Within each hemidesmosome, αβ₄ integrin plays a crucial role in hemidesmosome assembly by binding to laminin-5 in the basement membrane zone of epithelial tissue. Recent analyses have implicated “specificity-determining loops” (SDLs) in the I-like domain of β integrin in regulating ligand binding. Here, we investigated the function of an SDL-like motif within the extracellular I-like domain of β₁ integrin. We generated point mutations within the SDL of β₁ integrin tagged with green fluorescent protein (GFP-β₁K150A and GFP-β₁Q155L). We also generated a mutation within the I-like domain of the β₄ integrin, lying outside the SDL region (GFP-β₄V284E). We transfected constructs encoding the mutated β₁ integrins and a GFP-conjugated wild type β₄ integrin (GFP-β₄WT) into 804G cells, which assemble hemidesmosomes, and human endothelial cells, which express little endogenous β₄ integrin. In transfected 804G cells, GFP-β₄WT and GFP-β₄V284E colocalize with hemidesmosome proteins, whereas hemidesmosomal components in cells expressing GFP-β₁K150A and GFP-β₁Q155L are aberrantly localized. In endothelial cells, GFP-β₄WT and mutant proteins are co-expressed at the cell surface with α₄ integrin. When transfected endothelial cells are plated onto laminin-5 matrix, GFP-β₄WT and GFP-β₄V284E localize with laminin-5, whereas GFP-β₁K150A and GFP-β₁Q155L do not. GFP-β₄WT and GFP-β₄V284E expressed in endothelial cells associate with the adaptor protein Shc when the cells are stimulated with laminin-5. However, GFP-β₁K150A and GFP-β₁Q155L fail to associate with Shc even when laminin-5 is present, thus impacting downstream signaling. These results provide evidence that the SDL segment of the β₄ integrin subunit is required for ligand binding and is involved in outside-in signaling.

Integrins are members of a family of cell surface receptor proteins involved in cell-matrix and cell-cell interactions (1, 2). A primary function of many integrins is to adhere cells to extracellular matrix molecules and to provide a structural link between the outside of the cell and the cytoskeleton (1–3). In addition, integrins mediate inside-out and outside-in signaling pathways, thereby regulating growth, differentiation, and migration of cells during development, wound healing, and in a variety of pathological conditions including tumor metastasis (4).

Each integrin is a heterodimer containing an α and a β subunit. At present 18 α and 8 β subunits have been identified, which combine to form 24 integrins (1, 2). Both subunits have large extracellular amino-terminal domains, single membrane-spanning regions, and short cytoplasmic tails of about 50 residues that interact with components of the microfilament cytoskeleton system (1, 2). One notable exception is the β₄ integrin subunit, whose cytoplasmic domain comprises over 1000 amino acids. It links to the keratin intermediate filament network at the site of hemidesmosomes in certain epithelial tissues via interaction with a transmembrane molecule of 150 kDa (BP180), a cytoplasmic structural protein of 230 kDa (BP230) and the cytoskeletal linker protein plectin (5, 6).

The binding of integrins to their extracellular ligands triggers outside-in signaling events that may include activation of tyrosine, serine/threonine, and lipid kinases as well as small GTP-binding proteins, including RhoA, Rac1, and Cdc42 (4). Activation of these signaling pathways can lead to a variety of responses in the cell, including changes in cytoskeletal organization, cell division, differentiation, and apoptosis. Conversely, intracellular signals are believed to trigger conformational changes in the cytoplasmic domains of an integrin that are transmitted along the molecule to its extracellular domain. These conformational changes appear to regulate the affinity of integrins for their ligands (2).

Recent structural data, including crystallographic analyses, have shed new light onto how integrins bind ligand, how ligand-binding specificity is accomplished, and how signals are transmitted by integrin molecules across the membrane (2). For example, the extracellular domains of several of the α integrin subunits contain an “inserted” sequence, the I or A domain, of about 200 amino acids, that has been implicated in ligand binding (2). The β integrin subunit also possesses an extracellular I-like domain within which a short sequence of about 30 residues appears to specify ligand binding. This 30-residue domain has been termed the specificity-determining loop (SDL) (7).

In particular, Takagi et al. (7) have shown that removal of the SDL blocks the ability of the αβ₄ integrin to bind its ligand, fibronectin. Moreover, when a seven-residue sequence contained within the SDL of β₄ integrin is switched...
with a comparable sequence in β4 integrin, ligand-binding specificity of the mutated integrin is altered (8). In addition, the SDLs of the β1 and β2 integrin subunits appear to play an essential role in heterodimerization with specific α integrin subunits (namely α6β1, α4β1, and α6β2) (7).

The SDL of the β1 integrin does not appear to play a role in mediating its interaction with its α6 integrin (7), its only known partner (1, 2). The α6β1 integrin heterodimer is primarily expressed in epithelial cells and plays an essential role in the assembly of hemidesmosomes, certain basement membrane adhesions that stabilize the interaction of epithelia with the underlying connective tissue (5, 6, 9). The importance of the α6β1 integrin in the adhesion of epithelial cells to the basement membrane is underscored by the blistering that occurs at the dermal-epidermal border in the skin of patients afflicted with junctional epidermolysis bullosa associated with pyloric atresia, in which expression of α6β1 integrin is aberrant, and in genetically engineered mice in which expression of α6 or β1 integrin protein is inactivated by targeted disruption (5, 6, 10–12). The ligand of integrin α6β1 is laminin-5, a major structural component of the basement membranes of epithelial tissues (5, 6). Indeed, the binding of α6β1 integrin and laminin-5 is a necessary prerequisite to certain epithelial cell adhesion, migration, and morphogenetic events (5, 6). Yet, despite this importance, structural and molecular characterization of regions that regulate α6β1 integrin binding to laminin-5 have not been investigated. Takagi et al. (7) have suggested that the SDLs of all β integrin subunits are involved in regulating ligand binding. However, to date no studies have addressed whether the SDL of β1 integrin modulates α6β1 integrin/laminin-5 interaction. Hence, we have undertaken assays of the function of the SDL of β1 integrin with regard to both ligand binding and transduction of outside-in signals.

MATERIALS AND METHODS

Cell Culture and Transfection—Cells of the rat bladder epithelial cell line 804G were cultured and transiently transfected as previously reported (13–15). Briefly, 804G cells were cultured at 37 °C in minimum essential medium containing a final concentration of 2 mM l-glutamine, 10% fetal bovine serum, 50 units/ml penicillin, and 50 units/ml streptomycin. Prior to transfection, 804G cells were maintained for 48 h on 22-mm glass coverslips or 100-mm plastic dishes. They were transfected with 8.8 μg of plasmid DNA using the calcium phosphate protocol described elsewhere (14, 15). Immortalized and derived endothelial cells (TrHBMECs) were maintained in Dulbecco’s modified Eagle’s medium at about 1 × 10^5 cells/ml. The cells were electroporated with DNA at 950 microfarads, 196 ohms, and 210 V in a BTX Electro Cell Manipulator 600 (BTX, San Diego, CA) (17).

Construction of β1 Integrin Wild Type Protein and Generation of Point Mutations—A mammalian expression vector encoding the sequence of the green fluorescent protein (GFP) fused to a full-length human integrin β1 subunit (GFP-β1WT) was described elsewhere (15). Point mutations were generated in the β1 integrin sequence in this vector using the QuikChange XL site-directed mutagenesis kit from Stratagene (La Jolla, CA) following the procedure of the manufacturer. Plasmids were used to transform XL10-Gold Ultracompetent Cells (Stratagene), which were then plated on selective media and incubated overnight at 37 °C. DNA was prepared from the resultant colonies using the Wizard Plus Miniprep DNA Purification System (Promega, Madison, WI) and sequenced using Big Dye chemistry on an ABI Prism Elite PCS sorter (Beckman Coulter, Miami, FL).

Immunoblotting and Immunoprecipitation Protocols—For immunoblotting, confluent cell cultures of 804G cells and TrHBMECs were solubilized in sample buffer consisting of 8 M urea, 1% SDS in 10 mM Tris-HCl, pH 6.8, 15% β-mercaptoethanol. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and processed for immunoblotting (20, 21). Nitrocellulose-bound antibodies were detected by chemiluminescence using the ECL kit (Amersham Biosciences).

Antibodies were immunoprecipitated from 804G cells or TrHBMECs by first extracting the cells with immunoprecipitation buffer consisting of 25 mM HEPES, pH 7.5, 1% Brij 97, 150 mM NaCl, 5 mM MgCl2, 0.2% SDS, supplemented with a protease inhibitor mixture and 1 mM orthovandate (Sigma). Integrin or GFP antibodies were added to the extract and incubated at 4 °C for 2 h. Subsequently, protein G-agarose (Invitrogen) was added to the mix for an additional 2 h. The protein G-agarose was collected by centrifugation, washed three times in immunoprecipitation buffer, and then solubilized in Laemmli type SDS-PAGE sample buffer (20). The resulting protein solution was processed for Western immunoblotting as detailed above.

Flow Cytometry—Cells were trypsinized and resuspended in either PBS or tissue culture medium containing a 50% dilution of normal goat serum. The cell suspension was then incubated with the monoclonal antibody 3E1 for 45 min at room temperature. Cells were washed, and Cy5-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) was added to each of the samples. Following a 45-min incubation at room temperature, stained cells were washed, resuspended in 0.5% formaldehyde, and then analyzed by flow cytometry using a Beckman Coulter Elite PCS sorter (Beckman Coulter, Miami, FL).

Immunofluorescence Microscopy—Cells on glass coverslips were fixed in 3.7% formaldehyde in PBS for 1 min at room temperature and then permeabilized for 8 min in 0.1% Triton X-100 in PBS at 4 °C. Antibody was overlaid onto the cells, and the preparations were incubated for 37 °C for 60 min. The cells on coverslips were washed in three changes of PBS and then overlaid with fluorochrome-conjugated secondary antibody, placed at 37 °C for an additional 60 min, washed extensively, and then mounted on slides. All preparations were viewed in a Zeiss laser-scanning microscope (LSM) 510 confocal microscope (Zeiss Inc., Thornwood, NY). Microscope images were captured with a cooled charge-coupled device camera (CoolSNAP) mounted on an Axioskop microscope (Zeiss), and figures were generated using Adobe Photoshop 7.0 software. At least 50 cells were examined for each experimental condition.

RESULTS

Comparison of the SDLs of β Integrin Subunits—Fig. 1 shows the alignment of a relatively conserved sequence of amino acids within the extracellular domain of β integrin subunits. This region is located within the I-like domain and contains the so-called SDL, which is proposed to regulate ligand interaction (7). The residues at the amino-flanking region of the SDLs of all of the β integrins share a high level of homology, each being FQXXVX. However, the sequences of the SDLs themselves show more diversity. Interestingly, all of the SDLs of β integrin subunits, with the exception of β1, possess a short region of 5–8 amino acids flanked by cysteine residues, which, at least in β2 integrin, appears to play an important role in regulating ligand binding (22) (Fig. 1).

The divergence of the sequence of the β1 integrin SDL, par-
particulary the absence of cysteine residues, motivated us to assess whether the integrin SDL is involved in the regulation of the integrin binding to its ligand laminin-5 and, if so, to determine which residues are important in this pivotal function for a matrix receptor. To begin to do so, we first prepared a series of expression vectors encoding human GFP-tagged integrin subunit, in which residues within the SDL (residues 150, 155, and 165) or within the I-like extracellular domain of the integrin in which lies outside the SDL region (residue 284), were mutated (Fig. 1). We selected one conserved and two nonconserved residues within the integrin subunit SDL region for our mutational analyses. Each of the polar residues at positions 150, 155, and 165 was replaced by a small, nonpolar amino acid. We mutated residue 125 in the non-SDL region to a charged residue to increase the possibility of mutational effects. Constructs encoding the resulting tagged mutated proteins (GFP-β4K150A, GFP-β4Q155L, GFP-β4E165A, and GFP-β4V284E) as well as a GFP-tagged wild type β4 integrin subunit (GFP-β4WT) were transfected into either rat bladder 804G cells or immortalized TrHBMECs (16, 23). We chose 804G cells for this analysis, since they assemble mature hemidesmosomes in vitro and express all known hemidesmosome structural proteins (23). Thus, using this cell line, we would be able to assess the impact of expression of the mutated integrin subunits on the localization of other hemidesmosome proteins. In addition, an immortalized endothelial cell line was used in our studies, since these cells possess barely detectable levels of integrin subunit but express α6 integrin, partnered with β1 integrin as determined by fluorescence-activated cell sorting (see below) (24). Furthermore, when such endothelial cells are induced to express wild type β1 integrin and are plated subsequently onto laminin-5-rich matrix derived from 804G cells, β1 integrin clusters in the membrane at sites of cell-substratum interaction in a pattern that reflects the organization state of laminin-5 polymer on the substrate (25, 26) (see below). In other words, this endothelial cell model system provides a morphological readout of integrin subunit/ligand binding.

At 48 h following transfection of 804G cells and TrHBMECs with vectors encoding GFP-tagged wild type and mutated integrin subunits, extracts of the transfected cells were prepared for immunoblotting using an antibody against GFP to confirm expression of the appropriately sized protein (Fig. 2). Proteins of ~227 kDa are detected in extracts of the transfected cells using the GFP antibody probe with one exception. The GFP antibody recognizes a 180-kDa polypeptide in extracts of cells expressing GFP-β4WT (lane WT), GFP-β4V284E (lane V284E), and GFP-β4Q155L (lane Q155L), whereas the same probe recognizes a 180-kDa polypeptide in extracts of cells expressing GFP-β4E165A (lane E165A). Note that with the exception of extracts of cells expressing GFP-β4E165A, comparable loadings of protein (20 μg) were loaded onto each lane. Approximately 50 μg of cells expressing GFP-β4E165A was processed for immunoblotting (IB) in order to assess whether full-length tagged protein could be detected.

![Fig. 2](image_url)

**Fig. 2. Western immunoblotting analysis of tagged β4 integrin proteins.** Expression of GFP-tagged wild type and mutated β4 integrin subunits in 804G cells (upper panels) and TrHBMECs (lower panels) was assessed by western immunoblotting of cell extracts using a GFP monoclonal antibody. The GFP antibody fails to recognize any polypeptides in mock-transfected cells (lane mock). In extracts derived from cells expressing GFP-β4WT (lane WT), GFP-β4V284E (lane V284E), GFP-β4K150A (lane K150A), and GFP-β4Q155L (lane Q155L), the GFP antibody recognizes a polypeptide of 227 kDa, whereas the same probe recognizes a 180-kDa polypeptide in extracts of cells expressing GFP-β4E165A (lane E165A). Note that with the exception of extracts of cells expressing GFP-β4E165A, comparable loadings of protein (20 μg) were loaded onto each lane. Approximately 50 μg of cells expressing GFP-β4E165A was processed for western blotting (IB) in order to assess whether full-length tagged protein could be detected.

![Table I](image_url)

**Table I.** Western Immunoblotting Analysis of Tagged β4 Integrin Proteins. Expression of GFP-tagged wild type and mutated β4 integrin subunits in 804G cells and TrHBMECs was assessed as described above. The polypeptides detected by western immunoblotting using a GFP antibody (mock lanes) and the GFP antibody precipitated the polypeptides recognized by the anti-GFP antibodies (WT lanes) are indicated. The polypeptide detected by the anti-GFP antibodies (lanes E165A) is comparable to the polypeptide detected by the GFP antibody (lanes WT), whereas with the exception of the K150A and Q155L mutants, the polypeptides detected by western immunoblotting using the GFP antibody (lanes WT) are not detected by western immunoblotting using the anti-GFP antibodies (lanes E165A). Note that with the exception of extracts of cells expressing GFP-β4E165A, comparable loadings of protein (20 μg) were loaded onto each lane. Approximately 50 μg of cells expressing GFP-β4E165A was processed for western blotting (IB) in order to assess whether full-length tagged protein could be detected.

![Fig. 3](image_url)

**Fig. 3.** Comparison of the sequences within and surrounding the SDLs of β4 integrin subunits. The putative sequence of each of the SDLs is demarcated by the brackets. The SDL of the β4 integrin subunit spans residues 150–174. Note that, with the exception of the β4/6 integrin subunit, cysteine residues border a series of between 5 and 8 residues (underlined) within each SDL. In our analyses, residues 150, 155, and 165 of the SDL of the β4 integrin subunit were mutated (indicated by the hatched boxes).

![Fig. 4](image_url)

**Fig. 4.** Comparison of the sequences within and surrounding the SDLs of β4 integrin subunits. The putative sequence of each of the SDLs is demarcated by the brackets. The SDL of the β4 integrin subunit spans residues 150–174. Note that, with the exception of the β4/6 integrin subunit, cysteine residues border a series of between 5 and 8 residues (underlined) within each SDL. In our analyses, residues 150, 155, and 165 of the SDL of the β4 integrin subunit were mutated (indicated by the hatched boxes).
sults from a representative trial). We scored the GFP signal in transfected cells as being in an extensive cat paw pattern (greater than 50% of the basal cell surface exhibits cat paw arrays), in a partial cat paw pattern (less than 50% of the basal cell surface exhibits cat paw arrays), or diffuse/spotty.

Next, populations of 804G cells expressing GFP-β4WT protein and cells expressing the various mutated integrins were analyzed by immunofluorescence microscopy to assess the impact of the protein products of the transgene on the organization of the endogenous cytoplasmic, transmembrane, and matrix proteins of hemidesmosomes. Specifically transfected cell populations were probed with antibodies against BP230, plectin, BP180, and the α6 laminin subunit of laminin-5. GFP-β4WT and GFP-β4V284E proteins distribute precisely with BP230, plectin, and BP180 in extensive cat paw arrays along the substratum attached surface in all transfected 804G cells we have analyzed (Fig. 5, A, B, and C and E, F, and G, respectively). GFP-β4WT and GFP-β4V284E protein also co-localize

### Table 1

Fluorescence-activated cell sorting analysis of 804G and TrHBMEC cells

The median fluorescence intensity for both GFP and Cy5 derived from the data in Fig. 3 is represented numerically. The values were gated on light scatter to include intact, homogenously distributed cells (excluding debris and cells with altered scatter) and then gated on the fluorescence value based on the mock-transfected, negative controls.

|          | 804G Left box | 804G Right box | TrHBMEC Left box | TrHBMEC Right box |
|----------|---------------|----------------|------------------|-------------------|
| Mock     | Cy5 0.2       | Cy5 0           | Cy5 1.7          | Cy5 4.7           |
| GFP      | 0.5           | 0.5            | 0.8              | 5.6               |
| GFP-β4WT | Cy5 0.6       | Cy5 37.7        | Cy6 2.7          | 13.3              |
| GFP-β4V284E | Cy5 0.5   | Cy5 27.8        | Cy6 2.3          | 8.7               |
| GFP-β4Q155L | Cy5 1.4     | GFP 23.8        | GFP 0.8          | 8                  |
| GFP-β4K150A | Cy5 0.9       | Cy5 20.8        | Cy6 2.3          | 11.1              |
| GFP-β4V284E | Cy5 1.5     | GFP 17.3        | GFP 0.8          | 8.9               |
| GFP-β4K150A | GFP 0.7       | GFP 3.7         | GFP 0.7          | 8                  |
with the α3 laminin subunit in the extracellular matrix secreted by 804G cells (Fig. 5, D and H). In sharp contrast, in 804G cells expressing GFP-β4K150A or GFP-β4Q155L, the GFP-tagged proteins show minimal co-localization with other hemidesmosomal proteins, and, in the majority of the same cells, the organization of BP230, plectin, and BP180 is also perturbed (Fig. 5, I, J, and K), only results where 804G cells are expressing GFP-β4Q155L are shown (Tables II and III). Although the degree of perturbation differs from cell to cell, in the majority of the transfected 804G cells, BP230, BP180, and plectin appear diffusely distributed or in a spotlike pattern (Fig. 5, I, J, and K). In all instances, expression of the mutated tagged β4 integrin subunit had little, if any, impact on the organization of laminin-5 in the matrix of the transfected cells (Fig. 5L). At least 50 cells were counted for each condition in each trial, and these immunofluorescence observations are quantified in Table III. We assessed whether the endogenous plaque and transmembrane hemidesmosome proteins in cells expressing the tagged β4 proteins organize into an extensive cat paw array or a partial cat paw array or appear diffusely/spotily distributed as above (Table III). These results indicate that both GFP-β4K150A and GFP-β4Q155L have a dominant negative impact on the ability of 804G cells to organize clusters of plaque and transmembrane hemidesmosome proteins.

**Table II**

Localization of tagged β4 integrin proteins in transfected 804G cells

| Protein            | Percentage distribution |
|--------------------|-------------------------|
| GFP-β4WT           |                         |
| Extensive cat paw  | 95                      |
| Partial cat paw    | 5                       |
| Diffuse/spotty     | 0                       |
| GFP-β4V284E        |                         |
| Extensive cat paw  | 89                      |
| Partial cat paw    | 11                      |
| Diffuse/spotty     | 0                       |
| GFP-β4K150A        |                         |
| Extensive cat paw  | 14                      |
| Partial cat paw    | 28                      |
| Diffuse/spotty     | 58                      |
| GFP-β4Q155L        |                         |
| Extensive cat paw  | 6                       |
| Partial cat paw    | 43                      |
| Diffuse/spotty     | 51                      |

**Table III**

Localization of endogenous hemidesmosomal protein in transfected 804G cells

| Protein | GFP-β4WT | GFP-β4V284E | GFP-β4K150A | GFP-β4Q155L |
|---------|----------|-------------|-------------|-------------|
| BP230   |          |             |             |             |
| Extensive cat paw | 85       | 89          | 8           | 4           |
| Partial cat paw    | 15       | 11          | 21          | 32          |
| Diffuse/spotty     | 0        | 0           | 71          | 64          |
| Plectin           |          |             |             |             |
| Extensive cat paw  | 92       | 81          | 2           | 4           |
| Partial cat paw    | 8        | 19          | 38          | 38          |
| Diffuse/spotty     | 0        | 0           | 60          | 60          |
| BP180             |          |             |             |             |
| Extensive cat paw  | 94       | 73          | 21          | 2           |
| Partial cat paw    | 6        | 27          | 31          | 44          |
| Diffuse/spotty     | 0        | 0           | 48          | 54          |

the organization of the laminin-5 on the substrate immediately underlying the transfected cell (18, 25) (Fig. 6, A–C). We used this *in vivo* morphological assay system to assess whether our
of this region to ligand is regulated by a series of amino acids, namely, the SDL, within the extracellular domain of the integrin subunits we analyzed within 15 min of plating the transfected cells onto a laminin-5-rich matrix of 804G cells. At 0 and 60 min after plating, extracts of the cells were made and then processed for immunoprecipitation (IP) using GFP antibodies. The precipitated proteins were then subjected to immunoblotting (IB) using antibodies against Shc and GFP (Aii) or antibodies against phosphotyrosine and GFP (Aiii). The GFP blots were performed to confirm successful precipitation of tagged β4 integrin protein. In B, mock-transfected endothelial cells or endothelial cells expressing wild type, and the mutated β4 integrin subunits were plated for 15 min onto β1 antibody (3E1)-coated surfaces. Extracts of the cells were then subjected to immunoprecipitation (IP) using antibodies against GFP. The precipitates were then prepared for immunoblotting (IB) using antibodies against Shc and GFP (Bii) and antibodies against phosphotyrosine and GFP (Biii, as indicated).

We also assayed the ability of wild type and mutated β4 integrin to recruit Shc when α5β1 integrin was clustered with the β4 integrin monoclonal antibody 3E1. The 66-kDa isoform of Shc is clearly immunoprecipitated from extracts of endothelial cells expressing wild type and all of the mutated β4 integrin subunits we analyzed within 15 min of plating the transfected cells onto a 3E1 antibody-coated surface (Fig. 7B). In addition, the β4 integrin precipitates derived from such cells contain a 105-kDa tyrosine phosphorylated protein (Fig. 7B). Together these results indicate that the point mutations have no secondary, inhibitory effect on the ability of the cytoplasmic domain of β4 integrin subunits to bind Shc protein and mediate signaling.

**DISCUSSION**

Recent structural analyses of integrin heterodimers, primarily of the α5β1 integrin, have provided new clues to the structural basis and regulation of integrin-ligand binding. For example, the N-terminal halves of both the α and β integrin subunits comprise the region that binds ligand (2, 31). Binding of this region to ligand is regulated by a series of amino acids, termed the SDL, within the extracellular domain of the β integrin subunit (2, 31–33). In particular, Takagi et al. have reported that a motif, composed of CYDMKHTC, within the
SDL of the $\beta_4$ integrin subunit sequence is critically involved in specifying ligand binding of integrin $\alpha_4\beta_4$ (8). Moreover, a series of eight residues (CPNKEKEC) within the SDL region of the $\beta_4$ integrin subunit regulates binding of the $\alpha_6\beta_4$ heterodimer to its ligand (22).

In this work, we studied the functions of the I-like region in the $\beta_4$ integrin SDL. To do so, we generated point mutations in the SDL and in the $\alpha_6$ extracellular domain of the $\beta_4$ integrin subunit. Wild type and mutated $\beta_4$ integrin subunits were then expressed in 804G cells or an immortalized endothelial cell type. In both cell types, wild type and mutated $\beta_4$ integrin is expressed at the cell surface and partners with $\alpha_6$ integrin. In addition, point mutations within the $\beta_4$ subunit SDL have no impact on $\alpha_6\beta_4$ integrin heterodimer assembly. This is consistent with previous work showing that the SDLs of $\beta_4$, $\beta_1$, and $\beta_3$ integrin are not required for formation of $\alpha_6\beta_4$, $\alpha_6\beta_1$, $\alpha_4\beta_1$, and $\alpha_6\beta_3$ integrin complexes (7). In contrast, the SDLs of the $\beta_1$ and $\beta_2$ integrin subunits do appear to play an essential role in heterodimer formation with their $\alpha$ integrin partners (namely $\alpha_6\beta_2$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$) (7).

We used endothelial cells to assess the ability of the $\beta_4$ integrin subunit-containing integrin complexes to interact with laminin-5 ligand. We did so by plating endothelial cells expressing wild type or mutated $\beta_4$ integrins onto the laminin-5-rich matrix secreted and organized by 804G cells into exquisite arrays on their substrate. Our analyses show that wild type $\beta_4$ integrin protein and $\beta_4$ integrin protein possessing a point mutation in a non-SDL region of the extracellular domain cluster in the endothelial cells in patterns that mirror the organized arrays of 804G cell laminin-5 to which they adhere. In sharp contrast, $\beta_1$ integrins that possess point mutations in their SDLs do not cluster and fail to organize in patterns that mimic the organization of laminin-5 ligand in the extracellular matrix. In other words, the SDL of $\beta_4$ integrin regulates $\alpha_6\beta_4$ integrin/laminin-5 interaction.

A number of mutations in the $\beta_4$ integrin extracellular domain have been linked to the pathogenesis of the blistering skin disease junctional epidermolysis bullosa. Most if not all of these mutations result in lack of expression of the protein. Interestingly, no mutations in the SDL have been detailed. We suggest that expression of a $\beta_4$ subunit with mutations in its SDL may result in a more devastating phenotype when than no $\beta_4$ protein is produced at all. We provide some support for this, since we show that $\beta_4$ possessing mutations within its SDL act as dominant negatives in 804G cells.

Previous studies have shown that a chimeric $\beta_4$ integrin, whose extracellular domain has been replaced by the extracellular domain of the IL-2 receptor (i.e. a “headless” $\beta_4$ integrin), is able to cluster along sites of cell-substrate interaction and recruits plectin to such sites in a ligand-independent manner (34). Headless $\beta_4$ integrin also incorporates into formed hemidesmosome-like protein arrays in 804G cells and, unlike our mutated $\beta_4$ integrin, does not disrupt existing hemidesmosome-like structures (27, 34, 35). These and other results have led to a proposal that hemidesmosome assembly is driven primarily from within the cell (34, 35). These ideas, however, are not supported by the results we present here. Mutated $\beta_4$ integrin, which is incapable of binding ligand because of mutations in its SDL, destabilizes hemidesmosome protein arrays or prevents their assembly in 804G cells. Indeed, mutated $\beta_4$ integrin that does not interact with ligand acts as a “dominant negative” in transfected 804G cells and is not recruited into hemidesmosome-like protein arrays in either 804G cells or endothelial cells. How do we rationalize our results with those of Nievers et al. (34, 35)? We concur with the suggestion that headless $\beta_4$ integrin, which binds neither ligand nor $\alpha_6$ integrin, is either “activated” via the action of intracellular regulators or is constitutively active and hence can both cluster and transduce signals when expressed as a chimeric or truncated protein at the cell surface (34–37). The data we present here, however, support the notion that “activation” of full-length wild type $\beta_4$ integrin by ligand is a necessary prerequisite to its incorporation into assembled and/or assembling hemidesmosome-like protein arrays, since mutated $\beta_4$ integrin (which fails to interact with ligand) does not cluster in endothelial cells. The latter provides support for the notion that ligand interaction is dominant in $\beta_4$ integrin activation, since intracellular regulators clearly fail to mediate clustering and “activation” of mutated $\beta_4$ integrin. That the cytoplasmic tail of the mutated $\beta_4$ integrin is in an “inactive” state of conformation is supported by those studies where we analyzed the ability of wild type and mutated $\beta_4$ integrin to mediate outside-in signaling. Mutated $\beta_4$ integrin only recruits the adaptor protein Shc to its cytoplasmic tail and mediates signaling that results in tyrosine phosphorylation of a 105-kDa protein when it is clustered by human $\beta_4$ integrin antibodies. Indeed, taken together, our results suggest that $\beta_4$ interaction with ligand on the outside of the cells has a dramatic impact on the binding properties of its cytoplasmic tail and is essential for outside-in signaling.

Since we also show that mutated $\beta_4$ integrin binds rat $\alpha_6$ integrin, we suggest that mutated, “inactive” $\beta_4$ integrin partially or completely destabilizes hemidesmosome protein arrays in 804G cells by competing with endogenous wild type $\beta_4$ integrin for its $\alpha_6$ partner. The degree of disruption induced by the mutated protein appears to reflect the amount of the protein product of the transgene expressed in the cells (not shown). In summary, our results support the idea that signals emanating from matrix on the outside of a cell predominate over cytoplasmic “cues” with regard to induction of hemidesmosome protein array assembly. This is consistent with the conclusions of some of our previous studies (18, 19, 38).

It is becoming clear that the diverse SDLs of $\beta$ integrin subunits possess an essential role in regulating integrin function. The $\beta_4$ integrin subunit is no exception, since our findings emphasize the importance of its SDL in specifying ligand interaction, integrin clustering, and downstream signaling. Our data also imply that the activation of the cytoplasmic domain of the $\beta_4$ integrin is tightly controlled by its interaction with ligand. This phenomenon is all the more remarkable because of the size of the $\beta_4$ cytoplasmic tail, which is over 1000 amino acids. How signals emanating in the matrix influence the activation/conformation of this tail remains unclear, but we have provided the first evidence that the SDL is involved in regulating such signaling events.

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\(\beta_4\) Integrin-Ligand Interaction

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