Tetraspanin CD82 Attenuates Cellular Morphogenesis through Down-regulating Integrin α6-Mediated Cell Adhesion*

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Tetraspanin CD82 has been implicated in integrin-mediated functions such as cell motility and invasiveness. Although tetraspanins associate with integrins, it is unknown if and how CD82 regulates the functionality of integrins. In this study, we found that Du145 prostate cancer cells underwent morphogenesis on the reconstituted basement membrane Matrigel to form an anastomosing network of multicellular structures. This process entirely depends on integrin α6, a receptor for laminin. After CD82 is expressed in Du145 cells, this cellular morphogenesis was abolished, indicating a functional cross-talk between CD82 and α6 integrins. Interestingly, antibodies against other tetraspanins expressed in Du145 cells such as CD9, CD81, and CD151 did not block this integrin α6-dependent morphogenesis. We further found that CD82 significantly inhibited cell adhesion on laminin 1. Notably, the level of α6 integrins on the cell surface was down-regulated upon CD82 expression, although total cellular α6 protein levels remained unchanged in CD82-expressing cells. This down-regulation indicates that the diminished cell adhesiveness of CD82-expressing Du145 cells on laminin likely resulted from less cell surface expression of α6 integrins. As expected, CD82 physically associated with the integrin α6 in Du145-CD82 transfectant cells, suggesting that the formation of the CD82-integrin α6 complex reduces α6 integrin cell surface expression. Finally, the internalization of cell surface integrin α6 is significantly enhanced upon CD82 expression. In conclusion, our results indicate that 1) CD82 attenuates integrin α6 signaling during a cellular morphogenic process; 2) the decreased surface expression of α6 integrins in CD82-expressing cells is likely responsible for the diminished adhesive- ness on laminin and, consequently, results in the attenuation of α6 integrin-mediated cellular morphogenesis; and 3) the accelerated internalization of integrin α6 upon CD82 expression correlates with the down-regulation of cell surface integrin α6.

CD82 belongs to the tetraspanin superfamily, in which members are involved in biological events ranging from cell fusion, cell adhesion, and cell migration to cell proliferation, synapse formation, and neurite outgrowth (1–5). Originally, CD82 was identified as either a membrane protein that could induce the intracellular calcium mobilization in lymphocytes, an accessory molecule in T-cell activation, or the target of a monoclonal antibody (mAb)1 that inhibits human T-cell leukemia virus-induced syncytium formation (6–8). Studies have indicated that CD82 regulates cell aggregation (9–12), cell motility (10, 11, 13–17), cancer metastasis (11, 15, 18), and apoptosis (13, 19). Clustering CD82 on the plasma membrane with its mAb induces the tyrosine phosphorylation of Vav-1, SLP76, and Cas-L (20, 21), actin cytoskeletal rearrangement (20, 22), and dendritic cellular protrusions (8, 22). These observations strongly suggest that CD82 directly or indirectly solicits outside-in signaling to modulate cellular behaviors. Signaling molecules such as Rho GTPases, cAMP-dependent kinase, p130CAS/Crk, and Src kinases are involved in CD82-initiated or -mediated signaling (12, 17, 19, 20, 22). One putative mechanism by which CD82 regulates cellular signal transduction is that CD82 per se directly initiates signaling to attenuate or amplify other cellular signal transductions. The biochemical structure of tetraspanins, however, implies that CD82 is less likely to function as an independent signal initiator. Another putative mechanism is that CD82 regulates the signal transduction initiated from its associated proteins. For example, recent studies revealed that CD82 attenuates epidermal growth factor (EGF) signaling by accelerating EGF receptor endocytosis through a physical interaction with EGF receptor and also by inhibiting the ligand-induced dimerization of the EGF receptor (23, 24). It has been reported that integrin α3β1, α4β1, α5β1, α6β1, or αLβ2 associates with CD82 in cells such as leukemia cells, rhabdomysarcoma cells, hamster ovary cells, and colon carcinoma cells (9, 13, 21, 25–28). Thus, CD82 is also likely to regulate integrin-mediated signaling. Although the CD82-integrin complex is prevalent, and the role of CD82 in integrin-mediated cell migration is well documented, it still remains to be assessed 1) whether or not CD82 directly regulates the functional status of its associated integrins and 2) if CD82 indeed affects integrin activity, what regulatory mechanism exists.

To address these questions, we analyzed the effect of CD82 on integrin-dependent cellular morphogenic process on three-dimensional extracellular matrices (ECM). Previously this morphogenesis assay has been used to evaluate the functional cross-talk between tetraspanin CD151 and laminin-binding integrins (29). In this morphogenesis assay, cells attached on the ECM gel migrate and align to form cell-cell contacts and are subsequently organized into multicellular cables that intersect each other (30–37). In the process of this pattern formation, the three-dimensional extracellular cues play a critical role (37, 38). This cellular morphogenesis com-

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1 The abbreviations used are: mAb, monoclonal antibody; BSA, bovine serum albumin; ECM, extracellular matrix; EGF, epidermal growth factor; FCS, fetal calf serum; MFI, mean fluorescence intensity; pAb, polyclonal antibody; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; Mes, 4-morpholineethanesulfonic acid.

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bines cell-ECM adhesion, cell-cell adhesion, cell migration, differentiation (in the case of endothelial cells), and the branching or intersecting process. Thus, the assay is versatile and can be used to study these biological events. Second, cellular functions such as adhesion and migration are evaluated in a three-dimensional environment, which provides a more physiological setting than the traditional in vitro experiments carried out on two-dimensional matrices. Furthermore, the assay is feasible and is a relatively short term morphogenesis assay. Finally, formation of cellular cable networks on a three-dimensional substratum appears to be a common morphogenic process for many cells ranging from endothelial cells, fibroblast cells, smooth muscle cells, epithelial cells, to cancer cells (29, 33, 35, 37, 39). This assay, therefore, could be widely applied to most cells commonly used for molecular and cellular studies as a morphogenetic readout, although the physiological relevance of this morphogenesis for many cells still remains unclear. By taking these advantages, we have used this cellular morphogenesis assay as an experimental model to assess if and how the effect of CD82 on integrin-mediated adhesion or migration is linked to cellular morphogenesis.

The goals of this study were multiple. First, we sought to determine whether CD82 directly regulates the primary function of integrins, i.e. cell-ECM adhesion. If so, second, could we identify the mechanism responsible for the regulatory activity of CD82? Third, we aimed to analyze the functional interplay between CD82 and integrins during cellular morphogenesis. Through this study, we found that CD82 functions as a negative regulator of integrin-dependent cell adhesion and morphogenesis. More importantly, we demonstrated that CD82 down-regulates integrin α6-dependent cell adhesion by accelerating the internalization of cell surface integrin α6. Although this integrin-tetraspanin interactions have been described for more than a decade, our discovery illustrates an important mechanism by which tetraspanins regulate integrin function.

**MATERIALS AND METHODS**

*Antibodies and Extracellular Matrix Proteins—The mAbs against human proteins used in this study were integrin α2 subunit mAb IE10 (40), integrin α3 subunit mAb X1 (41), integrin α5 subunit mAb PUJ2-42, integrin α6 subunit mAbs GoH3 (BD Pharmingen) and A6-ELE (43), integrin β1 subunit mAb TS2/16 (44), integrin β4 subunit mAb 439-9B (BD Pharmingen), integrin α6 tytoplasmic tail pAb (28), CD9 mAb Du-all (Sigma), CD95 mAb A6-ELE (7), CD81 mAb M38 (7), CD82 mAbs M104 (7), 8E4 (8), and 4F9 (8), CD61 mAb 5C11 (45), and CD109 mAb 8E11 (43). A mouse IgG2 (clone MOPC 141) was used as a negative control antibody (Sigma). The secondary antibodies used in the study were horseradish peroxidase-conjugated goat-anti-mouse or goat-anti-rabbit IgG (Sigma) and fluorescein isothiocyanate-conjugated goat-anti-mouse or goat-anti-rabbit IgG antibody (BIBSIO/RGCE International, Camarillo, CA). The ECMs used in this study were reconstituted mouse basement membrane Matrigel (BD Biosciences), rat tail collagen I (BD Biosciences), and mouse laminin 1 (Invitrogen). For the ligand-dependent internalization assay (see below), laminin 1 proteins were labeled covalently with the fluorescent dye Alexa 488 (Molecular Probe, Eugene, OR) using the Alexa Fluor 488 protein labeling kit (Molecular Probe) per the manufacturer’s instructions.

*Cell Culture and Transfectants—Du145 prostate cancer cells were obtained from ATCC (Rockville, MD) and cultured in DMEM supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 μg/ml streptomycin. The full-length human CD82 cDNA was obtained from Dr. Christopher Class (German Cancer Research Center) and subcloned into a eukaryotic expression vector pCDNA3.1 (Invitrogen). Du145 cells were transfected with pCDNA3.1-CD82 plasmid DNA using SuperFectin (Qiagen, Valencia, CA) and selected with 1 mg/ml G418 (Invitrogen). Hundreds of G418-resistant clones were pooled, and stable CD82 transfecants were established by collecting CD82-positive Du145 cells using flow cytometric cell sorting. Prostate epithelial cells were obtained from Cambrex (Walkersville, MD) and cultured in FrEGM medium (Cambrex) containing 100 units/ml penicillin and 100 μg/ml streptomycin.

**Flow Cytometry—Du145 transfectant cells were incubated with primary mAb and then stained with fluorescein isothiocyanate-conjugated goat-anti-mouse or -rat IgG as described previously (46). Stained cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences). Fluorescence intensity obtained with the negative control mAb was subtracted from each individual mAb staining to give a specific mean fluorescence intensity (MFI) unit of each individual staining.

**Immunoprecipitation and Western Blot—**Immunoprecipitations were carried out basically as described previously (47). Briefly, identical numbers of Du145 transfectant cells were lysed with lysis buffer at 4 °C for 1 h. The lysis buffer contained 1% Brij 98 or 1% Nonidet P-40 (Sigma), 150 mM NaCl, 25 mM HEPES, 2 mM methylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM sodium vanadate, and 2 mM sodium fluoride. In some experiments, cell surface was labeled with 0.5 mg/ml EZlink sulfo-NHS-LC-biotin (Pierce) in phosphate-buffered saline (PBS) at 4 °C for 2 h before cell lysis. The lysates were pre-cleared with a combination of protein A-Sepharose and protein G-Sepharose beads (Amersham Biosciences) two times at 4 °C after removing the insoluble material by 14,000 × g centrifugation. Then mAb-preabsorbed protein A-Sepharose and protein G-Sepharose beads were incubated with cell lysate from 3 h to overnight at 4 °C. The precipitates were washed with the lysis buffer 3 times, dissolved in Laemmli sample buffer, heated at 95 °C for 5 min, separated by SDS-PAGE, and then transferred to nitrocellulose membranes (Schleicher & Schuell). The nitrocellulose membranes were sequentially immunoblotted with primary antibody and horseradish peroxidase-conjugated anti-rabbit IgG (Sigma) and then detected with chemiluminescent reagent (PerkinElmer Life Sciences). For biotinylation experiments, the membranes were blotted with horseradish peroxidase-conjugated extravidin (Sigma) followed with chemiluminescence. In some cases, the immunoblots were stripped and rebotted with mAbs according to the manufacturer’s instruction.

For Western blotting of total cellular proteins, an identical number of cells were lysed with radioimmunoprecipitation assay buffer, the protein concentrations of samples were normalized, equal amounts of proteins were loaded, and lysates were then separated by SDS-PAGE. The following procedures are the same as described above for immunoblotting.

**Cell adhesion Assay—**Adhesion assays were performed quantitatively as described previously (48). Briefly, Falcon 96-well plates were coated with mouse laminin 1 at different concentrations at 37 °C for 3 h or at 4 °C overnight. The coated wells were then blocked with 1% heat-inactivated bovine serum albumin (Sigma) at 37 °C for 45 min. Cells were incubated at 37 °C for 35 min at a density of 1 × 10⁴ cells/well in serum-free DMEM. For prostate epithelial cells, cells were incubated at 37 °C for 6 min at density of 2 × 10⁴ cells/well in PrEGM media supplemented with 2 mM Mg²⁺. After removing non-adherent cells with three washes, adherent cells were visually quantitated. Background binding was assessed using uncoated wells and subtracted from experimental values. Results were expressed as the adjusted number of adherent cells on the plate/mm² and reported as mean ± S.D. of triplicate determinations.

**In Vitro Cellular Morphogenesis Assay—**The spontaneous formation of intersecting cable-like structures by Du145 transfectant cells on Matrigel was used to assess cellular morphogenetic process under different treatments. Matrigel or collagen I was placed into 24-well plates (0.4 ml/well) and allowed to solidify for 2 h at 37 °C. Du145-Mock or -CD82 transfectant cells were then seeded into each well onto the gel at a concentration of 1 × 10⁴ cells/well in 0.6 ml of DMEM containing 10% FCS. The cells on Matrigel or collagen gel were cultured at 37 °C in 5% CO₂ and photographed after an overnight or a 24-h incubation. Images were captured using Scion Image 1.60 (Scion Corp., Frederick, MD) software through a video camera (TM-7AS, PULNiX America, Inc., Sunnyvale, CA) attached to an inverted phase contrast microscope. All results represented at least three independent and reproducible experiments.

**Internalization Assay—**Ligand-dependent internalization assays were performed according to standard protocols (49, 50). Instead of iodinated ligand, fluorochrome Alexa 488-labeled laminin 1 was used in the experiments. Du145 transfectant cells were detached from cell culture plates using 2 mM EDTA in PBS and washed with PBS and the ligand binding buffer sequentially. Alexa 488-labeled laminin 1 was added at a concentration of 0.1 μg/ml at 4 °C on ice for 1 h. The cells were centrifuged at 4 °C to remove the supernatants containing unbound laminin. After resuspending cells with PBS, cell suspensions were incubated at 37 °C for different periods of time to allow cells to internalize the surface-bound laminin. After the 37 °C incubation, cells were treated with 2 mM EDTA in PBS to remove surface-bound laminin that had not yet been internalized. Then the cells were subjected to flow cytometric analysis of the internalized...
laminin. The cells that were not incubated with Alexa 488-labeled laminin 1 were used as a negative control for the flow cytometry analysis; the cells without EDTA treatment after the ligand incubation were used as a positive control. The fraction of internalized ligand was presented as the percentage of intracellular fluorescent intensity at each time point relative to the total cell-associated fluorescent intensity prior to transfer to 37 °C. The percentage of internalized laminin was calculated with the formula (MFI of a certain time point – MFI of negative control)/MFI of positive control × 100%.

The internalization of integrin α6 was determined following the established protocol (49, 51). Du145 transfectants 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 2 h to allow maximal biotinylation of the cell surface proteins. The free biotin was removed by 3 washes with PBS. The labeled cells were incubated at 37 °C for various time for the internalization and then treated with a reducing solution containing 20 mM Mes-Na, 100 mM NaCl, and 50 mM Tris (pH 8.6) at 4 °C to remove cell surface-bound biotin. Mes-Na was quenched by the addition of 20 mM iodonatocendate. The cells were lysed in radiolimune precipitation assay lysis buffer, and integrin α6 was immunoprecipitated. After SDS-PAGE and electric transferring, the biotinylated integrin α6 was detected by peroxidase-conjugated extravidin and visualized by chemiluminescence. The ratio of integrin α6 internalization at each time point was calculated from the internalized integrin at that time point as a percentage of the total surface integrin.

RNA Interference—Small interfering RNA duplexes against CD82 cDNA sequence were designed and synthesized by Dharmacon (Lafayette, CO). A nonspecific sequence, which had previously been found to be ineffective for knockdown experiments by Dharmacon, was used as a control. Prostate epithelial cells were transfected with small interfering RNA duplexes using Mirus Trans IT-TK0 transfection reagent (Mirus, Madison, WI) and the Prostate Boost Reagent (Mirus) as specified by the manufacturer. In some experiments, two transfections were performed 2 days apart for more efficient knockdown, and the experiments were carried out 48 h after the second transfection.

RESULTS

The Cellular Morphogenesis of Du145 cells on Matrigel Depends on Integrin α6—Du145 is an epithelial cell line originally isolated from the brain metastatic lesion of a prostate cancer patient (52) and is commonly used as an in vitro experimental model in the cellular behavior studies of prostate cancer. Du145 cells preserve some epithelial features of the original tissue and proliferate in an androgen-independent manner (52). To study the cellular behaviors of Du145 cells in various extracellular environments, we found that Du145 cells formed an anastomosing multicellular structure when they were plated on the top of the reconstituted basement membrane Matrigel (Fig. 1A). The structure was formed at 6–8 h after the cells were seeded and lasted 2–3 days. Du145 cells formed this web-like structure only on Matrigel, not on collagen I gel, indicating that a specific interaction between the integrin on Du145 cells and the ECM component in Matrigel is required for this morphogenetic process.

Matrigel contains ECM proteins including laminin 1, entactin/nidogen, collagen IV, and proteoglycan (30, 53, 54). To identify which pair of integrin-ECM protein interactions is responsible for this process, we used function-blocking antibodies against integrin α2β1, α3β1, α5β1, and α6, which are the cellular ligands for collagen, laminins, fibronectin, and laminins, respectively (55). Integrin α3β1 binds laminin 5, 10, and 11, whereas integrin α6 binds all laminins (56). As shown in Fig. 1B, only integrin α6 mAb GoH3 inhibited the formation of the anastomosing multicellular structure of Du145 cells, indicating that integrin α6 is responsible for this morphogenetic process. Function-blocking mAbs against integrin α2, α3, or α5 (40, 41, 57, 58), the major integrins expressed in Du145 cells, did not affect this morphogenetic process, confirming that only the laminin 1-integrin α6 engagement is required for this cellular morphogenesis.

CD82 Accelerates Integrin α6-Mediated Cell Adhesion—CD82 is ubiquitously expressed in normal tissues but frequently loses expression in advanced cancer cells, such as Du145 cells (16, 17). To test the role of CD82 in integrin α6-mediated morphogenesis, we stably expressed CD82 in Du145 cells. Flow cytometry analysis showed that the MFI of CD82 staining in Du145-Mock and -CD82 cells was 4.5 and 124.0 (following subtraction of the MFI of a negative control mAb), respectively (Table I). The expression of CD82 completely abolished the formation of an anastomosing structure on Matrigel (Fig. 2A), and the degree of inhibition correlated with the surface expression level of CD82 on Du145 cells (data not shown). These data indicated that CD82 molecules on the cell surface were directly involved in inhibiting of this morphogenic process. We compared the numbers of viable and apoptotic cells between Du145-Mock and -CD82 transfectants after incubating overnight on Matrigel and found that CD82 did not significantly alter cell proliferation or viability on Matrigel (Fig. 2B), confirming that the disrupted cellular morphogenesis did not result from less proliferation or survival of CD82-expressing Du145 cells on Matrigel.

We also tested effects of the antibodies against other tetraspanins on the morphogenic process. Tetraspanin CD151 has been reported to specifically regulate integrin α6β1- or α3β1-mediated cellular morphogenesis in NIH3T3 and COS-7 cells (29, 39). CD151 is well expressed in Du145 cells (Table I). To our surprise, the CD151 mAb 5C11 did not block the morphogenesis in Du145 cells (Fig. 2C). Antibodies against CD9 or CD81, which are also prominent tetraspanins expressed on Du145 cells, did not affect morphogenesis (Fig. 2C). These tetraspanin mAbs have been used in other studies to perturb specific tetraspanin-mediated functions (7, 45, 59). For Du145-CD82 cells, these tetraspanin mAbs could not reverse the morphogenesis-inhibitory effect of CD82 (Fig. 2C).

CD82 Down-Regulates Integrin α6-Mediated Cell Adhesion—Because cellular morphogenesis on Matrigel consists of at least adhesion, migration, and cellular branching/intersecting, disruption of any of these events, theoretically, will attenuate the morphogenic process on three-dimensional matrices. Given the fact that cell adhesion is the primary
function of integrins, and this cellular morphogenic process depends entirely upon integrin α6, the effect of CD82 on cell adhesion becomes an immediate issue to be addressed even though tetraspanins do not usually affect integrin-dependent cell-ECM adhesion (4, 5). Thus, we next asked whether CD82 directly affects the function of integrin α6, i.e. adhesion to its specific ligand. As shown in Fig. 3, cell adhesion on laminin 1, a ligand for integrin α6 and a component of Matrigel, was substantially reduced in Du145-CD82 cells as compared with Mock cells at all ligand-coating concentrations. We found that CD82 also down-regulated cell adhesion on fibronectin (data not shown).

CD82 Decreases the Integrin α6 Level on Cell Surface—We then questioned how integrin α6-dependent adhesion was attenuated by CD82. We compared the quantities of integrin α6 between the Mock and CD82 transfectant cells. Total cellular level of integrin α6 proteins was not altered upon CD82 expression as shown in Fig. 4A by Western blot analysis of Du145-Mock and Du145-CD82 cell lysates. However, the cell surface expression of integrin α6β1 and α6β4, analyzed by immunoprecipitation using biotin-labeled transfectant cells, was substantially reduced upon CD82 expression. To confirm the results, we further quantitated cell surface integrin α6 by flow cytometry using α6 mAb A6-ELE and GoH3. Again, the cell surface integrin α6 was significantly down-regulated in the CD82-expressing Du145 cells (Table I). Collectively, these data indicate that there are either fewer functional integrin α6 or fewer integrin α6 expressed on the cell surface in Du145-CD82 cells available for laminin engagement. These data also reflect that the steady-state distribution of α6 at the cell surface becomes markedly less upon CD82 expression, because both biotinylation and the antibody incubation in flow cytometry were performed under saturation conditions. Because both α6β1 and α6β4 are receptors for laminin 1, we further analyzed levels of the individual α6 integrins by flow cytometry. The integrin β4 subunit on the cell surface was completely lost upon CD82 expression (Table I), although a reduced β4 was seen in immunoprecipitation (Fig. 4B). By subtracting β4 staining from α6 staining (Table I), we found that the cell surface level of integrin α6β1 was also decreased in Du145-CD82 cells. Together, CD82 down-regulates both α6β1 and α6β4 integrins from the cell surface. The cell surface expression levels of other major integrins and tetraspanins were also measured in Du145-Mock and -CD82 transfectant cells. The level of integrin β1 subunit remained unchanged (Table I). Integrin α3β1, the receptor for laminin 5, and integrin α5β1, a receptor for fibronectin, showed equivalent expression on both transfectants (17). Tetraspanins such as CD9 and CD151 were also equivalently expressed on the cell surfaces of both Mock and CD82 transfectants (Table I). To this end, CD82 appears to specifically down-regulate integrin α6 from the plasma membrane. Consistent with this observation, we found that CD82 also down-regulated the cell surface expression of integrin α6 in the

| Table I | Comparison of the surface expression of integrins and tetraspanins between Du145-Mock and Du145-CD82 cells |
|-----------------|-----------------|
|                 | Mock            | CD82            |
| Integrin α6 (GoH3) | 78.4            | 23.6            |
| Integrin α6 (A6-ELE) | 35.1            | 7.3             |
| Integrin β1 | 338.1           | 331.9           |
| Integrin β4 | 6.0             | 0.0             |
| CD9 | 32.9            | 45.5            |
| CD82 | 4.5             | 124.0           |
| CD151 | 145.2           | 159.1           |

CD82 Associates with Integrin α6 in Du145 Cells—To address whether the possible association of CD82 to integrin α6 is involved in the down-regulation of integrin α6-dependent adhesion by CD82, we analyzed the CD82-integrin α6 association in Du145 cells. As shown in Fig. 5, integrin α6 co-immunoprecipitates with CD82 in 1% Brij 98 detergent in Du145-CD82 PC3 metastatic prostate cancer cells that were transfected with CD82 (data not shown).
transfectant cells, which is consistent with the results observed in other cells (4). The CD82-integrin α6 complex was disrupted in a more stringent detergent 1% Nonidet P-40 (Fig. 5), which is a typical kind of association seen in most tetraspanin-integrin complexes (3). As previously reported (4, 5, 45), integrin is a typical kind of association seen in most tetraspanin-integrin complexes (3). As previously reported (4, 5, 45), integrin α6 associates with tetraspanin CD151 in both mild and stringent cell lysis conditions in Du145-Mock as well as -CD82 transfectant cells but not with another cell surface protein CD109. Notably, the CD151-integrin α6 association became less evident upon CD82 expression (Fig. 5).

**CD82 Accelerates the Internalization of Integrin α6**—The presence of CD82-integrin α6 complexes in Du145 cells provides insight into the mechanism of how CD82 decreases the cell surface level of integrin α6. CD82 is contained in two subcellular pools; cell surface and endosomal/lysosomal vesicles. Thus, CD82 likely traffics between the cell surface and intracellular vesicles. We hypothesized that CD82, through initial association with integrin α6, ultimately decreases the cell surface level of integrin α6 by accelerating its internalization. To test this hypothesis, we performed the ligand-dependent internalization assay to assess whether integrin α6 is internalized at an accelerated pace upon CD82 expression. In this assay, fluorochrome-conjugated laminin 1 is allowed to bind cell surface integrin α6 on ice and then be internalized when cells are incubated at 37 °C for different periods of time.

As we predicted, the internalization of cell surface-bound laminin 1 was markedly increased in Du145-CD82 cells at each time point when compared with that of Mock cells (Fig. 6A). Because cells may have non-integrin receptors of laminin, we further assessed how much laminin 1-binding depends upon integrin α6. A function-blocking mAb to integrin α6 inhibited approximately two-thirds internalization activity of Du145 cells (Fig. 6B), indicating that the internalization of laminin 1 is mainly mediated by integrin α6. Although cells may not uptake soluble laminin under physiological conditions, a previous study showed that the internalization of laminin receptor integrin α6 is not appreciably affected by the conditions under which cells were kept, such as suspension or adherence (60). Thus, the ligand-dependent internalization could be measured when cells are kept in suspension with soluble laminin, be-

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2 X. A. Zhang, unpublished data.

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**CD82 Expression Increases the Internalization Rate of Integrin α6**—Because cells may have non-integrin receptors of laminin, we further assessed how much laminin 1-binding depends upon CD82 expression (Fig. 5).

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inhibition of laminin-1 binding by integrin α6. We found that some integrin molecules actually underwent endocytosis from the cell surface time 0 in Du145-CD82 cells (Fig. 6C), indicating that these molecules were internalized at time 0 in Du145-CD82 cells (Fig. 6C), indicating that these molecules actually underwent endocytosis from the cell surface during the incubation period at 37 °C which internalization is typically stopped (49).

CD82 Knockdown Promotes Surface Expression of Integrin α6 and Integrin α6-Mediated Cell Adhesion—To confirm that CD82 regulates the cell surface expression of integrin α6, we down-regulated CD82 expression in prostate epithelial cells, which constitutively express endogenous CD82 (73), using RNA interference. As shown in Fig. 7A, upon the specific knockdown of CD82, the cell surface level of integrin α6, exclusively α6β4, was enhanced. Subsequently, cell adhesion to laminin 1, a substrate of integrin α6, was increased upon the knockdown of CD82 (Fig. 7B), which is consistent with the conclusion we reached from the Du145 transfectants, i.e., CD82 negatively regulates cell surface expression of integrin α6. Because prostate epithelial cells readily form a well developed web-like structure on Matrigel, the knockdown of CD82 did not further alter the formation of the web-like structure (data not shown).

DISCUSSION

CD82 Attenuates the Cellular Morphogenesis by Initially Diminishing Integrin α6-Mediated Cell Adhesion—The cellular morphogenesis on Matrigel, namely the formation of an anastomosing cable-like multicellular structure, is composed of...
multiple but concerted events including cell-ECM adhesion, directional cell migration, formation of cell-cell contact and adhesion, retraction and remodeling of ECMs, and branching or intersecting process. It also requires cellular differentiation for endothelial cells. Because cell-ECM adhesion is the initial step of the morphogenic process, integrin is expected to play a critical role in cellular morphogenesis. However, the precise role of integrin-mediated cell-ECM adhesion in morphogenesis has not been well defined, because integrins also participate in cell migration, proliferation, and survival (62). We demonstrated that integrin-dependent cell adhesiveness on laminin 1 is diminished upon CD82 expression, and this attenuated cell adhesion correlates with the abrogation of laminin-dependent cellular morphogenesis. Because the morphogenic process begins with cell-ECM adhesion, the deficient cell adhesiveness on laminin is the initial cause, as well as very likely the major cause, for the abrogated cellular morphogenesis. Recently, Lammerding et al. (63) found that mutations in the C-terminal cytoplasmic domain of CD151 result in the loss of cell adhesion strengthening to laminin 1 and impair the cellular morphogenesis of fibroblasts on basement membrane matrix. Our study is consistent with their results and with the notion that appropriate cell adhesiveness is essential for cellular morphogenesis.

Not only is the integrin α6-laminin 1 engagement critical for cell attachment, but it is also important for cell migration on this matrix protein. During cellular morphogenesis, directional cell migration is the subsequent step after cell attachment to allow cells to align to one another and form cell-cell contacts. It is well established that CD82 inhibits cell migration (10, 11, 13–17). We previously reported that CD82 inhibits both random and directional migration of Du145 cells on laminins (16, 17). The deficient motility of Du145-CD82 cells clearly contributes to the disruption of Du145 cellular morphogenesis on Matrigel by CD82. Because optimal cell migration requires an intermediate level of cell adhesiveness (64), the suppressed cell motility in Du145-CD82 cells could be the immediate consequence of the deficient cell adhesion and is more directly responsible for the attenuated morphogenic process. This conclusion is reflected by the results shown in Fig. 2A, in which Du145-CD82 cells scattered or formed only small cell aggregates on the surface of Matrigel, indicating that Du145-CD82 cells did not move much compared with Mock cells from the place where they were originally plated. Because tetraspanin CD151 was found to play a positive role in matrix retraction during the same morphogenic process (63), the role of CD82 in matrix retraction remains to be further investigated. Predictably, the attenuated cell adhesiveness on Matrigel by CD82 will eventually hinder the matrix retraction.

Although Matrigel is composed mainly of ECMs, it also contains various growth factors such as EGF, platelet-derived growth factor, and fibroblast growth factor (30, 53, 54). Growth factor-solicited signaling is also important for this cellular morphogenesis (65). Notably, CD82 has been reported to attenuate EGF activity (23, 24). We observed that the disruptive effects on cellular morphogenesis by CD82 were alleviated when more FCS was used in the morphogenic assay (data not shown), suggesting that an attenuation of growth factor receptor-initiated signaling in CD82-expressing cells is involved in this morphogenesis. Thus, the disruption of cellular morphogenesis by CD82 could result from a combinatorial effect of the down-regulation of both integrin and growth factor activities. Because both pathways essential for development can be down-regulated by CD82 and CD82 inhibits morphogenic processes in vitro, it becomes very interesting to understand the role that CD82 plays in animal development.

Among cell adhesion molecules, the laminin-binding integrins are required for the morphogenesis on Matrigel, because this process depends on laminin 1. Although integrin α3β1 is one of the major laminin receptors, it does not bind to laminin 1 or the EHS tumor laminin (56, 66, 67), which is the laminin in Matrigel. Thus, α6 integrins (α6β1 and α6β4) are apparently essential for laminin 1-dependent morphogenesis (29). To our surprise, in contrast to previous reports using NIH3T3 and COS-7 cells (29, 39), the same CD151 mAb did not disrupt the cellular morphogenic process of Du145 cells on Matrigel. Although CD151 is indeed associated with α3 and α6 integrins in Du145 cells, the lesser involvement of CD151 in the α6 integrin functions in CD82-expressing cells may be because of the fact that the α6 integrins can be regulated by multiple tetraspanins. Consequently, the regulatory role of the α3 and α6 integrins tightly associated CD151 could become less evident when the α6 integrins are recruited to complex with the predominant
tetraspanin present in cells like CD82 in Du145-CD82 cells.

CD82 Inhibits Integrin-mediated Cell-ECM Adhesions—Although the integrin-tetraspanin complexes were discovered more than a decade ago and are prevalent, the question of whether or not tetraspanins regulate integrin-dependent cell-ECM adhesion remains controversial. At least, the regulation of integrin-mediated cell-ECM adhesiveness is not seen as a primary function for tetraspanins (5). Many studies indicate that tetraspanins do not substantially alter integrin-dependent static cell-ECM adhesion (4). Although Lammerding et al. (63) demonstrated that CD151 specifically regulates integrin αβ1-mediated cell adhesive strengthening, the static adhesion to laminin 1 was not affected by CD151. Feigelson et al. (68) recently found that tetraspanin CD81 promotes integrin αβ1- and α5β1-mediated instantaneous cell adhesion strengthening under shear flow in a ligand occupancy-independent manner. In our study, the expression of CD82 in Du145 cells down-regulated integrin-mediated cell-laminin adhesions, measured by the 35-min “static” adhesion assays, and the knockdown of CD82 in prostate epithelial cells promoted the static cell adhesion on laminin 1. Notably, CD82 has not been reported to regulate cell-ECM adhesion. Although a recent study indicated that cells expressing an alternatively spliced CD82 variant showed increased cell-extracellular matrix adhesion compared with wild type CD82-expressing cells, whether wild type CD82 affects integrin-mediated cell adhesion was not thoroughly investigated and discussed (27). Thus, to our knowledge, our study demonstrated for the first time that tetraspanin CD82 regulates integrin-mediated cell-ECM adhesiveness. Interestingly, CD82 not only inhibits cell adhesion on laminin but also that on fibronectin, although the relatively more robust integrin-tetraspanin associations usually occur between laminin-binding integrins and tetraspanins (5). If deficient cell adhesiveness in CD82-expressing cells is indeed the cause of suppressed cell motility, the diminished adhesiveness on laminin and fibronectin explains CD82-mediated inhibition of Du145 cell migration on both matrix proteins as previously reported (16, 17).

Given a specific matrix protein, cell adhesion can be regulated by the expression level and the affinity/avidity of a specific integrin. For CD82, the down-regulation of cell adhesion on laminin 1 appears to be primarily due to less cell surface expression of integrin α6. No evidence so far suggests that tetraspanins alter integrin affinity (5). However, tetraspanin CD81 reportedly regulates integrin α4β1 avidity (68). Because CD82 regulates cytoskeleton reorganization (20), whether CD82 affects cell adhesiveness by also modulating integrin avidity remains to be tested.

CD82 Down-regulates Integrin α6-Dependent Cell Adhesion by Accelerating Its Internalization—Odintsova et al. (23) reported that CD82 attenuates EGF signaling by accelerating EGF receptor endocytosis through a physical interaction with EGF receptor. In our study, we demonstrated that CD82 down-regulates integrin α6-mediated cell adhesion by associating with it and accelerating its internalization. In both cases, CD82 regulates the functionality of transmembrane proteins by using this “touch and down” mechanism. The functional cross-talk between CD82 and transmembrane proteins is apparently not limited to EGF or integrin α6. For example, CD82 down-regulates integrin α5β1-mediated cell migration (14, 17). Although the mechanism by which CD82 down-regulates integrin α5β1 function remains to be investigated, we extrapolate that the touch and down mechanism could also apply to integrin α5β1, because CD82 has been reported to associate with integrin α5β1 (14). Thus, we propose that the touch and down could serve as a general mechanism for CD82. How CD82 down-regulates the functionality of the above proteins is an important and interesting issue that remains to be addressed. The clue for the answer to this question may come from the structure of CD82 molecules. The C-terminal cytoplasmic domain of CD82 contains a YXXΦ sequence (Φ is tyrosine, X could be any amino acid residues, Φ represents bulky hydrophobic amino acid residues such as Leu and Val), which is an endosome/lysosome targeting motif (69). Through this motif, CD82 likely facilitates the internalization of its associated proteins and then helps target these proteins into the lysosomes for degradation.

CD82 Is a Tetraspanin That Inhibits the Function of Its Associated Proteins—Tetraspanin CD151 up-regulates integrin α6-mediated cell adhesive strengthening (63) and plays a positive role in cell migration (45, 70, 71). Thus, CD151 appears to be a tetraspanin that promotes the function of their associated integrins. In contrast to CD151, CD82 is a prototype of the tetraspanins that inhibit the function of their associated proteins such as integrins and growth factor receptors. These observations support a notion that tetraspanins can be grouped into positive regulators and negative regulators. This classification may facilitate the interpretation of promiscuous and paradoxical literature reports regarding various tetraspanin biological functions and help to evaluate the cellular function of a particular tetraspanin by integrating the roles of other tetraspanins.

In summary, our study assured that integrin-dependent cell-ECM adhesion per se is essential for cellular morphogenesis and described that tetraspanin CD82 inhibits this morphogenic process. We also demonstrated that CD82 indeed attenuates integrin-dependent cell-ECM adhesions, which is likely to be the mechanistic interpretation for the inhibitory roles that CD82 plays in migration, invasion, and metastasis. We further found that CD82-diminished cell adhesion to laminin likely results from the accelerated internalization of integrin α6. Finally and also more importantly, our study provided the initial evidence that integrin-mediated cell adhesion can be regulated by integrin trafficking such as the internalization of integrin α6.

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