD151 (8C3), each coated on 96-well plates at 5 nM, for 6 h at room temperature. The amounts of bound liposomes were quantified by using a liquid scintillation counter and are expressed as relative values to the amount bound to P1B5. Each bar represents the mean of triplicate assays ± SD. (*B*) ^{3}H-labeled liposomes containing $\alpha3\beta1^+$ and $\alpha3\beta1^-$ were incubated with P1B5 (5 nM) or mAb AG89 recognizing activated $\beta1$ integrins (100 nM) coated on 96-well plates, followed by quantification of the bound liposomes. (*C*) Reassociation of purified CD151 with $\alpha3\beta1^-$. $\alpha3\beta1^-$ was incubated with purified CD151 at an equimolar ratio overnight at 4°C, and then subjected to precipitation with 3G8-conjugated agarose beads. Precipitates were immunoblotted with anti-$\alpha3$ (I-19) or anti-CD151 (8C3) antibodies under nonreducing conditions. The arrow indicates the $\alpha3$ band. The minor band below the $\alpha3$ band (asterisk) was derived from the 3G8-conjugated beads, and is considered to be nonspecific. (*D*) $\alpha3\beta1^+$ (open bars), $\alpha3\beta1^-$ (filled bars), and $\alpha3\beta1^-$ reassociated with purified CD151 at an equimolar ratio ($\alpha3\beta1^-$/CD151; shaded bars) were reconstituted in liposomes and incubated with laminin-10/11 (LN-10/11) or P1B5 coated on 96-well plates at 5 nM, followed by quantification of the bound liposomes as described above. The amounts of bound liposomes are expressed as relative values to the amount bound to P1B5. Each bar represents the mean of triplicate assays ± SD.

FRET efficiency was monitored by the YFP/CFP emission ratio. Photobleaching of YFP resulted in an increase in CFP emission, demonstrating the presence of basal FRET in living cells (data not shown). Although the YFP/CFP emission ratio, indicative of FRET, remained unaffected when the cells were exposed to medium containing control IgG, a significant decrease in the ratio was observed within 1 min upon exposure to medium containing mAb 8C3 (Fig. 3*A*; see also Fig. 7, which is published as supporting information on the PNAS web site). Another mAb, 11G5, which is incapable of dissociating CD151 from integrin $\alpha3\beta1$, did not induce any significant decrease in the ratio. These results, reproduced in three separate experiments, indicated that 8C3, but not 11G5, was capable of dissociating CD151 from integrin $\alpha3\beta1$ on the surface of living cells. In support of this possibility, the amount of CD151 coprecipitated with integrin $\alpha3\beta1$ from cell lysates was significantly reduced after pretreatment of the cells with 8C3 (data not shown).

Because dissociation of CD151 from integrin $\alpha3\beta1$ was expected to destabilize the active conformation of the integrin, we examined the reactivity of mAb AG89 to A549 cells pretreated
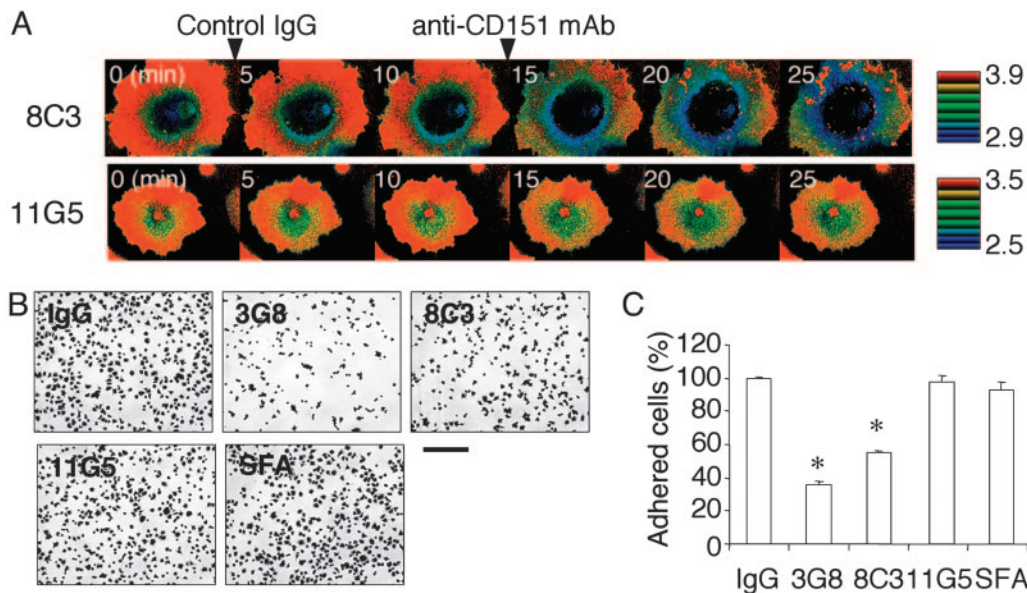
Fig. 3. mAb 8C3 dissociates CD151 from integrin $\alpha3\beta1$ on the cell surface. (*A*) Cos7 cells coexpressing CFP-tagged integrin $\alpha3$ chain and YFP-tagged CD151 were serially treated with control IgG and an anti-CD151 mAb (8C3 or 11G5) at the indicated time points, and fluorescence images for CFP and YFP were recorded at 1-min intervals by time-lapse microscopy. YFP/CFP ratio images were used to represent the FRET efficiency and are expressed in eight colors in the intensity-modulated display mode (red for highest and blue for lowest). YFP/CFP ratio images at 5-min intervals are shown. Complete sets of images are included in Fig. 7. (*B*) A549 cells were preincubated with the indicated mAbs for 20 min at room temperature, and plated on 24-well plates coated with laminin-10/11. After incubation for 20 min at 37°C, the attached cells were fixed and stained. (Scale bar, 200 μm.) SFA, anti-CD151 mAb SFA1.2B4. (*C*) The numbers of attached cells were counted in three separate fields and expressed as percentages of the number after preincubation with mouse control IgG. Each bar represents the mean $\pm$ SD. Statistical analyses were performed by Student's *t* test. $\ast$, $P < 0.001$ versus IgG.

with 8C3. Flow cytometric analyses showed that 8C3 pretreatment decreased the reactivity of cells toward AG89, whereas pretreatment with 11G5 did not (Fig. 8, which is published as supporting information on the PNAS web site). The decrease in AG89 reactivity was not due to a decrease in the surface expression of $\beta1$-containing integrins, because the reactivity toward TS2/16, another anti-integrin $\beta1$ mAb, remained unaffected after 8C3 pretreatment (Fig. 8).

We also examined the effect of 8C3 on cell adhesion to substrates coated with laminin-10/11. A549 cells have been shown to adhere to laminin-10/11 in an integrin $\alpha3\beta1$-dependent manner (13, 14). Cells pretreated with 8C3 were significantly less active in adhering to laminin-10/11 than untreated cells, as was the case for cells pretreated with a function-blocking mAb against the integrin $\alpha3$ chain (Fig. 3 *B* and *C*). In contrast, pretreatments with 11G5 or SFA1.2B4 did not exert any significant effects on the cell adhesion to laminin-coated substrates. These results provide further support for the 8C3-mediated dissociation of CD151 from integrin $\alpha3\beta1$ and resulting destabilization of the activated conformation of the integrin.

Effect of CD151 Knock-Down on Cell Adhesion to Laminin-10/11. Finally, we examined the role of CD151 in modulating integrin $\alpha3\beta1$ activity by RNA interference (RNAi)-mediated knockdown of CD151. Flow cytometric analyses showed that the expression level of CD151 on A549 cells was reduced by $\approx90\%$ upon introduction of siRNA targeted against CD151, whereas that of integrin $\alpha3\beta1$ remained unaffected (Fig. 4*A*; also Fig. 9,
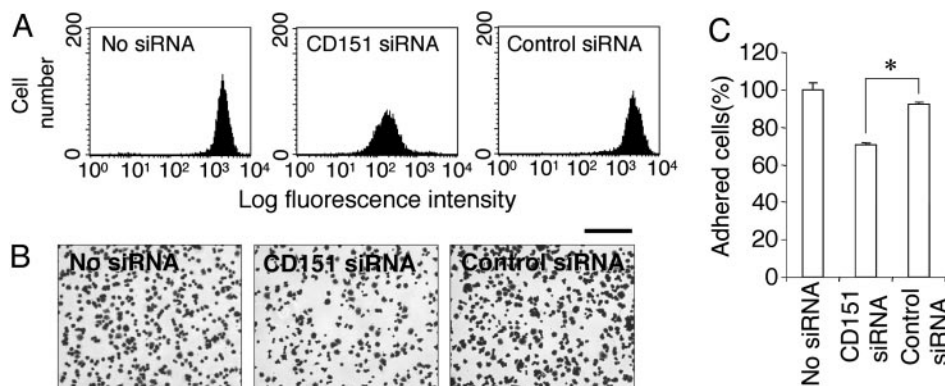

Fig. 4. Effect of CD151 knock-down on cell adhesion to laminin-10/11. (*A*) The CD151 expression levels in A549 cells transfected with siRNA against CD151 (CD151 siRNA) or control siRNA were examined by flow cytometry by using mAb 8C3. The expression of CD151 is knocked down by $\approx90\%$ by CD151 siRNA. (*B*) A549 cells transfected with control or CD151 siRNA were plated on 24-well plates coated with laminin-10/11. After a 20-min incubation at 37°C, the attached cells were fixed and stained. (Scale bar, 200 μm.) (*C*) The numbers of attached cells were counted in three separate fields and expressed as percentages of the number without siRNA transfection (No siRNA). Each bar represents the mean $\pm$ SD. $\ast$, $P < 0.001$.

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which is published as supporting information on the PNAS web site). CD151 knock-down by RNAi resulted in ≈20% reduction in the number of cells adhering to laminin-10/11, whereas cells treated with control siRNA did not exhibit any significant decrease. Although the effect of CD151 knock-down on cell adhesion was only partial, it was reproducible and statistically significant, supporting our conclusion that CD151 stabilizes the activated conformation of integrin $\alpha3\beta1$ in a physiological context.

Discussion

Despite the stable and stoichiometric association between CD151 and integrin $\alpha3\beta1$, the significance of their complex formation has been poorly understood at the molecular level. This result is largely due to the absence of biochemical analyses that directly compare the activities of the integrin with and without CD151 association. Due to the stability of integrin $\alpha3\beta1$–CD151 complexes, it is difficult to dissociate CD151 from the integrin under physiological conditions. Hence, treatment with antibodies against CD151 as well as transfection with CD151 mutants that are unable to associate with integrins have been used to address the role of CD151 association in integrin-dependent cellular functions (8–10, 22). Several lines of evidence have indicated that CD151 is involved in integrin $\alpha3\beta1/\alpha6\beta1$-dependent adhesive events, including migration, spreading, and mechanical force generation, but not in static cell adhesion (8, 22), although it has not been proven whether these agents can dissociate endogenous CD151 from $\alpha3\beta1$ and other laminin-binding integrins. For example, although the QRD mutant of CD151 lacks the ability to associate with integrins (8), there is no reason to assume that exogenous expression of this mutant can prevent the association of endogenous CD151 with $\alpha3\beta1$ and other integrins. Furthermore, no anti-CD151 antibodies have previously been demonstrated to dissociate CD151 from integrin $\alpha3\beta1$ on the cell surface. In the present investigation, we demonstrated that mAb 8C3 actively dissociates CD151 from integrin $\alpha3\beta1$ not only in purified CD151-integrin $\alpha3\beta1$ complexes but also on the surface of living cells, thereby allowing us to compare the ligand-binding activities as well as conformational states of integrin $\alpha3\beta1$ with and without CD151 association. Our results clearly showed that the ligand-binding activity of integrin $\alpha3\beta1$ was potentiated by association with CD151 through stabilization of the active conformation of the integrin. To the best of our knowledge, we present a previously uncharacterized demonstration of a regulatory role for CD151 in the adhesive interactions of integrin $\alpha3\beta1$ with its physiological ligands.

There is accumulating evidence that the ligand-binding activity of integrins is intimately linked with their conformational state (1). The crystal structure of the extracellular domain of integrin $\alpha v\beta3$ revealed a compact conformation with a severe bend in the middle of the receptor (23). Subsequent electron-microscopic as well as biochemical studies showed that the compact conformation represents the low-affinity default state, whereas activation and ligand binding result in global unbending of the receptor, leading to an extended conformation (19). mAb AG89 binds to the midregion of the $\beta1$ chain, which is partially buried in the bent conformation, and thereby preferentially recognizes activated $\beta1$-containing integrins (16, 21). Our results show that the reactivity of integrin $\alpha3\beta1$ toward AG89 was significantly reduced after depletion of CD151, with a concomitant reduction in the ligand-binding activity. This observation strongly suggests that integrin $\alpha3\beta1$ assumes a more extended conformation when it is associated with CD151. It is important to note that both the AG89 reactivity and the laminin-binding activity of CD151-free integrin $\alpha3\beta1$ were reduced, but not abolished. Integrins on cells can exist in multiple conformational states, and there seems to be a rather fast equilibrium among
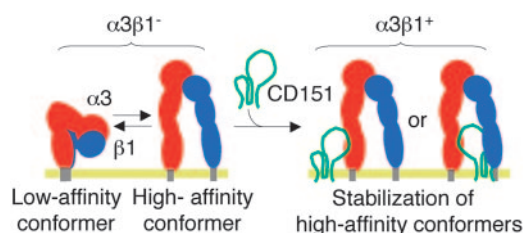

Fig. 5. Schematic model for conformational modulation of integrin $\alpha3\beta1$ by CD151 association. The extracellular domains of the $\alpha3$-(red) and $\beta1$-(blue) subunits are schematically depicted as connected ovals, with membrane-anchoring regions at their ends (gray rods). Integrin $\alpha3\beta1$ depleted of CD151 ($\alpha3\beta1^-$) assumes both low-affinity (bent) and high-affinity (extended) conformers, which are in fast equilibrium. Lateral association of CD151 (represented by a green line) with $\alpha3\beta1^-$ stabilizes the high-affinity conformers, thereby potentiating the ligand-binding activity of integrin $\alpha3\beta1$.

them (24). Therefore, CD151 may modulate the activity of integrin $\alpha3\beta1$ by shifting the position of the conformational equilibrium toward the more extended form, rather than strictly controlling the ligand-binding affinity (Fig. 5).

The association of integrin $\alpha3\beta1$ with CD151 is mediated by interaction of the $\alpha3$ chain with the large extracellular loop of CD151 (5). The region of the $\alpha3$ chain involved in the association with CD151 has been mapped to amino acid residues 570–705 (5), which correspond to the "thigh" and "calf-1" domains of the αv chain (23). This portion corresponds to the region where the integrin is most severely bent in its putative low-affinity conformation, and is thus likely to be the fulcrum point during the "switchblade-opening." Therefore, it is tempting to speculate that the laterally associated CD151 acts as a "prop" to stabilize the extended high-affinity conformation of $\alpha3\beta1$ and render the bending unfavorable (Fig. 5).

The observation that mAb 8C3 can dissociate CD151 from integrin $\alpha3\beta1$ was the strategic basis of our investigation into the biological significance of the stable association of CD151 with integrin $\alpha3\beta1$. Although the precise mechanism by which 8C3 dissociates CD151 from integrin $\alpha3\beta1$ remains to be defined, two possible scenarios can be postulated. One is the "wedge" model, in which 8C3 binds at or near the integrin-binding site on CD151, and sterically prevents CD151 from associating with integrin $\alpha3\beta1$. The other is the "allosteric" model, in which binding of 8C3 induces a conformational change in CD151, converting it to a low-affinity conformation for the integrin. According the wedge model, 8C3 would deplete CD151 from integrin $\alpha3\beta1$–CD151 complexes by capturing only the small fraction of CD151 that spontaneously dissociates from the integrin, and therefore would not deplete CD151 to completion in a short period. In contrast, the allosteric model predicts rapid dissociation of the integrin $\alpha3\beta1$–CD151 complexes, because the rate-limiting step would be binding of 8C3 to the complexes. Given that 8C3 was capable of dissociating CD151 from integrin $\alpha3\beta1$ on the surface of living cells within 1 min, the allosteric model seems more likely, although detailed kinetic analyses as well as epitope mapping of 8C3 will be needed to consolidate this model.

One approach for addressing the in vivo functions of CD151 is to produce animals deficient in CD151 expression and analyze their phenotypes. Wright et al. (25) successfully produced CD151-null mice and demonstrated that these mice were apparently healthy and fertile except for some minor abnormalities, including impaired outside-in signaling in platelets and reduced keratinocyte migration in skin explant cultures (25, 26). Despite the role of CD151 in the adhesive interactions of integrin $\alpha3\beta1/\alpha6\beta1$ integrins with laminins (8, 22), no apparent abnormalities in organogenesis of the skin and kidneys, in which cell-basement membrane interactions play critical roles, were

observed in the CD151-null mice (25). This finding is in striking contrast to mice deficient in integrin $\alpha 3$ and/or $\alpha 6$ chains, which exhibit severe phenotypes including malformation of the kidneys and lungs and skin blistering (27, 28). The absence of such severe phenotypes in CD151-null mice could result from functional compensation by other tetraspanins that are also capable of associating with integrin $\alpha 3\beta 1$ and other laminin-binding integrins. Recently, CD151 was found to be identical to the MER2 blood group antigen (29). MER2-negative individuals exhibited hereditary nephritis and pretibial epidermolysis bullosa, both of which were associated with thickening and splitting of the basement membranes (29). These abnormalities partially resemble the phenotypes of integrin $\alpha 3$- or $\alpha 6$-deficient mice (27, 28), consistent with a regulatory role for CD151 in the interactions of integrins $\alpha 3\beta 1/\alpha 6\beta 1$ with laminins, which in turn regulate the basement membrane assembly of laminins.

Tetraspanins have been considered to be molecular facilitators that organize integrins and other proteins into a network of membrane microdomains, called the "tetraspanin web," with which some signaling molecules, such as protein kinase C, are also associated (2, 30). Besides this organizational role, CD151 and possibly other tetraspanins play a regulatory role in the ligand binding of integrin $\alpha 3\beta 1$ (and possibly other laminin-binding integrins) through stabilizing its activated conformation. Although the structural basis of the CD151-dependent modulation of the ligand-binding activity of laminin-binding integrins remains to be elucidated, CD151 can be regarded as a "conformational facilitator" of integrin $\alpha 3\beta 1$ and other laminin-binding integrins. Further studies on the mechanism by which CD151 modulates the ligand-binding of integrin $\alpha 3\beta 1$ should provide clues for better understanding of the conformational regulation of laminin-binding integrin functions as well as the integrin-mediated outside-in signaling.

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