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Leslie M. Shaw  
University of Massachusetts Medical School

Arthur M. Mercurio  
University of Massachusetts Medical School

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Regulation of α6β1 Integrin Laminin Receptor Function by the Cytoplasmic Domain of the α6 Subunit

Leslie M. Shaw and Arthur M. Mercurio
Program in Cell and Developmental Biology and Deaconess Hospital, Harvard Medical School, Boston, Massachusetts 02115

Abstract. The α6β1 integrin is expressed on the macrophage surface in an inactive state and requires cellular activation with PMA or cytokines to function as a laminin receptor (Shaw, L. M., J. M. Messier, and A. M. Mercurio. 1990. J. Cell Biol. 110:2167-2174). In the present study, the role of the α6 subunit cytoplasmic domain in α6β1 integrin activation was examined. The use of P388D1 cells, an α6-integrin deficient macrophage cell line, facilitated this analysis because expression of either the α6A or α6B subunit cDNAs restores their activation responsive laminin adhesion (Shaw, L. S., M. Lotz, and A. M. Mercurio. 1993. J. Biol. Chem. 268:11401-11408). A truncated α6 cDNA, α6-ΔACYT, 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-ΔACYT/β1 integrin that was unable to mediate laminin adhesion or increase this adhesion in response to PMA under normal conditions, i.e., in medium that contained physiological concentrations of Ca**+ and Mg**+. The α6A-ΔACYT transfectants adhered to laminin, however, when Ca**+/Mg**+ was replaced with 150 μM Mn**+. We also assessed the role of serine phosphorylation in the regulation of α6Aβ1 integrin function by site-directed mutagenesis of the two serine residues present in the α6A cytoplasmic domain because this domain is phosphorylated on serine residues in response to stimuli that activate the laminin receptor function of α6Aβ1. Point mutations were introduced in the α6A cDNA that changed either serine residue #1064 (M1) or serine residue #1071 (M2) to alanine residues. In addition, a double mutant (M3) was constructed in which both serine residues were changed to alanine residues. P388D1 transfectants which expressed these serine mutations adhered to laminin in response to PMA to the same extent as cells transfected with wild-type α6A cDNA. These findings provide evidence for a novel mode of integrin regulation that is distinct from that reported for other regulated integrins (O'Toole, T. E., D. Mandelman, J. Forsyth, S. J. Shattil, E. E. Plow, and M. H. Ginsberg. 1991. Science (Wash. DC). 254:845-847. Hibbs, M. L., H. Xu, S. A. Stacker, and T. A. Springer. 1991. Science (Wash. DC). 251:1611-1613), and they demonstrate that serine phosphorylation of the α6A cytoplasmic domain is not involved in this regulation.

The rapid activation of integrin function by signal transduction pathways constitutes an important regulatory mechanism for cell–cell and cell–matrix interactions (for review see Ginsberg et al., 1992; Hynes, 1992). This hypothesis is substantiated by the finding that several integrins are expressed on the cell surface in an inactive state and require cellular activation with a variety of agonists to acquire the capability to mediate adhesion to their appropriate ligands (e.g., Du et al., 1991; Dustin and Springer, 1991; Shaw et al., 1990). This process has been termed “inside-out” integrin signaling, and the integrins that are the targets of signaling pathways are often referred to as “activation-dependent” integrins (Ginsberg et al., 1992; Hynes, 1992).

Examples of such activation-dependent integrins include the leukocyte specific β2 integrins (Dustin and Springer, 1991; Hermanowski-Vosatka et al., 1992), the αIIbβ3 platelet integrin (Du et al., 1991), and the α6β1 integrin on macrophages (Shaw et al., 1990, 1993) and T-cells (Shimizu et al., 1990). It is clear that kinase activation is a critical component of inside-out integrin signaling (Shaw et al., 1990; Dustin and Springer, 1991; Shattil and Brugge, 1991). G proteins (Shattil et al., 1992) and the production of specific lipids (Hermanowski-Vosatka et al., 1992) have also been implicated in this process. Although the details of these signaling pathways have not been elucidated, it appears that they may induce a conformational change in the integrin extracellular domain which facilitates ligand binding (Du et al., 1991; Neugebauer and Reichardt, 1991; Diamond and Springer, 1993).

The cytoplasmic domains of the activation-dependent inte-
grins are likely targets of intracellular signaling pathways. For example, the cytoplasmic domains of the β2 (Chatila et al., 1989), β3 (Hillery et al., 1991), and α6 (Shaw et al., 1990; Hogervorst et al., 1993) integrin subunits are phosphorylated on serine residues in response to the appropriate agonists. Such information, although suggestive, is correlative and it does not confirm a role for the cytoplasmic domain in integrin regulation. For this reason, mutagenesis of the cytoplasmic domains of αLβ2 and αIbbβ3 integrins has been used to examine their role in inside-out signaling. From these studies, it appears that the β2 cytoplasmic domain of the αLβ2 integrin is critical for its regulation because deletion of the β2 cytoplasmic domain, but not the αL cytoplasmic domain, resulted in an inactive receptor that could no longer respond to PMA activation (Hibbs et al., 1992). Site-directed mutagenesis of the serine residue in the β2 subunit that is phosphorylated in response to PMA did not alter the activation of the αLβ2 receptor, indicating that phosphorylation may not be an important mechanism for activation of receptor function (Hibbs et al., 1992). In contrast to the results obtained with αLβ2, it was observed for αIbbβ3 that deletion of the αIb cytoplasmic domain, but not the β3 cytoplasmic domain, created a constitutively active receptor that did not require cellular activation of its ligand-binding function (O'Toole et al., 1991). The existing data, therefore, indicate distinct modes of regulation for αLβ2 and αIbbβ3 because each of these integrins differ not only in which subunit cytoplasmic domain is critical for activation of function, but also in how the specific cytoplasmic domain influences receptor regulation.

We have reported that the α6β1 integrin requires inside-out signaling to function as a macrophage laminin receptor (Shaw et al., 1990). This activation-dependent function correlates with the association of this integrin with the actin cytoskeleton and serine phosphorylation of the α6 subunit (Shaw et al., 1990). Based on these findings and the disparate results obtained with the αLβ2 (Hibbs et al., 1991a,b) and αIbbβ3 integrins (O'Toole et al., 1991), we thought it was important to investigate the role of the α6 subunit cytoplasmic domain in the regulation of α6β1 function. This analysis was facilitated by our recent finding that the activation responsive adhesion of the α6β1 integrin is maintained when the α6 subunit is expressed by cDNA transfection in P388D1 cells, an α6-deficient macrophage cell line (Shaw et al., 1993). In this report, we provide evidence for a mechanism of integrin regulation that is distinct from αLβ2 and αIbbβ3 because deletion of the α6 cytoplasmic domain abolished laminin adhesion under physiological conditions as well as in response to PMA. We also observed that site-directed mutagenesis of the two serine residues in the α6 cytoplasmic domain did not inhibit cell attachment to laminin.

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 15% certified FBS (GIBCO BRL, Gaithersburg, MD). Thioglycollate-elicited (TG)1 macrophages were obtained from C57BL/6J mice (Jackson ImmunoResearch Labs., Inc., West Grove, PA) as described previously (Shaw et al., 1990).

Adhesion Assays

Adhesion assays were performed as described previously (Shaw and Mercurio, 1989; Shaw et al., 1990). Briefly, multiwell tissue culture plates (11.3-mm diam) were coated overnight at 4°C with 0.2 ml of PBS containing either 20 μg/ml of murine Englebreth-Holm-Swarm (EHS) laminin or 20 μg/ml human fibronectin (Boehringer Mannheim Corp., Indianapolis, IN). Laminin was purified from the EHS sarcoma as described (Kleinman et al., 1982). The wells were then washed with PBS and 1-2 × 105 cells in RPMI-H (GIBCO BRL) or Puck's Saline A (Sigma Chem. Co., St. Louis, MO) were added per well. Divalent cations were included in the Puck's Saline A at the concentrations indicated in the individual figure legends. PMA (50 ng/ml) was added to some of the wells and the cells were incubated at 37°C for 30 min to 1 h. The wells were washed three times with RPMI-H 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 SDS and adhesion was quantitated by measuring the absorbance at 600 nm.

To examine inhibition of laminin adhesion, cells were preincubated in suspension for 30 min at 4°C with 2B7, a mAb specific for the human α6 subunit (Shaw et al., 1993), and murine IgG Fc fragment (20 μg/ml; Jackson ImmunoResearch Labs., Inc.). Subsequently, the cells were assayed as described above for laminin adhesion.

Surface Labeling

Cells were washed twice with PBS containing 1 mM each of CaCl2 and MgCl2. After washing, the macrophages were resuspended in the same buffer at a concentration of 5 × 10⁵ cells/ml. NHS-LC-biotin (Pierce, Rockville, IL) was resuspended in DMSO and added to the cells at a concentration of 0.1 mg/ml. Cells were incubated in the presence of biotin for 15 min at 4°C at which time the cells were spun down, resuspended in fresh biotin, and incubated for another 15 min at 4°C. Subsequently, the cells were washed several times with PBS containing 50 mM NH₄Cl to remove unincorporated biotin.

Cell Extraction and Immunoprecipitation

Surface biotinylated cells were solubilized at 4°C for 15 min in a 50 mM Tris buffer, pH 7.5, containing 0.15 M NaCl, 1% Triton-X-100, 1 mM each of CaCl2 and MgCl2, and 2 mM PMSF. Nuclei were removed by centrifugation at 12,000 g for 10 min. Aliquots of labeled cell extracts were incubated overnight at 4°C with α6 specific antibodies. Immune complexes were recovered with protein G agarose (Pharmacia LKB Biotechnology, Piscataway, NJ). The agarose beads were added for 1 h at 4°C with constant agitation. The beads were washed two times with a 50 mM Tris buffer, pH 7.5, containing 0.1% Tween 20 and 0.15 M NaCl, two times with the same buffer containing 0.5 M NaCl, and one time with 0.05 M Tris, pH 6.8. Laemmli sample buffer was added to the samples which were then incubated at 100°C for 5 min. Surface biotinylated immunoprecipitates were resolved by SDS-PAGE (12%) and transferred to nitrocellulose filters. The filters were blocked for 30 min using a 50 mM Tris buffer, pH 7.5, containing 0.1% Tween 20, 0.5 M NaCl, and 5% (wt/vol) Carnation dry milk. The filters were incubated for 1 h in the same buffer containing streptavidin conjugated to horseradish peroxidase (3 μg/ml; Pierce). After three ten-minute washes in blocking buffer lacking dry milk, protein was detected by enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL).

Unlabeled cells were solubilized as described above and cell extracts were immunoprecipitated with the α6 specific mAb, 2B7. Immunoprecipitates were resolved by SDS-PAGE (10%) and transferred to nitrocellulose filters. The filters were blocked for 30 min using a 50 mM Tris buffer, pH 7.5, containing 0.05% Tween-20, 0.15 M NaCl, and 5% (wt/vol) Carnation dry milk. The filters were then incubated for 2 h in the same buffer without milk containing a 1:100 dilution of a polyclonal Ab for β1 (Marcantonio and Hynes, 1988). After washing, the filters were incubated with a goat anti-rabbit IgG conjugated to horseradish peroxidase (0.2 μg/ml; Kirkegaard and Perry). Protein was detected by enhanced chemiluminescence (Amersham Corp.).

Site-directed Mutagenesis

The human α6 cDNA was cloned by PCR and subcloned into the eukaryotic expression vector pRC/CMV as described previously (Shaw et al., 1990).

1. Abbreviations used in this paper: EHS, Englebreth-Holm-Swarm; TG, thioglycollate-elicited.

The Journal of Cell Biology, Volume 123, 1993
The resulting PCR fragment was subcloned into pCRII using the TA cloning system (Invitrogen). An XbaI-HindlII fragment was removed by digestion and inserted into the ot6A cDNA in pRC/CMV after removal of the corresponding wild-type XbaI-HindlII fragment.

Site-directed mutagenesis of the ot6A cytoplasmic domain was carried out by overlap extension. Individual serine 1064 to alanine 1064 and serine 1071 to alanine 1071 mutations in the ot6A cDNA were generated by using pairs of complementary mutagenic oligonucleotide primers (5'-TGCTCA-GCCAACATGATAAAG-3' and 5'-GAGCTCCTGCTGCTAGCTA-3', respectively) representing nucleotides 3326-3346 and 3347-3367 (Tamura et al., 1990). The underlined letters identify the nucleotide changes that were introduced. The outer set of primers were 5'-CAATTACAGCTAAAGC-3' and 5'-AGTTGCTGCTGCTAGCTA-3' which correspond to nucleotides 2497-2512 was used as the upstream primer for this PCR reaction (Tamura et al., 1990). The resulting PCR fragment was subcloned into pCRII using the TA cloning system (Invitrogen).

Results

Construction of ot6 Cytoplasmic Domain Mutants

Previously, we reported that expression of the ot6A integrin cDNA in P388D1 cells restores their ability to adhere to laminin (Shaw et al., 1993). This adhesion is mediated by the transfected integrin because it is inhibited by an ot6 specific mAb (Fig. 1). The experimental approach taken in this study was to mutate the cytoplasmic domain sequence of ot6A and to express the mutated cDNA in P388D1 cells, an ot6-deficient macrophage cell line. Initially, we deleted the cytoplasmic domain sequence to ot6A subunits (Tamura et al., 1990; Hogervorst et al., 1991), as well as in all other integrin ot subunits (Hemler, 1990). The deletion was made after this pentapeptide to facilitate expression of the truncated subunit because very low levels of ot6A expression were observed when this sequence was included in a deletion of the ot6B subunit cytoplasmic domain (O'Toole et al., 1991). The ot6 insertional mutation resulted in a cDNA, termed ot6-ACYT, that lacked any ot6A or ot6B specific cytoplasmic domain sequences (Fig. 2).

We also assessed the role of serine phosphorylation in the regulation of ot6 integrin function by site-directed mutagenesis of the two serine residues present in the ot6A cytoplasmic domain. Point mutations were introduced in the ot6A cDNA that changed either serine residue #1064 (M1) or serine residue #1071 (M2) to alanine residues (Fig. 2). In addition, a double mutant (M3) was constructed in which both serine residues were changed to alanine residues (Fig. 2).

The ot6 cDNAs were subcloned into the eukaryotic expression vector pRC/CMV and transfected into P388D1 cells. After selective growth in medium containing G418, the population of cells that expressed the human-mouse chimera ot6 integrin was isolated by sequential cycles of FACScan using 2B7, a mAb that is specific for the human ot6 integrin subunit (Shaw et al., 1993).
The complete amino acid sequence of the human α6A cytoplasmic domain is shown (see Tamura et al., 1990; Hogevoorst et al., 1991; Shaw et al., 1993 for details). The three possible serine to alanine mutations that were constructed are indicated by solid arrows. The cytoplasmic domain was deleted after the GFFKR pentapeptide (residue #1044) to create the α6-ΔCYT mutant subunit.

Figure 2. Construction of integrin cytoplasmic domain mutations. The complete amino acid sequence of the human α6A cytoplasmic domain is shown (see Tamura et al., 1990; Hogevoorst et al., 1991; Shaw et al., 1993 for details). The three possible serine to alanine mutations that were constructed are indicated by solid arrows. The cytoplasmic domain was deleted after the GFFKR pentapeptide (residue #1044) to create the α6-ΔCYT mutant subunit.

Figure 3. Surface expression of the human α6A and α6-ΔCYT integrins in P388D1 transfectants. Populations of transfected P388D1 cells expressing either the α6A or α6-ΔCYT cDNAs were isolated by sequential FACS using 2B7, a mAb specific for the human α6 integrin subunit (Shaw et al., 1993), and then analyzed by flow cytometry. (A) Secondary Ab alone; (B) Wild-type α6A; (C) α6-ΔCYT; (D) Overlay of α6A (solid line) and α6-ΔCYT (dotted line) FACS

The Journal of Cell Biology, Volume 123, 1993
Surface expression of the human/mouse α6β1 and α6-ΔCYTβ1 integrin chimeras in P388D1 cells. The transfected cells shown in Fig. 2 were surface labeled with biotin, and aliquots of detergent extracts from equal numbers of cells were immunoprecipitated with the 2B7 mAb. Immunoprecipitates were resolved by 12% SDS-PAGE under reducing conditions and transferred to nitrocellulose filters. Proteins were visualized with streptavidin conjugated to horseradish peroxidase and enhanced chemiluminescence. 

(A) The migration positions of the light chains of the wild-type α6 and α6-ACYT subunits are shown in the right margin. Both are doublets. The light chain of the α6-ACYT subunit migrates faster than the wild-type α6 light chain due to the deletion of ~3kD. The extra bands between 97 and 45 kD result from non-specific binding to protein G-sepharose (data not shown). (Arrowhead) The light chain of the 2B7 mAb. 

(B) Shorter exposure of the blot shown in A. The α6 and β1 subunits are resolved in this exposure and are indicated in the right margin. (C) Unlabeled cell extracts were immunoprecipitated with the 2B7 mAb, resolved by 10% SDS-PAGE under reducing conditions, transferred to nitrocellulose filters, and blotted with a polyclonal antiserum specific for the β1 subunit.

Adhesive properties of P388D1 cells transfected with the α6A and α6-ΔCYT cDNAs. Transfected cells were enriched for α6Aβ1 and α6-ΔCYTβ1 integrin surface expression by FACS using 2B7, and then assayed for their ability to adhere to laminin and fibronectin substrata. Tissue culture wells were coated with either EHS laminin (20 μg/ml) or human fibronectin (20 μg/ml). Transfected cells (1.5 x 10⁵) were resuspended in RPMI and added to the protein coated wells. PMA (50 ng/ml) was added to some of the wells. After 1 h at 37°C, non-adherent cells were removed by washing, and 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. (Solid bars) control transfectants; (hatched bars) PMA-stimulated transfectants; (Neo) P388D1, cells transfected with the vector alone.

Divalent cation modulation of laminin adhesion. Cells were resuspended in Puck's Saline A containing either 1.8 mM Ca⁺⁺ and 0.8 mM Mg⁺⁺ or 150 μM Mn⁺⁺, and added to laminin-coated wells at a concentration of 1.5 x 10⁵ cells per well. PMA (50 ng/ml) was added to some of the wells and the multiwell plates were incubated for 1 h at 37°C. Non-adherent cells were removed by washing with Puck's Saline A and 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. (A) TG-elicited macrophages; (B) Human α6A, α6-ΔCYT, and Neo P388D1 transfectants.
Figure 8. Adhesive properties of P388D1 cells transfected with the α6A serine mutant subunit cDNAs. Transfected cells were enriched for α6A, α6A-M1, α6A-M2, and α6A-M3 integrin surface expression by FACS as described in Fig. 7, and then assayed for their ability to adhere to laminin. Transfected cells (1.5 x 10⁶) were resuspended in RPMI and added to the laminin coated wells. PMA (50 ng/ml) was added to some of the wells. After 1 h at 37°C, non-adherent cells were removed by washing and adherent cells were fixed, stained, and quantitated as described in Materials and Methods. The data shown are the mean values (±SEM) from five experiments, each done in triplicate. (Solid bars) control transfectants; (hatched bars) PMA-stimulated transfectants.

of the transfectants increased their adhesion to laminin in response to PMA activation. The levels of laminin adhesion observed for the serine mutants were comparable to those observed for the wild-type α6A transfectants. This result provides evidence that serine phosphorylation is not essential for the ability of α6β1 to mediate laminin adhesion.

**Discussion**

Integrin cytoplasmic domains can regulate the ligand-binding function of their extracellular domains (for review see Ginsberg et al., 1992; Hynes, 1992). However, the mechanisms involved in this regulation are not well understood and, in fact, may differ for individual integrins. In the present study, the role of the α6 integrin cytoplasmic domain in the laminin receptor function of the α6β1 integrin was examined. The use of P388D1 cells, an α6-integrin deficient cell line, facilitated this analysis because, as we have shown previously, expression of either the α6A or α6B cDNAs restores their activation responsive laminin adhesion (Shaw et al., 1993). Deletion of the α6 cytoplasmic domain resulted in the surface expression of a truncated α6β1 integrin that
was unable to mediate laminin adhesion under normal conditions, i.e., in medium that contained physiological concentrations of Ca\(^{2+}\) and Mg\(^{2+}\), even after activation with PMA. This finding provides evidence for a novel mode of integrin regulation that is distinct from that reported for other regulated integrins such as \(\alpha I\beta3\) (O'Toole et al., 1991) and \(\alpha L\beta2\) (Hibbs et al., 1991a). Because the \(\alpha 6\alpha\) cytoplasmic domain is phosphorylated on serine residues in response to stimuli that activate the laminin receptor function of \(\alpha 6\beta 1\) (Shaw et al., 1990; Hogerworst et al., 1993), we examined the role of the two serine residues in this cytoplasmic domain by site-directed mutagenesis and subsequent expression in P388D cells. The results obtained indicate that serine phosphorylation is not essential for adhesion to laminin.

Previous studies have focused on the role of integrin subunit cytoplasmic domains in the regulated function of the \(\alpha I\beta3\) and \(\alpha L\beta2\) integrins. Deletion of the \(\alpha 1\) cytoplasmic domain resulted in the generation of a constitutively active \(\alpha 1\beta3\) receptor (O'Toole et al., 1991), but deletion of the \(\alpha L\beta2\) cytoplasmic domain had no effect on the \(\alpha L\beta2\) receptor function (Hibbs et al., 1991a). In contrast, deletion of the \(\beta 3\) cytoplasmic domain had no effect on the \(\alpha I\beta3\) receptor function (O'Toole et al., 1991), but deletion of the \(\beta 2\) cytoplasmic domain generated an inactive \(\alpha L\beta2\) receptor (Hibbs et al., 1991a). It has also recently been shown that deletion of the \(\alpha 1\) and \(\alpha 5\) cytoplasmic domains did not affect the ability of these mutant \(\alpha 1\beta 1\) or \(\alpha 5\beta 1\) receptors to mediate adhesion to their respective ligands (Briesewitz et al., 1993; Bauer et al., 1993). Our finding that deletion of the \(\alpha 6\) cytoplasmic domain resulted in the abolition of \(\alpha 6\beta 1\) receptor function and activation implies a mechanism of integrin regulation that is distinct from that of \(\alpha I\beta3\), \(\alpha L\beta2\), \(\alpha 1\beta 1\), and \(\alpha 5\beta 1\) because each of the \(\alpha\) subunit cytoplasmic domains contributes differently to the function of these integrin receptors.

Similarities exist, however, between the \(\alpha 6\) and \(\beta 2\) subunits because their deletion abolishes PMA-dependent receptor activation (cf Fig. 4 and Hibbs et al., 1991a). In addition, COS cells expressing the \(\alpha L\beta2\) integrin that contained a deleted \(\beta 2\) cytoplasmic domain were induced to mediate adhesion to ICAM-1 by a mAb, NKL-L16, that stimulates \(\alpha L\beta2\) function in the absence of PMA stimulation (Hibbs et al., 1991a). This result is similar to our finding that P388D cells expressing the truncated \(\alpha 6\Delta CYT\beta 1\) integrin adhered to laminin if \(Ca^{2+}\) and \(Mg^{2+}\) in the culture medium were replaced with Mn\(^{2+}\). Thus, although physiological regulation of \(\alpha 6\beta 1\) function probably occurs through signaling pathways that affect the \(\alpha 6\) cytoplasmic domain, it is possible to induce receptor function by modulating the extracellular domain with divalent cations.

A key question that arises from this study is how the \(\alpha 6\) cytoplasmic domain regulates the function of the \(\alpha 6\beta 1\) integrin. Because the \(\alpha 6\) cytoplasmic domain is required for receptor activation, it can be proposed that this domain associates with a "positive regulator" upon cell activation that alters the function of the receptor, either through changes in extracellular ligand binding affinity (Ginsberg et al., 1992) or avidity (Danilov and Juliano, 1989). The use of mAbs that recognize only "activated" forms of the receptors provide evidence that, in many cases, a conformational change in the integrin heterodimer occurs after activation (Dransfield and Hogg, 1989; O'Toole et al., 1991; Diamond and Springer, 1993). In fact, Sims et al. (1991) were able to directly demonstrate a change in conformation of the \(\alpha I\beta3\) integrin heterodimer upon activation using resonance energy transfer. mAbs that promote adhesion have also been described and these antibodies are presumed to mimic the effects of physiological activation on receptor function, this important point has not been clearly demonstrated. Changes in conformation could also facilitate clustering of receptors (Dettmers et al., 1987) or their association with heterologous proteins (Brown et al., 1990; Shaw et al., 1990) that would increase the avidity of integrins for their ligands. Changes in affinity and avidity are not mutually exclusive and may work in concert to facilitate integrin-mediated adhesion. In the case of \(\alpha 6\beta 1\), physiological activation could increase the affinity of this integrin for laminin and it could also promote the linkage of the \(\alpha 6\) cytoplasmic domain with the cytoskeleton. This latter possibility is supported by our previous finding that the activation-dependent adhesion of macrophages to laminin involves the association of the \(\alpha 6\beta 1\) integrin with the actin cytoskeleton (Shaw et al., 1990).

Although there have been no reports of cytoskeletal proteins binding directly to \(\alpha\) subunit cytoplasmic domains, the data presented in this paper, as well as other recent studies (Chan et al., 1992; Tawil et al., 1993; Ylanne et al., 1993; Briesewitz et al., 1993) suggest this possibility. Chan et al. (1992) constructed chimeric integrin subunits that consisted of the extracellular and transmembrane domains of the \(\alpha 2\) subunit and the cytoplasmic domains of either the \(\alpha 2\), \(\alpha 4\), or \(\alpha 5\) subunits. When transfected into a rhabdomyosarcoma cell line, RD, the wild-type \(\alpha 2\) subunit and the \(\alpha 2/\alpha 5\) chimera promoted contraction of collagen gels, while the \(\alpha 2/\alpha 4\) chimera promoted cell migration on a laminin substratum. However, adhesion to either substratum was not altered. Contraction and motility require markedly different cytoskeletal rearrangements, and these results suggest that each \(\alpha\) subunit cytoplasmic domain may interact with unique cytoskeletal components. In addition, \(\alpha\) subunit cytoplasmic domains have been implicated in the preferential association of laminin receptor integrins with either focal contacts or podosomes (Tawil et al., 1993). The \(\alpha\) subunits may also play a role in regulating the recruitment of integrin receptors to focal contacts (Briesewitz et al., 1993; Ylanne et al., 1993). Comparison of these studies on \(\alpha\) subunit cytoplasmic domains with the data on \(\beta 1\) integrin cytoplasmic domains suggests that the \(\beta 1\) cytoplasmic domain provides a critical linkage with the cytoskeleton that is essential for integrin-mediated adhesion (Solowska et al., 1989; Hayashi et al., 1990; Marcantonio et al., 1990; Reszka et al., 1992). The \(\alpha\) subunit cytoplasmic domains may interact with a different cluster of cytoplasmic/cytoskeletal proteins that modulate specific aspects of integrin function subsequent to adhesion (e.g., the ability to promote cell migration or contraction). In addition, the results obtained in our study suggest that some \(\alpha\) subunit cytoplasmic domains may have important regulatory functions as targets of intracellular signaling pathways. Identification of proteins that interact with the \(\alpha 6\) cytoplasmic domain upon cell activation should provide considerable insight into the nature of \(\alpha\) subunit...
function. Such information would also be useful in addressing the unresolved issue of how cytoplasmic domains regulate the extracellular function of integrin receptors.

Although the α6β1 cytoplasmic domain is phosphorylated on serine residues in response to PMA and cytokine stimulation, the results obtained in this study demonstrate clearly that this phosphorylation is not required for α6β1-dependent laminin adhesion. In addition, it has been reported recently that the α6β1 integrin is not phosphorylated in response to PMA stimulation (Hogervorst et al., 1993), even though α6β1 can function as an activation-dependent laminin receptor (Shaw et al., 1993). Taken together, the conclusion can be drawn that serine phosphorylation is not essential for the ability of either α6β1 or α6β1 to function as a laminin receptor. This conclusion is in agreement with related studies that have been done on αLβ2 (Hibbs et al., 1991b). However, it would be premature to exclude any role for phosphorylation in α6β1 function at this point. Specifically, the possibility that phosphorylation is required for events that occur subsequent to attachment such as activation of cell motility or other such processes merits investigation. As discussed above, the contribution of α5 subunit cytoplasmic domains in “outside-in” signaling functions of integrin receptors has been demonstrated (Chan et al., 1992; Tawil et al., 1993). In this direction, determining the distinct functions mediated by α6β1 and α6β1 would be fruitful because α6β1 is not phosphorylated (Hogervorst et al., 1993).

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