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Regulation of Cellular Interactions with Laminin by Integrin Cytoplasmic Domains: The A and B Structural Variants of the $\alpha 6\beta 1$ Integrin Differentially Modulate the Adhesive Strength, Morphology, and Migration of Macrophages

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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 $\alpha 6\beta 1$ 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 $P388D_1$ 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 heterodimers 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 Mn^{2+}. The α6-ΔCYT transfectants migrated to a lesser extent than either the α6A or α6B transfectants in the presence of Mn^{2+}. The α6 transfectants differed significantly in the concentration of substratum bound laminin required for half-maximal adhesion in the presence of Mn^{2+}: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 [Ca^{2+}] and [Mn^{2+}] 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 $\alpha 6\beta 1$ 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 $\beta1$ (Altruda *et al.*, 1990; Languino and Ruoslahti, 1992), $\beta3$ (van Kuppevelt *et al.*, 1989), $\beta4$ (Hogervorst *et al.*, 1990; Suzuki and Naitoh, 1990; Tamura *et al.*, 1990; Clarke and Mercurio, 1993), $\alpha3$ (Tamura *et al.*, 1991), $\alpha6$ (Hogervorst *et al.*, 1991; Tamura *et al.*, 1991), and $\alpha7$ (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 $\alpha6$ and $\alpha7$ 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 $\alpha6$A and $\alpha6$B cytoplasmic domain variants. This work has involved the use of macrophages because adhesion to laminin is mediated by the $\alpha6\beta1$ 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 $\alpha6$A and $\alpha6$B cDNAs in an $\alpha6$-deficient macrophage cell line, P388D$_1$, enabled us to determine that both the $\alpha6$A$\beta1$ and $\alpha6$B$\beta1$ integrin variants can be activated by inside-out signaling pathways (Shaw *et al.*, 1993). A truncated $\alpha6$ cDNA, $\alpha6$-ΔCYT, was constructed in which the human cytoplasmic domain sequence was deleted after the GFFKR pentapeptide. Expression of this cDNA in P388D$_1$ cells resulted in the surface expression of a chimeric $\alpha6$-ΔCYT$\beta1$ 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 $\alpha6$A-ΔCYT transfectants adhered to laminin, however, when extracellular Ca^{2+}/Mg^{2+} was replaced with Mn^{2+}. A logical question that emerged from these studies is whether the $\alpha6$A$\beta1$ and $\alpha6$B$\beta1$ variants, as well as the $\alpha6$-ΔCYT$\beta1$ mutant, exhibit differences in their ability to initiate specific cellular functions subsequent to laminin attachment.

In the present study, we observed that P388D$_1$ cells transfected with either the human $\alpha6$A, $\alpha6$B, or $\alpha6$-ΔCYT integrin cDNAs differed markedly in their morphology on laminin and in their ability to migrate toward a laminin gradient. Subsequent analysis of these $\alpha6$ 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: $\alpha6A\beta1 \gg \alpha6B\beta1 > \alpha6\Delta CYT\beta1$. These data suggest that specific sequences within the $\alpha6$A and $\alpha6$B cytoplasmic domains differentially modulate the functional activity of the $\alpha6\beta1$ extracellular domain.

MATERIALS AND METHODS

Cells

The P388D$_1$ mouse macrophage cell line was obtained from the American Type Tissue Collection (Rockville, MD). Cells were maintained in RPMI containing 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (RPMI-H) and 15% certified fetal bovine serum (GIBCO, Grand Island, NY).

cDNA Transfections

The human $\alpha6$A and $\alpha6$B cDNAs were cloned by polymerase chain reaction and subcloned into the eukaryotic expression vector pRc/CMV as described previously (Shaw *et al.*, 1993). The $\alpha6$-ΔCYT mutant cDNA was constructed as described previously (Shaw and Mercurio, 1993). The pRc/CMV vectors containing either the $\alpha6$A, $\alpha6$B, or $\alpha6$-ΔCYT cDNAs, as well as the vector alone, were transfected into the P388D$_1$ 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 $\alpha6$ subunits on the cell surface were isolated by fluorescence-activated cell sorter (FACS). A human $\alpha6$ integrin specific monoclonal antibody (mAb), 2B7, was used for this sorting and for subsequent analysis of the transfectants (Shaw *et al.*, 1993). The sorting was repeated sequentially for each transfectant to enrich for homogeneous populations of cells expressing equivalent levels of the transfected $\alpha6$ subunits on the cell surface.

Flow Cytometry

Transfected P388D$_1$ cells were washed twice with phosphate-buffered saline containing 0.1% bovine serum albumin (PBS/BSA). Aliquots of cells (3×10^5) 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, multiwell 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 10^5 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 $MnCl_2$, 0.1 mM $CaCl_2$, 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 10^6/ml, and 10^5 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 mm^2. Five separate fields were counted for each filter.

To examine the Ca^{2+} 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 $MgCl_2$ 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, $P388D_1$ 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 equivalents 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 $P388D_1$ 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).

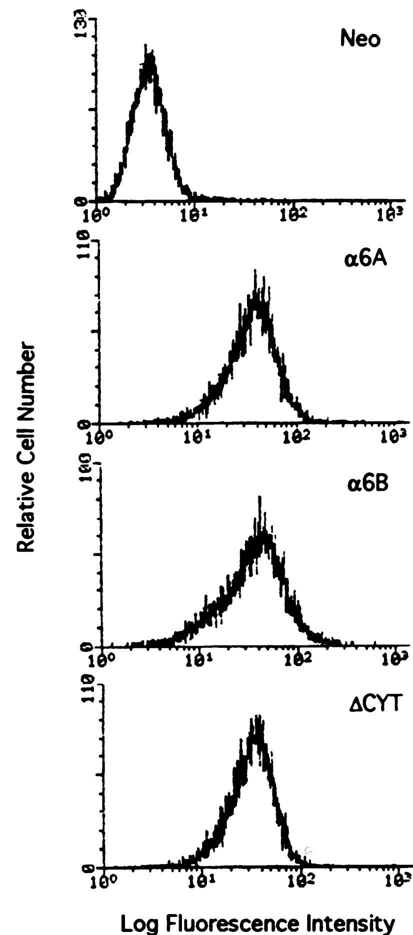

Figure 1. Surface expression of the human α6A, α6B, and α6-ΔCYT integrin subunits in $P388D_1$ transfectants. Populations of transfected $P388D_1$ cells expressing either the α6A, α6B, or α6-ΔCYT subunits on the cell surface were isolated by sequential FACS using 2B7, a mAb specific for the human α6 integrin subunit, and then analyzed by flow cytometry. Neo, $P388D_1$ cells transfected with the vector alone.

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

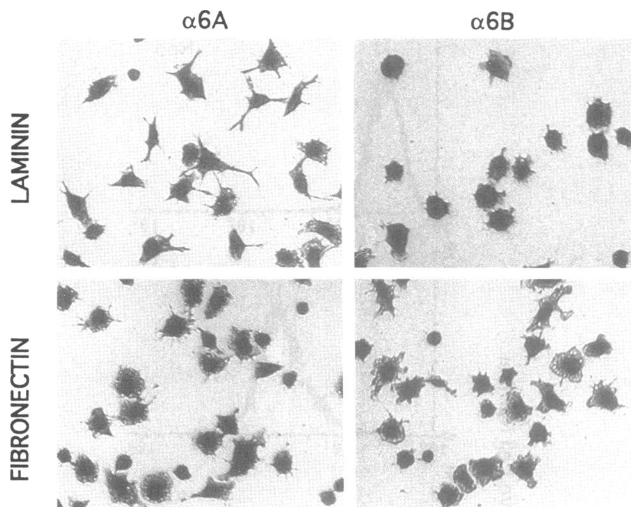

Figure 2. Photomicrographs of adherent $P388D_1$ transfectants. α6A-$P388D_1$ and α6B-$P388D_1$ transfectants were allowed to adhere to either laminin or fibronectin substrata for 45 min. After washing, adherent cells were fixed and stained as described in MATERIALS AND METHODS. Stained cells were photographed using brightfield optics. Magnification, ×2000.

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 α6A and α6B transfectants differed significantly ($p < 0.01$) in their ability to migrate toward a laminin gradient. As shown in Figure 3A, the α6A transfectants were three- to fourfold more migratory toward laminin than the α6B transfectants in a 24-h assay.

PMA did not increase the number of either the α6A or α6B 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 α6A and α6B transfectants is α6β1 dependent because it was completely inhibited by 2B7 under all of the conditions examined (Figure 3A). $P388D_1$ cells that were transfected with the α6 cytoplasmic deletion mutant, α6-ΔCYT, did not migrate toward laminin under these conditions. This observation is consistent with our previous finding that the α6-ΔCYT transfectants did not adhere to laminin in RPMI-H (Shaw and Mercurio, 1993). $P388D_1$ 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 α6A, α6B, and α6-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 refects their ability to migrate on vitronectin.

The α6 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 α6A and α6B 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 microtiter wells, and the possibility existed that differences in binding may not have been apparent at this concentration.

To determine if the α6 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 Mn^{2+}) 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 α6-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 α6 transfectants were used that expressed equivalent levels of α6 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 α6A transfectants exhibited the strongest adhesion with half-maximal adhesion occurring at a concentration of ~2.1 μg/ml of laminin. The α6B transfectants required 6.3 μg/ml of laminin for half-maximal adhesion, and the α6-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 α6A and α6B transfectants are significant ($p < 0.05$) (Table 1). These data indicate that the α6 transfectants differ in their relative adhesive strength for laminin.

In response to PMA activation, the relative adhesive strength of the α6A transfectants increased only slightly

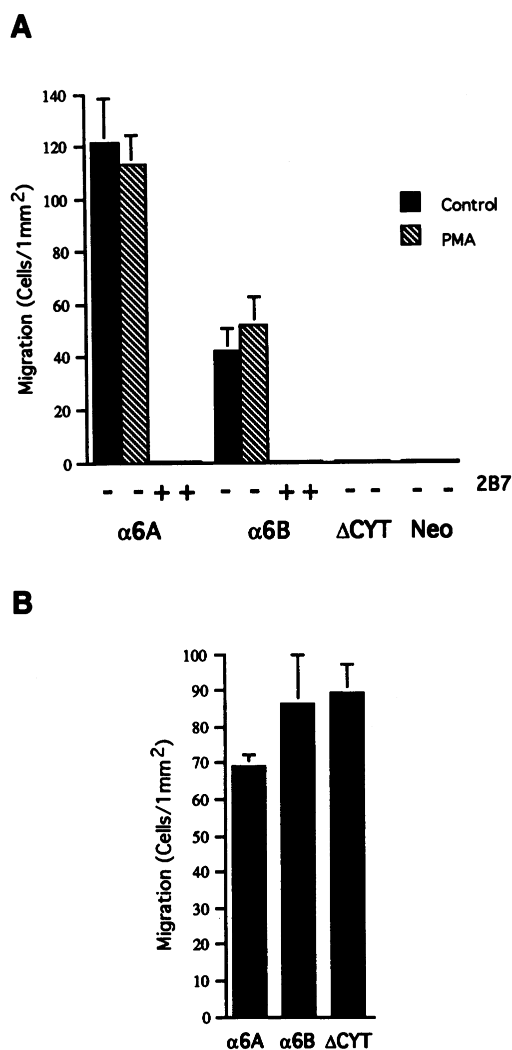

Figure 3. (A) Migration of α6A-$P388D_1$, α6B-$P388D_1$, and α6ΔCYT-$P388D_1$ cells toward a laminin substratum. RPMI-H containing laminin (15 μg/ml) was added to the bottom well of a Transwell chamber (8 μM pore filters), and 10^5 cells were added to the top well. After a 24-h incubation, the cells 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. 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-$P388D_1$, α6B-$P388D_1$, and α6ΔCYT-$P388D_1$ 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, $P388D_1$ cells transfected with the expression vector alone; ■ control transfectants; ▨, PMA-stimulated transfectants.

(Figure 4B). However, the concentration of laminin required for half-maximal adhesion of both the α6B and α6-ΔCYT transfectants decreased markedly to ~2 μg/ml, a value similar to that observed for the α6A transfectants (Figure 4B). Thus, all of the transfectants exhibited similar adhesive strengths for laminin in response to PMA. The behavior of the α6-ΔCYT transfectants in this experiment does not conflict with our previous conclusion that this mutant cannot respond to PMA because in the present experiment adhesion was induced by the presence of Mn^{2+}. Most likely, other targets of PMA such as cytoskeletal proteins strengthen Mn^{2+}-induced adhesion of these cells.

Ca^{2+} Is Required for α6-mediated Migration but Not Adhesion

Because integrin heterodimers can differ in their Ca^{2+} requirements for adhesion and migration (Grzesiak *et al.*, 1992; Leavesley *et al.*, 1993), we thought it important to examine the role of Ca^{2+} in the behavior of the α6A and α6B transfectants on laminin. For this purpose, both adhesion and migration assays were performed in the presence of 0.5 mM EGTA to chelate extracellular Ca^{2+}.

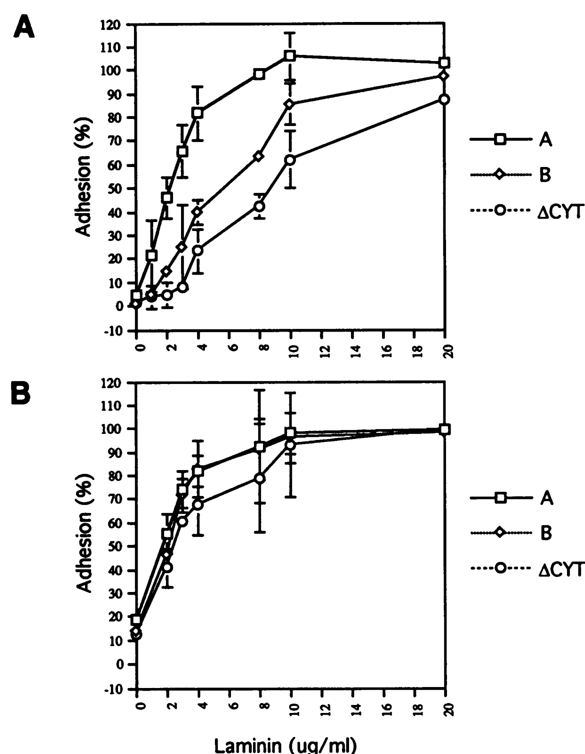

Figure 4. Laminin titration adhesion assays. (A) α6A-$P388D_1$, α6B-$P388D_1$, and α6ΔCYT-$P388D_1$ transfectants were assayed for their ability to adhere to a range of laminin substratum concentrations. Tissue culture wells were coated with EHS laminin (1–20 μg/ml) overnight and counter-coated with 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 α6A or α6B transfectants to a laminin substratum (Figure 5A). In fact, chelation of Ca^{2+} enhances the constitutive adhesion of both the α6A and α6B transfectants. This finding suggests that Ca^{2+} is a negative regulator of α6β1 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 α6A or α6B 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 α6A and α6B transfectants was evident in the presence of Ca^{2+} (Figure 5B).

The α6 Transfectants Differ in Their Cation Sensitivity for Adhesion to Laminin

Extracellular Mn^{2+} (150 μM) markedly increases the ability of the α6A-, α6B-, and α6-ΔCYT P388D$_1$ transfectants to adhere to laminin (Shaw and Mercurio, 1993). Presumably, the interaction of Mn^{2+} with divalent cation binding sites in the α6 extracellular domain increases the affinity of the α6β1 integrin for laminin (Sonnenberg *et al.*, 1988; Shaw and Mercurio, 1991). In contrast, extracellular Ca^{2+} negatively regulates the ability of these α6 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 α6 transfectants for Ca^{2+} may be apparent in such an assay. As shown in Figure 6A, the α6A, α6B, and α6-ΔCYT 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 α6A transfectants exhibited half-maximal adhesion at a $[Ca^{2+}]$ of 5.5 mM, the α6B transfectants at a $[Ca^{2+}]$ of 1.8 mM, and the α6-ΔCYT transfectants at a $[Ca^{2+}]$ of 0.8 mM (Figure 6B). The difference in the $[Ca^{2+}]$ required for half-maximal adhesion of the α6A and α6B transfectants is significant ($p < 0.05$) (Table 1). In the presence of PMA, the α6A and α6B transfectants were both resistant to Ca^{2+} titration at the concentrations examined. However, although PMA increased the resistance of the α6-ΔCYT 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 α6A, α6B, and α6-ΔCYT transfectants differed in the $[Mn^{2+}]$ required to obtain half-maximal adhesion. The α6A transfectants exhibited half-maximal adhesion at a $[Mn^{2+}]$ of 14.7 μM, the α6B transfectants at a $[Mn^{2+}]$ of 33.5 μM, and the α6-ΔCYT transfectants at a $[Mn^{2+}]$ of 39.0 μM (Figure 7). The differences in the $[Mn^{2+}]$ required for half-maximal adhesion of the α6A and α6B transfectants and the α6B and α6-ΔCYT transfectants are significant ($p < 0.05$) (Table 1).

Analysis of α6-ΔCYT Migration

The ability of the α6-ΔCYT 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 α6-ΔCYT transfectants adhered to laminin as well as the α6A and α6B transfectants. Under these conditions, the α6A transfectants were two- to threefold more migratory than the α6B transfectants (Figure 8), a difference similar to that observed in RPMI-

Table 1. Summary of laminin and divalent cation titration data

	Control	PMA
[Laminin] required for half-maximal adhesion (μg/ml)		
α6A	2.1 ± 0.4	1.8 ± 0.3
α6B	6.3 ± 0.3[a]	2.0 ± 0.2
ΔCYT	8.8 ± 0.6[b]	2.2 ± 0.8
$[Ca^{2+}]$ required for half-maximal adhesion (mM)		
α6A	5.5 ± 0.4	>10
α6B	1.8 ± 0.3[a]	>10
ΔCYT	0.8 ± 0.3	8.1 ± 1.9
$[Mn^{2+}]$ required for half-maximal adhesion (mM)		
α6A	14.7 ± 2.9	N.D.
α6B	33.5 ± 0.5[a]	N.D.
ΔCYT	39.0 ± 1.0[b]	N.D.

Mean values values (±SEM) for the [laminin], $[Ca^{2+}]$, and $[Mn^{2+}]$ required for half-maximal adhesion of the α6 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.

[a] $p < 0.05$ in comparison to α6A.

[b] $p < 0.05$ in comparison to α6B.

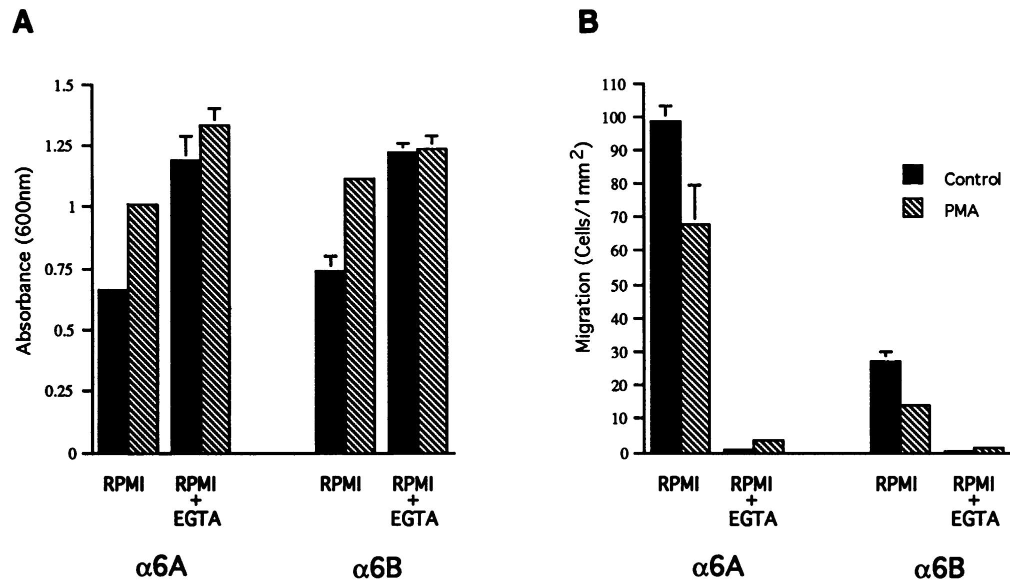

Figure 5. Ca^{2+} requirements for α6β1 dependent adhesion and migration. (A) α6A-P388D₁ and α6B-P388D₁ transfectants were resuspended in RPMI-H or RPMI-H containing 0.5 mM EGTA and 2 mM Mg^{2+} and added to laminin-coated wells at a concentration of 10^5 cells per well. PMA (50 ng/ml) was added to some 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-P388D₁ and α6B-P388D₁ transfectants were resuspended in RPMI-H or RPMI-H containing 0.5 mM EGTA and 2 mM Mg^{2+} 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, P388D₁ cells transfected with the vector alone; ■, control transfectants; ▧, PMA-stimulated transfectants.

H (Figure 3). Interestingly, the α6-ΔCYT transfectants exhibited some migration toward laminin in the presence of these divalent cations, but the amount of this migration was low in comparison to that observed for the α6A and α6B transfectants (Figure 8). The differences that were observed for the migration of the α6A and α6B transfectants under both control and PMA conditions are significant ($p < 0.01$). The differences in migration between the α6B and α6-ΔCYT transfectants were not found to be significant ($p > 0.05$).

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 P388D₁ 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; Kassner 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 (αIIb [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

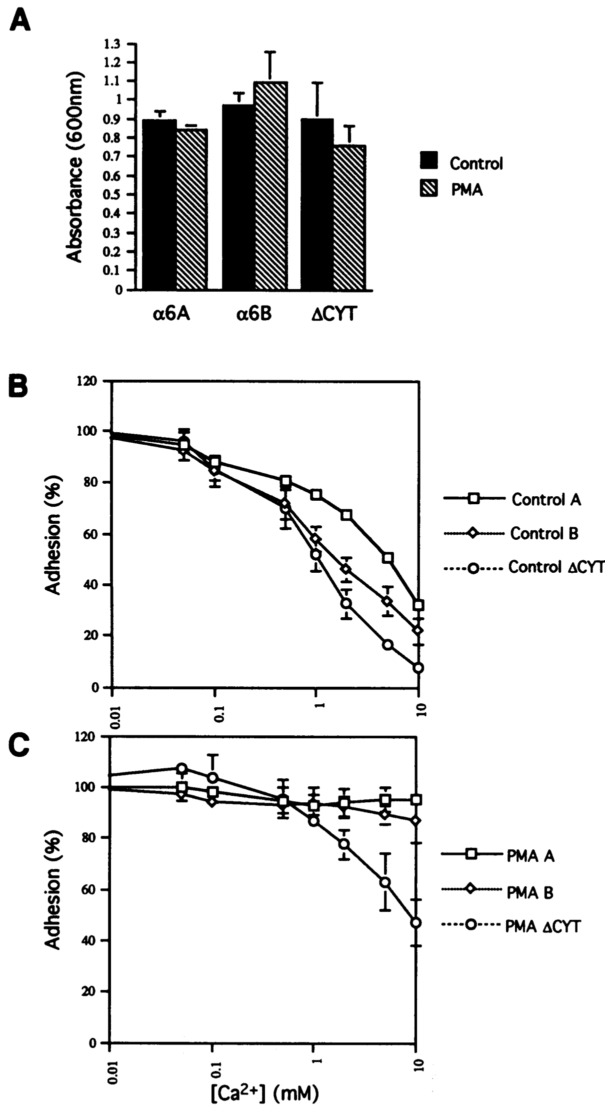

Figure 6. Ca^{2+} titration adhesion assays. Tissue culture wells were coated overnight with 20 μg/ml laminin. α6A-P388D$_1$, α6B-P388D$_1$, and α6ΔCYT-P388D$_1$ transfectants were resuspended in Puck's Saline A containing 0.5 mM Mn^{2+} and Ca^{2+} (0–10 mM) and added to the protein-coated wells at a concentration of 10^5 cells per well. PMA (50 ng/ml) was added to some of the wells, and the 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 (±SEM) from three experiments done in duplicate. (A) Adhesion in the presence of 0.5 mM Mn^{2+}; (B) adhesion in the presence of 0.5 mM Mn^{2+} and increasing [Ca^{2+}]; (C) same conditions as in B but in the presence of PMA. ■, control transfectants; ▧, PMA-stimulated transfectants.

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,

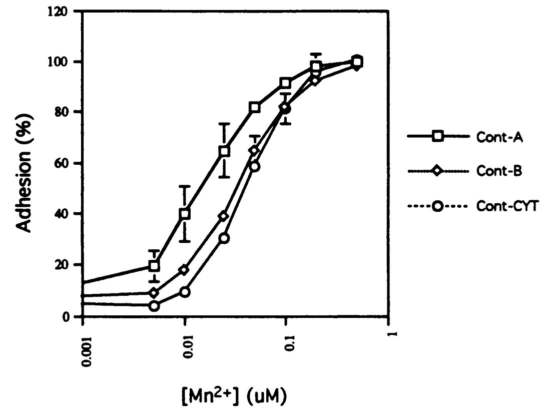

Figure 7. Mn^{2+} titration adhesion assays. Tissue culture wells were coated overnight with 20 μg/ml laminin. α6A-P388D$_1$, α6B-P388D$_1$, and α6ΔCYT-P388D$_1$ transfectants were resuspended in Puck's Saline A containing Mn^{2+} (0–1000 μM) and added to the protein-coated wells at a concentration of 10^5 cells per well. 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 (±SEM) from two representative experiments done in duplicate.

the conclusion was drawn that the cytoplasmic domain of the α subunit is necessary for regulating receptor ligand binding but that the specific sequence of the α subunit cytoplasmic domain is not critical for this regulation. In contrast, our data demonstrate that sequence differences between the α6A and α6B variants account for differences in the adhesive strength, morphology,

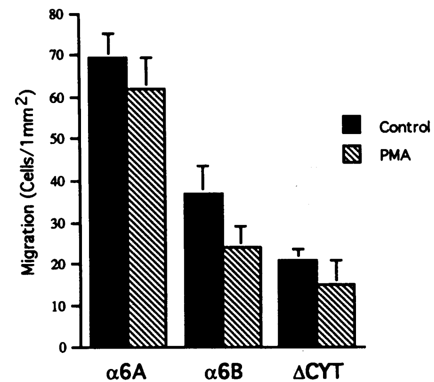

Figure 8. Effect of Mn^{2+} on the migration of the α6A-P388D$_1$, α6B-P388D$_1$, and α6ΔCYT-P388D$_1$ transfectants toward laminin. Puck's Saline A containing 0.5 mM Mn^{2+}, 0.1 mM Ca^{2+}, and laminin (15 μg/ml) was added to the bottom well of a Transwell chamber (8 μM pore filters). The transfectants 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.

and migration of the $\alpha6$ 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 $\alpha6A$, $\alpha6B$, and $\alpha6$-ΔCYT 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 $\alpha6A$ transfectants exhibited a stronger adhesive strength for laminin than the $\alpha6B$ transfectants, and they migrated three- to fourfold better than the $\alpha6B$ transfectants. This result suggests that the $\alpha6A$ transfectants are closer to their optimal adhesive strength for migration than the $\alpha6B$ transfectants. This correlation is strengthened by the finding that deletion of the $\alpha6$ cytoplasmic domain resulted in a decrease in both attachment strength and ability to migrate toward laminin in comparison to the $\alpha6A$ and $\alpha6B$ transfectants. Although PMA activation increased the adhesive strength of both the $\alpha6A$ and $\alpha6B$ 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 $\alpha6A$ and $\alpha6B$ transfectants for laminin to the same level, but the $\alpha6A$ transfectants still migrated three- to fourfold better than the $\alpha6B$ transfectants. This result suggests that the $\alpha6A$ and $\alpha6B$ 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 $\alpha6$-ΔCYT transfectants in this study demonstrate that the $\alpha6$ cytoplasmic domain plays a critical role in $\alpha6\beta1$-mediated migration. The $\alpha6$-ΔCYT transfectants were significantly impaired in their ability to migrate toward a laminin gradient even in the presence of Mn^{2+}, 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 $\alpha6$-ΔCYT transfectants can be obviated by Mn^{2+}, the $\alpha6$ cytoplasmic domain is still necessary to transmit outside-in signals. Our observation that some migration of the $\alpha6$-ΔCYT transfectants occurs in the presence of Mn^{2+} suggests that the $\beta1$ cytoplasmic domain is sufficient to support a basal level of migration but that the presence of an $\alpha6$ cytoplasmic domain significantly enhances this migration. Additional evidence to support a critical role for the $\alpha6$ 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 $\alpha6$-ΔCYT transfectants to levels that were observed for the $\alpha6A$ 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 $\alpha6$ 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 $\alpha6A\beta1$ and $\alpha6B\beta1$ receptors in K562 cells that concluded that the $\alpha6B$ transfectants adhered better to laminin than the $\alpha6A$ 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 $\alpha6A$ and $\alpha6B$ 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 $\alpha\beta$ 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 $\alpha4$ 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 $\alpha6$ transfectants. The data we obtained indicate that the $\alpha6A$ and $\alpha6B$ cytoplasmic domains can differentially modulate the Ca^{2+}- and Mn^{2+}-binding properties of the $\alpha6A$ and $\alpha6B$ 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 $\alpha 6$ cytoplasmic domains influence the activity of the extracellular domains.

An important question that arises from the data presented is how the $\alpha 6$ cytoplasmic domain sequences influence adhesive strength and cation binding activity. In the simplest of models, it can be postulated that the $\alpha 6$ cytoplasmic domain sequence confers a specific conformation on the extracellular domain. It would be premature, however, to conclude that the $\alpha 6$ cytoplasmic domains regulate the laminin-binding affinity of $\alpha 6\beta 1$ 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 $\alpha 6$ cytoplasmic domains alters receptor conformation. However, the only known modification of $\alpha 6$ is serine phosphorylation of the $\alpha 6$ 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 $\alpha 6A\beta 1$ or $\alpha 6B\beta 1$ 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 $\alpha 6A$ and $\alpha 6B$ 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 $\alpha 6B$ to $\alpha 6A$ expression (Cooper *et al.*, 1991). In one recent study of particular interest, $\alpha 6A$ and $\alpha 6B$ expression was examined by immunohistochemistry in the developing chick retina (de Curtis and Reichardt, 1993). A spatial distribution of the two $\alpha 6$ variants was observed; the $\alpha 6B$ subunit was expressed throughout the retina, whereas the $\alpha 6A$ 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 $\alpha 6A$ 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 $\alpha 6A\beta 1$ variant (Shaw *et al.*, 1993). It appears from these observations that a correlation between $\alpha 6A$ expression and motility may exist, and this correlation is supported by the mechanistic studies presented here.

Two other integrins with laminin receptor function, $\alpha 3\beta 1$ and $\alpha 7\beta 1$, have structural variants that differ only in their α subunit cytoplasmic domain sequences (Tamura *et al.*, 1991; Collo *et al.*, 1993). The $\alpha 3$, $\alpha 6$, and $\alpha 7$ 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 $\alpha 3$ and $\alpha 7$ subunits differ in their adhesive strength and ability to mediate migration or other cellular behaviors given the results obtained with the $\alpha 6$ variants in this report.

This paper is dedicated to the memory of Eric Holtzman.

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