Regulation of Cellular Interactions with Laminin by Integrin Cytoplasmic Domains: The A and B Structural Variants of the α6β1 Integrin Differentially Modulate the Adhesive Strength, Morphology, and Migration of Macrophages

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Submitted February 22, 1994; Accepted May 5, 1994
Monitoring Editor: Erkki Ruoslahti

Several integrin α subunits have structural variants that are identical in their extracellular and transmembrane domains but that differ in their cytoplasmic domains. The functional significance of these variants, however, is unknown. In the present study, we examined the possibility that the A and B variants of the α6β1 integrin laminin receptor differ in function. For this purpose, we expressed the α6A and α6B cDNAs, as well as a truncated α6 cDNA (α6-ΔCYT) in which the cytoplasmic domain sequence was deleted after the GFFKR pentapeptide, in P388D1 cells, an α6 deficient macrophage cell line. Populations of stable α6A, α6B, and α6-ΔCYT transfectants that expressed equivalent levels of cell surface α6 were obtained by fluorescence-activated cell sorter and shown to form hetero-dimers with endogenous β1 subunits. Upon attachment to laminin, the α6A transfectants extended numerous pseudopodia. In contrast, the α6B transfectants remained rounded and extended few processes. The transfectants were also examined for their ability to migrate toward a laminin substratum using Transwell chambers. The α6A transfectants were three- to fourfold more migratory than the α6B transfectants. The α6-ΔCYT transfectants did not attach to laminin in normal culture medium, but they did attach in the presence of Mn2+. The α6-ΔCYT transfectants migrated to a lesser extent than either the α6A or α6B transfectants in the presence of Mn2+. The α6 transfectants differed significantly in the concentration of substratum bound laminin required for half-maximal adhesion in the presence of Mn2+: α6A (2.1 μg/ml), α6B (6.3 μg/ml), and α6-ΔCYT (8.8 μg/ml). Divalent cation titration studies revealed that these transfectants also differed significantly in both the [Ca2+] and [Mn2+] required to obtain half-maximal adhesion to laminin. These data demonstrate that the A and B variants of the α6 cytoplasmic domain can differentially modulate the function of the α6β1 extracellular domain.

INTRODUCTION

The role of integrin adhesion receptors as conduits for signaling information in and out of cells has been established in recent years (for review see Ginsberg et al., 1992; Hynes, 1992; Sastry and Horwitz, 1993; Schwartz, 1993). Although the mechanisms of integrin signaling are poorly understood, one insightful set of findings is that integrin cytoplasmic domains play important regulatory roles in both inside-out and outside-in signaling pathways (reviewed in Sastry and Horwitz, 1993). This conclusion has been derived largely from mutational analyses of integrin cytoplasmic domains. Such studies have shown that the β1 integrin cytoplasmic domain provides a critical linkage with the cytoskeleton that is essential for adhesion (Solowska et al., 1989; Hayashi et al., 1990; Marcantonio et al., 1990; Reszka et al., 1992). Similar studies have indicated that integrin α subunits

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influence both receptor function and subsequent post-ligand binding events such as focal contact formation, migration, and gel contraction (Hibbs et al., 1991; O'Toole et al., 1991; Bauer et al., 1993; Briesewitz et al., 1993; Kassner and Hemler, 1993; Kawaguchi and Hemler, 1993; Shaw and Mercurio, 1993; Ylanne et al., 1993). One key problem that is highlighted by these observations is how information in integrin cytoplasmic domains is transmitted to both integrin extracellular domains and to signaling molecules inside the cell.

Functional analyses of integrin cytoplasmic domains must consider the finding that many integrin subunits have structural variants that are identical in their extracellular and transmembrane domains but that differ in their cytoplasmic domains. This group includes the β1 (Altamura et al., 1990; Languino and Ruoslahti, 1992), β3 (van Kuppevelt et al., 1989), β4 (Hogervorst et al., 1990; Suzuki and Naitoh, 1990; Tamura et al., 1990; Clarke and Mercurio, 1993), α2 (Tamura et al., 1991), α6 (Hogervorst et al., 1991; Tamura et al., 1991), and α7 (Collo et al., 1993) subunits. At least two structural variants exist for each of these integrin subunits. To date, most studies on integrin cytoplasmic domain variants have not focused directly on function but have examined such things as their relative patterns of expression. For example, the A and B variants of both the α6 and α7 integrin subunits exhibit developmentally regulated expression patterns (Cooper et al., 1991; Collo et al., 1993; Ziober et al., 1993). The central issue that now needs to be addressed is whether cytoplasmic domain variants of the same integrin exhibit differences in function.

We have focused our efforts on the function and regulation of the α6a and α6B cytoplasmic domain variants. This work has involved the use of macrophages because adhesion to laminin is mediated by the α6β1 integrin in these cells and this adhesion is markedly increased in response to inside-out signals (Mercurio and Shaw, 1988; Shaw et al., 1990). Expression of the α6A and α6B cDNAs in an α6-deficient macrophage cell line, P388D1, enabled us to determine that both the α6Aβ1 and α6Bβ1 integrin variants can be activated by inside-out signaling pathways (Shaw et al., 1993). A truncated α6 cDNA, α6-ΔCYT, was constructed in which the human cytoplasmic domain sequence was deleted after the GFFKR pentapeptide. Expression of this cDNA in P388D1 cells resulted in the surface expression of a chimeric α6-ΔCYT/β1 integrin that was unable to mediate laminin adhesion or increase this adhesion in response to phorbol 12-myristate 13-acetate (PMA) (Shaw and Mercurio, 1993). The α6A-ΔCYT transfectants adhered to laminin, however, when extracellular Ca2+/Mg2+ was replaced with Mn2+. A logical question that emerged from these studies is whether the α6Aβ1 and α6Bβ1 variants, as well as the α6-ΔCYT/β1 mutant, exhibit differences in their ability to initiate specific cellular functions subsequent to laminin attachment.

In the present study, we observed that P388D1 cells transfected with either the human α6A, α6B, or α6-ΔCYT integrin cDNAs differed markedly in their morphology on laminin and in their ability to migrate toward a laminin gradient. Subsequent analysis of these α6 transfectants using a combination of laminin and divalent cation titrations revealed that they differed in their relative adhesive strength for laminin. Specifically, we found the following order of relative adhesive strengths: α6Aβ1 > α6Bβ1 > α6ΔCYTβ1. These data suggest that specific sequences within the α6A and α6B cytoplasmic domains differentially modulate the functional activity of the α6β1 extracellular domain.

**MATERIALS AND METHODS**

**Cells**

The P388D1 mouse macrophage cell line was obtained from the American Type Tissue Collection (Rockville, MD). Cells were maintained in RPMI containing 25 mM N-2-hydroxyethylpiperezine-N'-2-ethanesulfonic acid (HEPES) buffer (RPMI-H) and 15% certified fetal bovine serum (GIBCO, Grand Island, NY).

**cDNA Transfections**

The human α6A and α6B cDNAs were cloned by polymerase chain reaction and subcloned into the eukaryotic expression vector pRC/CMV as described previously (Shaw et al., 1993). The α6-ΔCYT mutant cDNA was constructed as described previously (Shaw and Mercurio, 1993). The pRC/CMV vectors containing either the α6A, α6B, or α6-ΔCYT cDNAs, as well as the vector alone, were transfected into the P388D1 cell line with Lipofectin (GIBCO). Neomycin-resistant cells were isolated by selective growth in medium containing G418 (0.4 mg/ml) (GIBCO). The stable transfectants were pooled and populations of cells that expressed the human α6 subunits on the cell surface were isolated by fluorescence-activated cell sorter (FACS). A human α6 integrin specific monoclonal antibody (mAb), 2B7, was used for this sorting and for subsequent analysis of the transfectants (Shaw et al., 1993).

**Flow Cytometry**

Transfected P388D1 cells were washed twice with phosphate-buffered saline containing 0.1% bovine serum albumin (PBS/BSA). Aliquots of cells (3 x 10^6) were incubated for 30 min at 4°C with PBS/BSA containing murine IgG Fc fragment (6 μg/ml) (Jackson Immunoresearch, Avondale, PA). The mAb 2B7 was added at a concentration of 2 μg/ml, and the cells were incubated for an additional hour at 4°C. The cells were washed three times with PBS/BSA and then incubated with goat F(ab')2 anti-mouse IgG coupled to fluorescein (Tago, Burlingame, CA) for 1 h at 4°C. After washing three times with PBS/BSA, the cells were resuspended in PBS and analyzed using a FACScan (Becton Dickinson, Lincoln Park, NJ).

**Adhesion Assays**

Adhesion assays were performed as described previously (Shaw and Mercurio, 1993). Briefly, multilayer tissue culture plates (11.3 mm diameter) were coated overnight at 4°C with 0.2 ml of PBS containing murine Englebreth-Holm-Swarm (EHS) laminin at the concentrations indicated in the individual figure legends. Laminin was purified from...
the EHS sarcoma as described (Kleinman et al., 1982). The wells were then washed with PBS and 105 cells in Puck’s Saline A (200 μl) (Sigma, St. Louis, MO) containing 25 mM HEPES, and divalent cations were added to each well. For the laminin titration adhesion assays, 96-well nontissue culture-treated plates (Falcon, Lincoln Park, NJ) were used, and the wells were counter-coated for 3 h at room temperature with Puck’s Saline A containing 1% BSA. BSA was also included in the incubation buffer during the laminin titration adhesion assays. After a 45-min incubation at 37°C, the wells were washed three times with Puck’s Saline A at 37°C, fixed for 15 min with methanol, and stained with a 0.2% solution of crystal violet in 2% ethanol. The crystal violet stain was solubilized with a 1% solution of sodium dodecyl sulfate, and adhesion was quantitated by measuring the absorbance at 600 nm. For photomicrographs, the adherent cells were fixed in RPMI-H containing 3.7% formaldehyde for 15 min, permeabilized with methanol for 10 min, and then stained with crystal violet.

Migration Assays
Cell migration assays were performed using 6.5-mm Transwell chambers (8 μm pore size) (CoStar, Cambridge, MA). RPMI-H containing 15 μg/ml laminin (0.6 ml) or Puck’s Saline A containing 25 mM HEPES, 0.5 mM MnCl₂, 0.1 mM CaCl₂, 1 mM L-Glutamine, and 15 μg/ml laminin were added to the bottom well, and the filters were coated for ~30 min at 37°C. Cells were resuspended in the appropriate buffer at a concentration of 10⁶/ml, and 10⁵ cells were added to the top well of the Transwell chambers. After a 24-h incubation, the cells that had not migrated were removed from the upper face of the filters using cotton swabs, and the cells that had migrated to the lower surface of the filters were fixed in methanol and then stained with a 0.2% solution of crystal violet in 2% ethanol. Migration was quantitated by counting using brightfield optics with a Nikon Diaphot microscope (Garden City, NY) equipped with a 16-square reticle. The surface area of this grid was determined to be 1 mm². Five separate fields were counted for each filter.

To examine the Ca²⁺ requirements for cell migration, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) was added to the RPMI-H at a concentration of 0.5 mM and the MgCl₂ concentration was increased to 2 mM. 2B7 was included in some assays at a concentration of 8 μg/ml to examine inhibition of migration.

RESULTS
In the studies described in this report, P388D₁ cells were used that had been transfected with either the human α6A, α6B, or mutant α6-ΔCYT integrin cDNAs. Populations of cells that expressed equivalent levels of cell surface α6 were obtained by FACS using 2B7, a mAb specific for the human α6 integrin subunit (Figure 1). The levels of α6 expression on the cell surface of the transfectants were monitored by FACS analysis frequently, and only populations that expressed equivalent levels were used for comparative experiments. The α6A, α6B, and α6-ΔCYT subunits formed heterodimers with endogenous β1 subunits (Shaw and Mercurio, 1993).

The α6A and α6B Transfectants Differ in Their Morphology on Laminin
Both the α6A and α6B cDNAs are capable of restoring the ability of P388D₁ cells to adhere to a laminin substratum (Shaw et al., 1993). In the present study, however, we observed that these two populations of transfectants differed markedly in their morphology after attachment to a laminin substratum (Figure 2). Specifically, 68% of the α6A transfectants extended pseudopodia compared to 20% of the α6B transfectants. In addition, the pseudopodia extended by the α6A transfectants were considerably longer than those extended by the α6B transfectants. The α6B transfectants exhibited a more rounded appearance than the α6A transfectants. This difference in morphology is specific to laminin because these transfectants exhibited a similar morphology when attached to a fibronectin substratum (Figure 2).

The α6A and α6B Transfectants Differ in Their Migration Toward Laminin
Extensive pseudopod formation is characteristic of motile cells (Trinkaus, 1984). For this reason, the α6A and α6B transfectants were examined for their ability to migrate toward a laminin substratum using Transwell
chambers. These migration assays were performed in the same medium, RPMI-H, that had been used to examine their adhesion and morphology. The results obtained indicated that the a6A and a6B transfectants differed significantly (p < 0.01) in their ability to migrate toward a laminin gradient. As shown in Figure 3A, the a6A transfectants were three- to fourfold more migratory toward laminin than the a6B transfectants in a 24-h assay.

PMA did not increase the number of either the a6A or a6B transfectants that migrated toward laminin (Figure 3A). In fact, a slight decrease in the number of cells that migrated was often observed with PMA. This finding is in contrast to the marked increase in adhesion of these transfectants to laminin in response to PMA stimulation that we previously reported (Shaw et al., 1993). The migration of both the a6A and a6B transfectants is a6β1 dependent because it was completely inhibited by 2B7 under all of the conditions examined (Figure 3A). P388D1 cells that were transfected with the a6 cytoplasmic deletion mutant, a6-ΔCYT, did not migrate toward laminin under these conditions. This observation is consistent with our previous finding that the a6-ΔCYT transfectants did not adhere to laminin in RPMI-H (Shaw and Mercurio, 1993). P388D1 cells transfected with the pRc/CMV vector alone also did not migrate (Figure 3A).

The difference in migration that was observed for the transfectants was specific for laminin because the a6A, a6B, and a6-ΔCYT transfectants migrated to the same extent when fetal calf serum (15%) was included in the bottom well of the Transwell chamber (Figure 3B). It is interesting to note that these cells did not migrate toward fibronectin or collagen I in this assay. Presumably, the migration in serum reflects their ability to migrate on vitronectin.

The a6 Transfectants Differ in Their Relative Binding Strengths for Laminin

One hypothesis that can be formulated to explain the data presented in the preceding sections is that the a6A and a6B transfectants differ in their relative adhesive strength for laminin. Such differences in adhesive strength can be detected by measuring the attachment of the transfectants as a function of increasing laminin concentration. Recent quantitative studies have demonstrated that the slope of the relationship between the strength of cell substratum attachment and the concentration of matrix protein increases with both increasing adhesion receptor number and affinity between adhesion receptor and matrix protein (Cozen-Roberts et al., 1990; DiMilla et al., 1993). In our previous adhesion assays, we had used saturating laminin concentrations (20 µg/ml) to coat the microwell plates, and the possibility existed that differences in binding may not have been apparent at this concentration.

To determine if the a6 transfectants differed in their relative adhesive strength for laminin, adhesion assays were performed using a range of laminin substratum concentrations. The assays were carried out using conditions that promote maximal adhesion (i.e., 0.5 mM Mn2+) to discount the possibility that any observed differences could be attributed to differences in the response of these transfectants to inside-out signals. In addition, because the a6-ΔCYT transfectants can adhere to laminin in the presence of this cation, they could be examined in this assay as well (Shaw and Mercurio, 1993). Populations of a6 transfectants were used that expressed equivalent levels of a6 on the cell surface (Figure 1) to exclude the possibility that any differences in adhesion could be attributed to differences in receptor number. At the highest concentration of laminin used (20 µg/ml), all three of the transfectant populations adhered at equivalent levels. However, the transfectants differed in the concentration of laminin that was required for half-maximal adhesion (Figure 4A and Table 1). The a6A transfectants exhibited the strongest adhesion with half-maximal adhesion occurring at a concentration of ~2.1 µg/ml of laminin. The a6B transfectants required 6.3 µg/ml of laminin for half-maximal adhesion, and the a6-ΔCYT transfectants exhibited the weakest adhesion with a half-maximal requirement of 8.8 µg/ml of laminin. The differences in the concentration of laminin required for half-maximal adhesion of the a6A and a6B transfectants are significant (p < 0.05) (Table 1). These data indicate that the a6 transfectants differ in their relative adhesive strength for laminin.

In response to PMA activation, the relative adhesive strength of the a6A transfectants increased only slightly
in methanol. Vol. The cells that had not migrated were removed, and the cells that had migrated onto the lower surface of the filters were fixed in methanol and then stained. Antibodies specific for α6 (8 μg/ml) were included in the assays as indicated (±2B7). Migration was quantitated by counting. The data shown are the mean values (±SD) of two separate experiments done in duplicate. (B) Migration of α6A-P388D1, α6B-P388D1, and α6ΔCYT-P388D1 cells toward fetal calf serum. RPMI-H containing serum (15%) was added to the bottom well of a Transwell chamber, and assays were performed as described for laminin. The data shown are the mean values (±SEM) of one experiment done in duplicate. 2B7, α6-specific mAb; Neo, P388D1 cells transfected with the expression vector alone; control transfectants; PMA-stimulated transfectants. 

Figure 3. (A) Migration of α6A-P388D1, α6B-P388D1, and α6ΔCYT-P388D1 cells toward a laminin substrate. RPMI-H containing laminin (15 μg/ml) was added to the bottom well of a Transwell chamber (8 μM pore filters), and 10^6 cells were added to the top well. After a 24-h incubation, the cells that had not migrated were removed, and the cells that had migrated onto the lower surface of the filters were fixed in methanol and then stained. Antibodies specific for α6 (8 μg/ml) were included in the assays as indicated (±2B7). Migration was quantitated by counting. The data shown are the mean values (±SD) of two separate experiments done in duplicate. (B) Migration of α6A-P388D1, α6B-P388D1, and α6ΔCYT-P388D1 cells toward fetal calf serum. RPMI-H containing serum (15%) was added to the bottom well of a Transwell chamber, and assays were performed as described for laminin. The data shown are the mean values (±SEM) of one experiment done in duplicate. 2B7, α6-specific mAb; Neo, P388D1 cells transfected with the expression vector alone; control transfectants; PMA-stimulated transfectants.

Figure 4. Laminin titration adhesion assays. (A) α6A-P388D1, α6B-P388D1, and α6ΔCYT-P388D1 transfectedants were assayed for their ability to adhere to a range of laminin substrate concentrations. Tissue culture wells were coated with EHS laminin (1–20 μg/ml) overnight and counter-coated with 1% BSA for several hours. Transfected cells (5 × 10^4) were resuspended in Puck’s Saline A containing 1% BSA and 0.5 mM Mn^2+ and added to the protein-coated wells. After 45 min at 37°C, nonadherent cells were removed by washing, and adherent cells were fixed, stained, and quantitated as described in MATERIALS AND METHODS. (B) The adhesion assays were performed in the presence of PMA (50 ng/ml). The data shown are the mean values (±SD) from two experiments done in triplicate.
EGTA did not affect the viability of the cells (unpublished observation). The results obtained indicate that Ca$^{2+}$ is not required for adhesion of either the $\alpha$6A or $\alpha$6B transfectants to a laminin substratum (Figure 5A). In fact, chelation of Ca$^{2+}$ enhances the constitutive adhesion of both the $\alpha$6A and $\alpha$6B transfectants. This finding suggests that Ca$^{2+}$ is a negative regulator of $\alpha$6$\beta$1 function, a conclusion we deduced previously for peritoneal macrophages (Shaw and Mercurio, 1993). In contrast to their adhesion, however, no migration toward laminin was observed for either the $\alpha$6A or $\alpha$6B transfectants when extracellular Ca$^{2+}$ was chelated (Figure 5B). Similar to the data shown in Figure 3, a three- to fourfold difference in the migration of the $\alpha$6A and $\alpha$6B transfectants was evident in the presence of Ca$^{2+}$ (Figure 5B).

The $\alpha$6 Transfectants Differ in Their Cation Sensitivity for Adhesion to Laminin

Extracellular Mn$^{2+}$ (150 $\mu$M) markedly increases the ability of the $\alpha$6A-, $\alpha$6B-, and $\alpha$6-$\Delta$CYT P388D1 transfectants to adhere to laminin (Shaw and Mercurio, 1993). Presumably, the interaction of Mn$^{2+}$ with divalent cation binding sites in the $\alpha$6 extracellular domain increases the affinity of the $\alpha$6$\beta$1 integrin for laminin (Sonnenberg et al., 1988; Shaw and Mercurio, 1991). In contrast, extracellular Ca$^{2+}$ negatively regulates the ability of these $\alpha$6 transfectants to bind laminin (Figure 5). Based on these observations, we reasoned that adhesion of the transfectants to laminin at a fixed Mn$^{2+}$ concentration could be inhibited by increasing concentrations of Ca$^{2+}$. Moreover, differences in the relative affinities of the $\alpha$6 transfectants for Ca$^{2+}$ may be apparent in such an assay. As shown in Figure 6A, the $\alpha$6A, $\alpha$6B, and $\alpha$6-$\Delta$CYT transfectants exhibited the same amount of adhesion in the presence of 0.5 mM Mn$^{2+}$ and the absence of Ca$^{2+}$. However, these transfectants did differ in the [Ca$^{2+}$] required to obtain half-maximal adhesion in the presence of 0.5 mM Mn$^{2+}$. Specifically, the $\alpha$6A transfectants exhibited half-maximal adhesion at a [Ca$^{2+}$] of 5.5 mM, the $\alpha$6B transfectants at a [Ca$^{2+}$] of 1.8 mM, and the $\alpha$6-$\Delta$CYT transfectants at a [Ca$^{2+}$] of 0.8 mM (Figure 6B). The difference in the [Ca$^{2+}$] required for half-maximal adhesion of the $\alpha$6A and $\alpha$6B transfectants is significant ($p < 0.05$) (Table 1). In the presence of PMA, the $\alpha$6A and $\alpha$6B transfectants were both resistant to Ca$^{2+}$ titration at the concentrations examined. However, although PMA increased the resistance of the $\alpha$6-$\Delta$CYT transfectants to Ca$^{2+}$ inhibition, they still exhibited sensitivity to Ca$^{2+}$ under the conditions examined (Figure 6C).

In addition to the Ca$^{2+}$ titration experiment, we also performed a Mn$^{2+}$ titration to determine the concentration of Mn$^{2+}$ required for half-maximal adhesion of each of the transfectants. All three transfectant populations adhered to laminin to the same extent at the maximal [Mn$^{2+}$] examined, 0.5 mM Mn$^{2+}$ (Figure 6A). Similar to the Ca$^{2+}$ titration data, the $\alpha$6A, $\alpha$6B, and $\alpha$6-$\Delta$CYT transfectants differed in the [Mn$^{2+}$] required to obtain half-maximal adhesion. The $\alpha$6A transfectants exhibited half-maximal adhesion at a [Mn$^{2+}$] of 14.7 $\mu$M, the $\alpha$6B transfectants at a [Mn$^{2+}$] of 33.5 $\mu$M, and the $\alpha$6-$\Delta$CYT transfectants at a [Mn$^{2+}$] of 39.0 $\mu$M (Figure 7). The differences in the [Mn$^{2+}$] required for half-maximal adhesion of the $\alpha$6A and $\alpha$6B transfectants and the $\alpha$6B and $\alpha$6-$\Delta$CYT transfectants are significant ($p < 0.05$) (Table 1).

### Analysis of $\alpha$6-$\Delta$CYT Migration

The ability of the $\alpha$6-$\Delta$CYT transfectants to migrate in normal culture medium cannot be assayed because they do not attach to laminin in the presence of physiological concentrations of Ca$^{2+}$ and Mg$^{2+}$. Although these mutant transfectants adhere to laminin in the presence of Mn$^{2+}$ (Shaw and Mercurio, 1993), the use of this cation is complicated by the fact that Ca$^{2+}$ is required for migration (Figure 5), and Ca$^{2+}$ negatively regulates Mn$^{2+}$ adhesion (Figure 6). However, it became apparent from the data in Figure 6 that at divalent cation concentrations sufficient to promote migration (0.5 mM Mn$^{2+}$ and 0.1 mM Ca$^{2+}$), the $\alpha$6-$\Delta$CYT transfectants adhered to laminin as well as the $\alpha$6A and $\alpha$6B transfectants. Under these conditions, the $\alpha$6A transfectants were two- to threefold more migratory than the $\alpha$6B transfectants (Figure 8), a difference similar to that observed in RPMI-

### Table 1. Summary of laminin and divalent cation titration data

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PMA</th>
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<tr>
<td>[Laminin] required for half-maximal adhesion (µg/ml)</td>
<td></td>
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<tr>
<td>$\alpha$6A</td>
<td>2.1 ± 0.4</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>$\alpha$6B</td>
<td>6.3 ± 0.3*</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>$\Delta$CYT</td>
<td>8.8 ± 0.6*</td>
<td>2.2 ± 0.8</td>
</tr>
</tbody>
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| [Ca$^{2+}$] required for half-maximal adhesion (mM) | | |
| $\alpha$6A | 5.5 ± 0.4 | >10 |
| $\alpha$6B | 1.8 ± 0.3* | >10 |
| $\Delta$CYT | 0.8 ± 0.3 | 8.1 ± 1.9 |

| [Mn$^{2+}$] required for half-maximal adhesion (mM) | | |
| $\alpha$6A | 14.7 ± 2.9 | N.D. |
| $\alpha$6B | 33.5 ± 0.5* | N.D. |
| $\Delta$CYT | 39.0 ± 1.0* | N.D. |

Mean values (±SEM) for the [laminin], [Ca$^{2+}$], and [Mn$^{2+}$] required for half-maximal adhesion of the $\alpha$6 transfectants to laminin were determined from the data shown in Figures 4, 6, and 7. Statistical differences between pairs of transfectants were determined using Student's t test.

* $p < 0.05$ in comparison to $\alpha$6A.

$^b$ $p < 0.05$ in comparison to $\alpha$6B.
Figure 5. Ca2+ requirements for α6β1 dependent adhesion and migration. (A) α6A-P388D, and α6B-P388D, transfectants were resuspended in RPMI-H or RPMI-H containing 0.5 mM EGTA and 2 mM Mg2+ and added to laminin-coated wells at a concentration of 10^5 cells per well. PMA (50 ng/ml) was added to some of the wells, and the multiwell plates were incubated for 45 min at 37°C. After washing the adherent cells were fixed, stained, and quantitated as described in MATERIALS AND METHODS. The data shown are the mean values (±SD) from a representative experiment done in triplicate. (B) α6A-P388D, and α6B-P388D, transfectants were resuspended in RPMI-H or RPMI-H containing 0.5 mM EGTA and 2 mM Mg2+ and added to the top well of a Transwell chamber. RPMI-H containing laminin (15 μg/ml) was added to the bottom wells. After a 24-h incubation, the cells that had not migrated were removed, and the cells that had migrated onto the lower surface of the filters were fixed in methanol and then stained. Migration was quantitated by counting. The data shown are the mean values (±SD) from a representative experiment done in duplicate. Neo, P388D, cells transfected with the vector alone; ■, control transfectants; □, PMA-stimulated transfectants.

DISCUSSION

The finding that multiple cytoplasmic domain variants exist for several integrin subunits has suggested that such variants differ in function. We have studied this possibility using the two known structural variants of the α6 integrin, α6A and α6B. For this purpose, we used P388D, cells that had been transfected with either the α6A or α6B cDNAs and assessed their morphology on a laminin substratum and their ability to migrate toward a laminin gradient. The results obtained indicate that the α6A transfectants extend considerably more pseudopodia on laminin and are markedly better at migrating toward laminin than the α6B transfectants. Further investigation into the possible mechanism(s) responsible for these differences revealed that the α6A and α6B transfectants differ in their adhesive strength for laminin. The α6A and α6B transfectants also exhibited relative differences in their divalent cation requirements for adhesion. Taken together, these data indicate that sequences within the α6A and α6B cytoplasmic domains can differentially modulate the extracellular ligand and cation binding domains of the α6Aβ1 and α6Bβ1 receptors.

The importance of α subunit cytoplasmic domains in regulating integrin function has been established by several labs including ours (Hibbs et al., 1991; O'Toole et al., 1991; Bauer et al., 1993; Briesewitz et al., 1993; Kasnser and Hemler, 1993; Kawaguchi and Hemler, 1993; Shaw and Mercurio, 1993; Ylanne et al., 1993). Deletion of the α2, α4, and α6 cytoplasmic domains after the highly conserved GFFKR sequence abolished the ability of the mutant α2β1, α4β1, and α6β1 integrins to mediate constitutive adhesion to their respective ligands and to augment this adhesion in response to inside-out signals (Kassner and Hemler, 1993; Kawaguchi and Hemler, 1993; Shaw and Mercurio, 1993). Deletion of other α subunit cytoplasmic domains either increased (α1b [O'Toole et al., 1991]) or had no effect (αL, α1, α5 [Hibbs et al., 1991; Bauer et al., 1993; Briesewitz et al., 1993; Ylanne et al., 1993]) on receptor activity, but some of these differences may be attributed to the fact that not all of these deletions were made at the same position in the cytoplasmic domain (Hibbs et al., 1991; O'Toole et al., 1991). The data we have obtained in this study indicate that the α6 subunit cytoplasmic domain not only is necessary for regulating integrin ligand binding function but that this regulation is sequence specific. This conclusion differs from that obtained in recent studies that used chimeric integrin subunits, which differed only in their α subunit cytoplasmic domains, to
incubated for L.M. differ was wells and subunits exhibited different abilities

Figure 6. \( \text{Ca}^{2+} \) titration adhesion assays. Tissue culture wells were coated overnight with 20 \( \mu \text{g/ml} \) laminin. \( \alpha 6\text{A}-\text{P388D}_{1}, \alpha 6\text{B}-\text{P388D}_{1}, \) and \( \alpha 6\Delta\text{CYT-P388D}_{1} \) transfectants were resuspended in Puck's Saline A containing 0.5 mM Mn\(^{2+}\) and Ca\(^{2+}\) (0–10 mM) and added to the protein-coated wells at a concentration of \( 10^5 \) cells per well. PMA (50 ng/ml) was added to some of the wells, and the multwell plates were incubated for 45 min at 37\(^\circ\)C. After washing, the adherent cells were fixed, stained, and quantitated as described in MATERIALS AND METHODS. The data shown are the mean values (±SEM) from two representative experiments done in duplicate.

Figure 7. Mn\(^{2+}\) titration adhesion assays. Tissue culture wells were coated overnight with 20 \( \mu \text{g/ml} \) laminin. \( \alpha 6\text{A}-\text{P388D}_{1}, \alpha 6\text{B}-\text{P388D}_{1}, \) and \( \alpha 6\Delta\text{CYT-P388D}_{1} \) transfectants were resuspended in Puck's Saline A containing Mn\(^{2+}\) (0–1000 \( \mu \text{M} \)) and added to the protein-coated wells at a concentration of \( 10^5 \) cells per well. The multwell plates were incubated for 45 min at 37\(^\circ\)C. After washing, the adherent cells were fixed, stained, and quantitated as described in MATERIALS AND METHODS. The data shown are the mean values (±SEM) from two representative experiments done in duplicate.

Figure 8. Effect of Mn\(^{2+}\) on the migration of the \( \alpha 6\text{A}-\text{P388D}_{1}, \alpha 6\text{B}-\text{P388D}_{1}, \) and \( \alpha 6\Delta\text{CYT-P388D}_{1} \) transfectants toward laminin. Puck's Saline A containing 0.5 mM Mn\(^{2+}\), 0.1 mM Ca\(^{2+}\), and laminin (15 \( \mu \text{g/ml} \)) was added to the bottom well of a Transwell chamber (8 \( \mu \text{m} \) pore filters). The transfectants were resuspended in Puck's Saline A containing 0.5 mM Mn\(^{2+}\) and 0.1 mM Ca\(^{2+}\), and \( 10^5 \) cells were added to the top well. After a 24-h incubation, the cells that had not migrated were removed, and the cells that had migrated onto the lower surface of the filters were fixed in methanol and then stained. Migration was quantitated by counting. The data shown are the mean values (±SEM) of three separate experiments done in duplicate. ■ control transfectants; □, PMA-stimulated transfectants.

The conclusion was drawn that the cytoplasmic domain of the \( \alpha \) subunit is necessary for regulating receptor ligand binding but that the specific sequence of the \( \alpha \) subunit cytoplasmic domain is not critical for this regulation. In contrast, our data demonstrate that sequence differences between the \( \alpha 6\text{A} \) and \( \alpha 6\text{B} \) variants account for differences in the adhesive strength, morphology,

assess the contribution of these domains to integrin activity (Chan et al., 1992; Kassner and Hemler, 1993; Kawaguchi and Hemler, 1993). Although these chimeric subunits exhibited different abilities to mediate migration and gel contraction (Chan et al., 1992), they did not differ in their apparent ligand binding activity. Thus,
and migration of the α6 transfectants. These results are important because they provide a functional rationale for the existence of multiple cytoplasmic domain variants of a specific integrin subunit.

The relative adhesive strengths of the α6A, α6B, and α6ΔCYT transfectants correlate well with their ability to extend pseudopodia and to migrate toward a laminin gradient. This behavior is consistent with the recent work of DiMilla et al. (1993) that demonstrated that the ability of cells to migrate is a function of their initial attachment strength. More specifically, they observed a biphasic dependence of cell migration speed on substratum adhesive strength, i.e., maximal migration occurs at an intermediate attachment strength. The α6A transfectants exhibited a stronger adhesive strength for laminin than the α6B transfectants, and they migrated three- to fourfold better than the α6B transfectants. This result suggests that the α6A transfectants are closer to their optimal adhesive strength for migration than the α6B transfectants. This correlation is strengthened by the finding that deletion of the α6 cytoplasmic domain resulted in a decrease in both attachment strength and ability to migrate toward laminin in comparison to the α6A and α6B transfectants. Although PMA activation increased the adhesive strength of both the α6A and α6B transfectants for laminin, it caused a slight but reproducible decrease in the ability of these cells to migrate. Using the biphasic model, this observation could be explained by suggesting that PMA increases the attachment strength of the transfectants so that they exceed the intermediate strength that is optimal for migration. However, our data do not support this possibility because PMA activation increased the adhesive strengths of both the α6A and α6B transfectants for laminin to the same level, but the α6A transfectants still migrated three- to fourfold better than the α6B transfectants. This result suggests that the α6A and α6B transfectants may differ not only in adhesive strength but in other requirements for migration such as those associated with outside-in signaling.

The data obtained on the α6ΔCYT transfectants in this study demonstrate that the α6 cytoplasmic domain plays a critical role in α6β1-mediated migration. The α6ΔCYT transfectants were significantly impaired in their ability to migrate toward a laminin gradient even in the presence of Mn²⁺, which promotes their attachment to laminin. Most likely, this behavior can be attributed to the emerging concept that migration on a matrix substratum requires a cascade of inside-out and outside-in signaling events that trigger cycles of integrin-mediated attachment and detachment (e.g., Regen and Horwitz, 1992; Dickinson and Tranquillo, 1993). Although the inside-out signaling requirement for the α6ΔCYT transfectants can be obviated by Mn²⁺, the α6 cytoplasmic domain is still necessary to transmit outside-in signals. Our observation that some migration of the α6ΔCYT transfectants occurs in the presence of Mn²⁺ suggests that the β1 cytoplasmic domain is sufficient to support a basal level of migration but that the presence of an α6 cytoplasmic domain significantly enhances this migration. Additional evidence to support a critical role for the α6 cytoplasmic domain in signaling migration is provided by the laminin and divalent cation titration studies. PMA shifted both the divalent cation sensitivity and adhesive strength of the α6ΔCYT transfectants to levels that were observed for the α6A transfectants under constitutive conditions, but it did not increase their ability to migrate (Figures 4 and 8). As discussed above, such data suggest that the α6 cytoplasmic domain also facilitates the transmission of outside-in signals required for migration.

The results presented in this paper should be compared to a recent study of the α6Aβ1 and α6Bβ1 receptors in K562 cells that concluded that the α6B transfectants adhered better to laminin than the α6A transfectants after PMA activation (Delwel et al., 1993). The disparity between our data and those of Delwel et al. (1993) may be explained by the possibility that the cellular environments of K562 and P388D1 cells confer different properties on transfected integrins. This possibility is supported by several studies that have demonstrated a role for the cellular environment in regulating integrin function (reviewed in Hynes, 1992).

In addition to their differences in adhesive strength for laminin, the α6A and α6B transfectants differed in their divalent cation sensitivity for adhesion. All integrin α subunits contain three to five putative cation binding sites, and the activity of each αβ heterodimer can be modulated by divalent cations (Staatz et al., 1989; Loftus et al., 1990; Altieri, 1991; Kirchhofer et al., 1991; Hynes, 1992). The ability of cations to bind directly to these sites has been demonstrated for several integrin receptors (Gailit and Ruoslahti, 1988; Smith and Cheresh, 1991, Michishita et al., 1993). Two recent studies examined the functional significance of cation binding by mutating the divalent cation binding domains of the αM and α4 subunits (Masumoto and Hemler, 1993; Michishita et al., 1993). These mutant receptors exhibited decreased cation sensitivity and diminished functional activity. Additional evidence to support the importance of divalent cations for integrin receptor activation has been the identification of antibodies that recognize divalent cation-dependent epitopes that are present only on activated receptors (Dransfield and Hogg, 1989; van Kooyk et al., 1991). These findings suggest that divalent cations can influence receptor conformation and in so doing, contribute to activation of receptor function. In this study, we used divalent cations to examine the relative activation states of the α6 transfectants. The data we obtained indicate that the α6A and α6B cytoplasmic domains can differentially modulate the Ca²⁺- and Mn²⁺-binding properties of the α6A and α6B transfectants respectively. Moreover, the divalent cation data provide additional evidence to
support the conclusion drawn from the laminin titration data that specific sequences within the α6 cytoplasmic domains influence the activity of the extracellular domains.

An important question that arises from the data presented is how the α6 cytoplasmic domain sequences influence adhesive strength and cation binding activity. In the simplest of models, it can be postulated that the α6 cytoplasmic domain sequence confers a specific conformation on the extracellular domain. It would be premature, however, to conclude that the α6 cytoplasmic domains regulate the laminin-binding affinity of α6β1 in the absence of other molecules. This model, for example, would not account for the upregulation of receptor function observed in response to PMA stimulation. It could be argued that posttranslational modification of the α6 cytoplasmic domains alters receptor conformation. However, the only known modification of α6 is serine phosphorylation of the α6 cytoplasmic domain (Shaw et al., 1990; Hogervorst et al., 1993a), and we (Shaw and Mercurio, 1993) and others (Hogervorst et al., 1993b) have recently demonstrated that the two serine residues in this domain are not essential for upregulation of receptor function. For these reasons, the possibility that specific cytoplasmic/cytoskeletal proteins interact preferentially with either the α6Aβ1 or α6Bβ1 cytoplasmic domains to facilitate or restrict receptor function should be considered.

It is interesting to compare the results obtained in this study with several other studies that have examined the relative expression of the α6A and α6B variants. Such studies have noted, for example, tissue specific expression of these variants (Hogervorst et al., 1993a). Also, the differentiation of mouse embryonic stem cells, which involves alterations in cell migration, is associated with a change from α6B to α6A expression (Cooper et al., 1991). In one recent study of particular interest, α6A and α6B expression was examined by immunohistochemistry in the developing chick retina (de Curtis and Reichardt, 1993). A spatial distribution of the two α6 variants was observed; the α6B subunit was expressed throughout the retina, whereas the α6A subunit was expressed only in a small region of the retina proximal to the optic nerve and on the optic nerve itself. This pattern of α6A expression may correlate with its requirement for migration of the optic nerve. Also, inflammatory macrophages, which are characterized by their ability to migrate toward specific stimuli, express only the α6Aβ1 variant (Shaw et al., 1993). It appears from these observations that a correlation between α6A expression and motility may exist, and this correlation is supported by the mechanistic studies presented here.

Two other integrins with laminin receptor function, α3β1 and α7β1, have structural variants that differ only in their α subunit cytoplasmic domain sequences (Tamura et al., 1991; Collo et al., 1993). The α3, α6, and α7 integrins share a higher sequence homology to each other than to other integrin α subunits and, for this reason, it has been hypothesized that they arose from a common ancestral gene (Sastry and Horwitz, 1993). An obvious question that arises is whether the structural homology among these α subunits is reflected in functional similarities. Specifically, it will be interesting to determine whether the A and B structural variants of the α3 and α7 subunits differ in their adhesive strength and ability to mediate migration or other cellular behaviors given the results obtained with the α6 variants in this report.

This paper is dedicated to the memory of Eric Holtzman.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grant CA-42276. L. Shaw is a Ryan Fellow at Harvard Medical School. A. Mercurio is the recipient of an American Cancer Society Faculty Research Award.

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