

# Regulation of $\alpha 6\beta 1$ Integrin Laminin Receptor Function by the Cytoplasmic Domain of the $\alpha 6$ Subunit

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**Abstract.** The  $\alpha 6\beta 1$  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  $\alpha 6$  subunit cytoplasmic domain in  $\alpha 6\beta 1$  integrin activation was examined. The use of P388D<sub>1</sub> cells, an  $\alpha 6$ -integrin deficient macrophage cell line, facilitated this analysis because expression of either the  $\alpha 6A$  or  $\alpha 6B$  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  $\alpha 6$  cDNA,  $\alpha 6$ - $\Delta$ CYT, was constructed in which the human cytoplasmic domain sequence was deleted after the GFFKR pentapeptide. Expression of this cDNA in P388D<sub>1</sub> cells resulted in the surface expression of a chimeric  $\alpha 6$ - $\Delta$ CYT $\beta 1$  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<sup>++</sup> and Mg<sup>++</sup>. The  $\alpha 6A$ - $\Delta$ CYT transfectants adhered to laminin, however, when Ca<sup>++</sup>/Mg<sup>++</sup> was replaced with 150  $\mu$ M Mn<sup>++</sup>. We also assessed the role of

serine phosphorylation in the regulation of  $\alpha 6A\beta 1$  integrin function by site-directed mutagenesis of the two serine residues present in the  $\alpha 6A$  cytoplasmic domain because this domain is phosphorylated on serine residues in response to stimuli that activate the laminin receptor function of  $\alpha 6A\beta 1$ . Point mutations were introduced in the  $\alpha 6A$  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. P388D<sub>1</sub> transfectants which expressed these serine mutations adhered to laminin in response to PMA to the same extent as cells transfected with wild-type  $\alpha 6A$  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. F. 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  $\alpha 6A$  cytoplasmic domain is not involved in this regulation.

**T**HE 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  $\beta 2$  integrins (Dustin and Springer, 1991; Hermanowski-Vosatka et al., 1992), the  $\alpha IIb\beta 3$  platelet integrin (Du et al., 1991), and the  $\alpha 6\beta 1$  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-

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grins are likely targets of intracellular signaling pathways. For example, the cytoplasmic domains of the  $\beta 2$  (Chatila et al., 1989),  $\beta 3$  (Hillery et al., 1991), and  $\alpha 6$  (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  $\alpha L\beta 2$  and  $\alpha IIb\beta 3$  integrins has been used to examine their role in inside-out signaling. From these studies, it appears that the  $\beta 2$  cytoplasmic domain of the  $\alpha L\beta 2$  integrin is critical for its regulation because deletion of the  $\beta 2$  cytoplasmic domain, but not the  $\alpha L$  cytoplasmic domain, resulted in an inactive receptor that could no longer respond to PMA activation (Hibbs et al., 1991a). Site-directed mutagenesis of the serine residue in the  $\beta 2$  subunit that is phosphorylated in response to PMA did not alter the activation of the  $\alpha L\beta 2$  receptor, indicating that phosphorylation may not be an important mechanism for activation of receptor function (Hibbs et al., 1991b). In contrast to the results obtained with  $\alpha L\beta 2$ , it was observed for  $\alpha IIb\beta 3$  that deletion of the  $\alpha IIb$  cytoplasmic domain, but not the  $\beta 3$  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  $\alpha L\beta 2$  and  $\alpha IIb\beta 3$  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  $\alpha 6\beta 1$  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  $\alpha 6$  subunit (Shaw et al., 1990). Based on these findings and the disparate results obtained with the  $\alpha L\beta 2$  (Hibbs et al., 1991a,b) and  $\alpha IIb\beta 3$  integrins (O'Toole et al., 1991), we thought it was important to investigate the role of the  $\alpha 6$  subunit cytoplasmic domain in the regulation of  $\alpha 6\beta 1$  function. This analysis was facilitated by our recent finding that the activation responsive adhesion of the  $\alpha 6\beta 1$  integrin is maintained when the  $\alpha 6$  subunit is expressed by cDNA transfection in P388D<sub>1</sub> cells, an  $\alpha 6$ -deficient macrophage cell line (Shaw et al., 1993). In this report, we provide evidence for a mechanism of integrin regulation that is distinct from  $\alpha L\beta 2$  and  $\alpha IIb\beta 3$  because deletion of the  $\alpha 6$  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  $\alpha 6$  cytoplasmic domain did not inhibit cell attachment to laminin.

## Materials and Methods

### Cells

The P388D<sub>1</sub> 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). Thiolglycollate-elicited (TG)<sup>1</sup> macrophages were obtained from C57BL/6J mice

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

(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  $\mu$ g/ml of murine Englebreth-Holm-Swarm (EHS) laminin or 20  $\mu$ g/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  $1-2 \times 10^5$  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  $\alpha 6$  subunit (Shaw et al., 1993), and murine IgG Fc fragment (20  $\mu$ g/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 CaCl<sub>2</sub> and MgCl<sub>2</sub>. After washing, the macrophages were resuspended in the same buffer at a concentration of  $5 \times 10^6$  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 NH<sub>4</sub>Cl 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 CaCl<sub>2</sub> and MgCl<sub>2</sub>, 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  $\alpha 6$  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  $\mu$ g/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  $\alpha 6$  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  $\beta 1$  (Marcantonio and Hynes, 1988). After washing, the filters were incubated with a goat anti-rabbit IgG conjugated to horseradish peroxidase (0.2  $\mu$ g/ml; Kirkegaard and Perry). Protein was detected by enhanced chemiluminescence (Amersham Corp.).

### Site-directed Mutagenesis

The human  $\alpha 6A$  cDNA was cloned by PCR and subcloned into the eukaryotic expression vector pRc/CMV as described previously (Shaw et al.,

1993). Unless otherwise noted, all enzymes were purchased from New England Biolabs (Beverly, MA). The  $\alpha 6$  cytoplasmic truncation was generated by introducing a stop codon (bold face) into an oligonucleotide primer for PCR. This primer, 5'-AGCAGAAGCTTTCATCTCTTGAAGAAA-3' corresponds to nucleotides 3278 to 3266 (Tamura et al., 1990). A Hind III recognition sequence (underlined) was added to the 5' end of this primer to facilitate cloning. A second primer, 5'-CAATTACAGCTAAAGC-3', which corresponds 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). An XbaI-HindIII fragment was removed by digestion and inserted into the  $\alpha 6A$  cDNA in pRc/CMV after removal of the corresponding wild-type XbaI-HindIII fragment.

Site-directed mutagenesis of the  $\alpha 6A$  cytoplasmic domain was carried out by overlap extension. Individual serine 1064 to alanine 1064 and serine 1071 to alanine 1071 mutations in the  $\alpha 6A$  cDNA were generated by using pairs of complementary mutagenic oligonucleotide primers (5'-TGCTCAGCCAGCTGATAAAGA-3' and 5'-GAGGCTTACTGCTGATGCATA-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'-AGTTTGGGTACTGTGAAGCT-3' which correspond to nucleotides 2497-2512 and 3592-3573, respectively. Two segments (2497-3346 and 3326-3592 and 2497-3367 and 3347-3592) were generated in separate reactions, each containing one set of outer and inner mutagenic primers and 0.1  $\mu$ g plasmid containing the  $\alpha 6A$  cDNA. For overlap extension by PCR, amplified products from the first round of PCR were purified from an agarose gel and mixed in a subsequent PCR reaction containing additional outer primer pairs. The resulting PCR generated recombinant products were purified from an agarose gel and digested with XbaI and HindIII. This XbaI-HindIII fragment was purified from an agarose gel and sub-cloned into the  $\alpha 6A$  cDNA as described above for the cytoplasmic deletion. The double serine mutant was generated by overlap extension using one of the single serine mutant cDNAs as the starting template. All of the PCR reactions were performed using the following conditions: 1 cycle of 94°C for 4 min; 35 cycles of 94°C for 1 min and 50°C for 1.5 min; and 1 cycle of 50°C for 7 min. The nucleotide sequences of the XbaI-HindIII PCR products for the cytoplasmic deletion mutant and the serine mutants were confirmed by dideoxy sequencing.

### cDNA Transfections

The pRc/CMV vector containing the human  $\alpha 6A$  and mutant  $\alpha 6A$  subunits, and the vector alone were transfected into the P388D<sub>1</sub> cell line with lipofectin (GIBCO BRL). Neomycin resistant clones were isolated by selective growth in medium containing G418 (0.4 mg/ml; GIBCO BRL). The stable transfectants were pooled and the population of cells that express the human  $\alpha 6$  subunit on the cell surface was isolated by FACS. A human  $\alpha 6$  integrin specific 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 isolate a homogeneous population of cells expressing the transfected  $\alpha 6$  subunits.

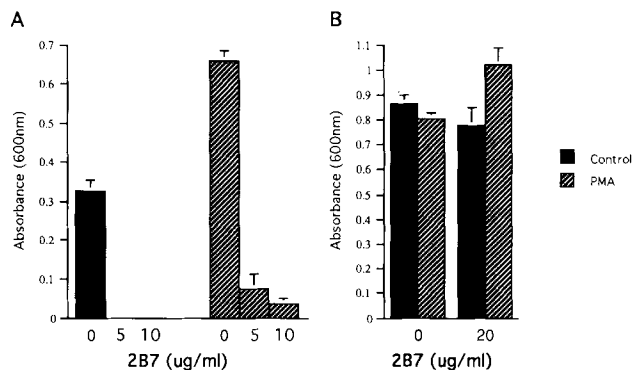
### Flow Cytometry

Transfected P388D<sub>1</sub> cells were washed twice with PBS containing 0.1% BSA (PBS/BSA). Aliquots of cells ( $3-5 \times 10^5$ ) were incubated for 30 min at 4°C with PBS/BSA containing murine IgG Fc fragment (6  $\mu$ g/ml; Jackson ImmunoResearch Labs, Inc.). The mAb 2B7 was added at a concentration of 2  $\mu$ g/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)<sub>2</sub> anti-mouse IgG coupled to fluorescein (Tago, Inc., 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 Immunocytometry Sys., Mountain View, CA).

## Results

### Construction of $\alpha 6$ Cytoplasmic Domain Mutants

Previously, we reported that expression of the  $\alpha 6A$  integrin cDNA in P388D<sub>1</sub> 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  $\alpha 6$



**Figure 1.** mAb inhibition of  $\alpha 6A$ -P388D<sub>1</sub> adhesion to laminin (A) and fibronectin (B). Tissue culture wells were coated with either EHS laminin (20  $\mu$ g/ml) or human fibronectin (20  $\mu$ g/ml). Transfected cells ( $1.5 \times 10^5$ ) were resuspended in RPMI containing 1% FCS and preincubated with the 2B7 mAb and murine IgG Fc fragment (20  $\mu$ g/ml) for 30 min at 4°C. The cells were then 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 ( $\pm$ SD) from a representative experiment done in triplicate. (Solid bars) control transfectants; (hatched bars) PMA-stimulated transfectants. In these assays,  $\sim 80\%$  of the  $\alpha 6A$ -P388D<sub>1</sub> transfectants added adhere to laminin.

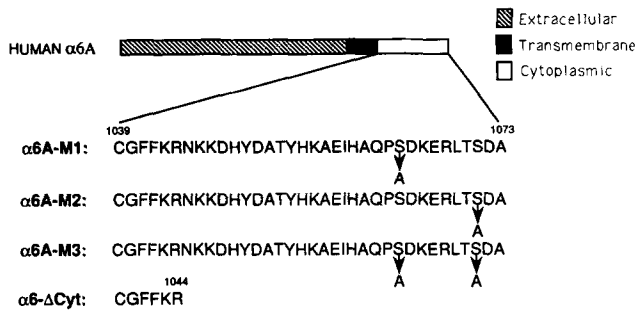
specific mAb (Fig. 1). The experimental approach taken in this study was to mutate the cytoplasmic domain sequence of  $\alpha 6A$  and to express the mutated cDNA in P388D<sub>1</sub> cells, an  $\alpha 6$ -deficient macrophage cell line. Initially, we deleted the cytoplasmic domain by introducing a stop codon in the  $\alpha 6A$  cDNA after the sequence that encodes the GFFKR pentapeptide. This sequence is conserved in both the  $\alpha 6A$  and  $\alpha 6B$  subunits (Tamura et al., 1990; Hogervorst et al., 1991), as well as in all other integrin  $\alpha$  subunits (Hemler, 1990). The deletion was made after this pentapeptide to facilitate expression of the truncated subunit because very low levels of  $\alpha$ IIb $\beta$ 3 expression were observed when this sequence was included in a deletion of the  $\alpha$ IIb subunit cytoplasmic domain (O'Toole et al., 1991). The  $\alpha 6$  insertional mutation resulted in a cDNA, termed  $\alpha 6$ - $\Delta$ CYT, that lacked any  $\alpha 6A$  or  $\alpha 6B$  specific cytoplasmic domain sequences (Fig. 2).

We also assessed the role of serine phosphorylation in the regulation of  $\alpha 6A\beta 1$  integrin function by site-directed mutagenesis of the two serine residues present in the  $\alpha 6A$  cytoplasmic domain. Point mutations were introduced in the  $\alpha 6A$  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 mutant  $\alpha 6$  cDNAs were subcloned into the eukaryotic expression vector pRc/CMV and transfected into P388D<sub>1</sub> cells. After selective growth in medium containing G418, the population of cells that expressed the human-mouse chimeric  $\alpha 6\beta 1$  integrin was isolated by sequential cycles of FACS using 2B7, a mAb that is specific for the human  $\alpha 6$  integrin subunit (Shaw et al., 1993).

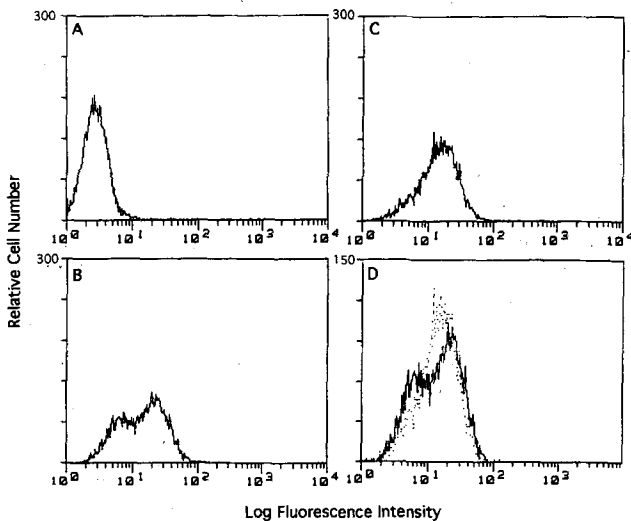
### Analysis of the $\alpha 6$ Cytoplasmic Deletion Subunit

Populations of P388D<sub>1</sub> cells were obtained that expressed



**Figure 2.** Construction of integrin cytoplasmic domain mutations. The complete amino acid sequence of the human  $\alpha 6A$  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 GFFFKR pentapeptide (residue #1044) to create the  $\alpha 6$ - $\Delta$ CYT mutant subunit.

levels of surface  $\alpha 6$ - $\Delta$ CYT comparable to those obtained after transfection of the wild-type  $\alpha 6A$  cDNA (Fig. 3). To confirm that the cytoplasmic domain of the  $\alpha 6$ - $\Delta$ CYT subunit was deleted, the transfected cells were surface labeled by biotinylation and detergent extracts were immunoprecipitated with the 2B7 mAb (Fig. 4). As expected from the cDNA sequence, the light chain of the  $\alpha 6$ - $\Delta$ CYT subunit migrates slightly faster ( $\sim 3$  kD) than the light chain of the wild-type  $\alpha 6A$  subunit on reducing gels because it is missing 29 amino acids of its cytoplasmic domain (Fig. 4 A). The light chain of the  $\alpha 6A$  subunit appears as a doublet as previously reported (Hogervorst et al., 1993). A shorter exposure of the same blot reveals that the  $\beta 1$  subunit coimmunoprecipitates with both the  $\alpha 6A$  and  $\alpha 6$ - $\Delta$ CYT subunits (Fig. 4 B). The identity of the  $\beta 1$  subunit was confirmed by immu-



**Figure 3.** Surface expression of the human  $\alpha 6A$  and  $\alpha 6$ - $\Delta$ CYT integrins in P388D<sub>1</sub> transfectants. Populations of transfected P388D<sub>1</sub> cells expressing either the  $\alpha 6A$  or  $\alpha 6$ - $\Delta$ CYT cDNAs were isolated by sequential FACS using 2B7, a mAb specific for the human  $\alpha 6$  integrin subunit (Shaw et al., 1993), and then analyzed by flow cytometry. (A) Secondary Ab alone; (B) Wild-type  $\alpha 6A$ ; (C)  $\alpha 6$ - $\Delta$ CYT; (D) Overlay of  $\alpha 6A$  (solid line) and  $\alpha 6$ - $\Delta$ CYT (dotted line) FACS scans.

noblotting the 2B7 immunoprecipitates with a polyclonal antiserum specific for the  $\beta 1$  cytoplasmic domain (Marcantonio and Hynes, 1988). This antiserum recognized the  $\beta 1$  subunit in 2B7 immunoprecipitates from both the wild-type  $\alpha 6A$  and the  $\alpha 6$ - $\Delta$ CYT transfectants (Fig. 4 C).

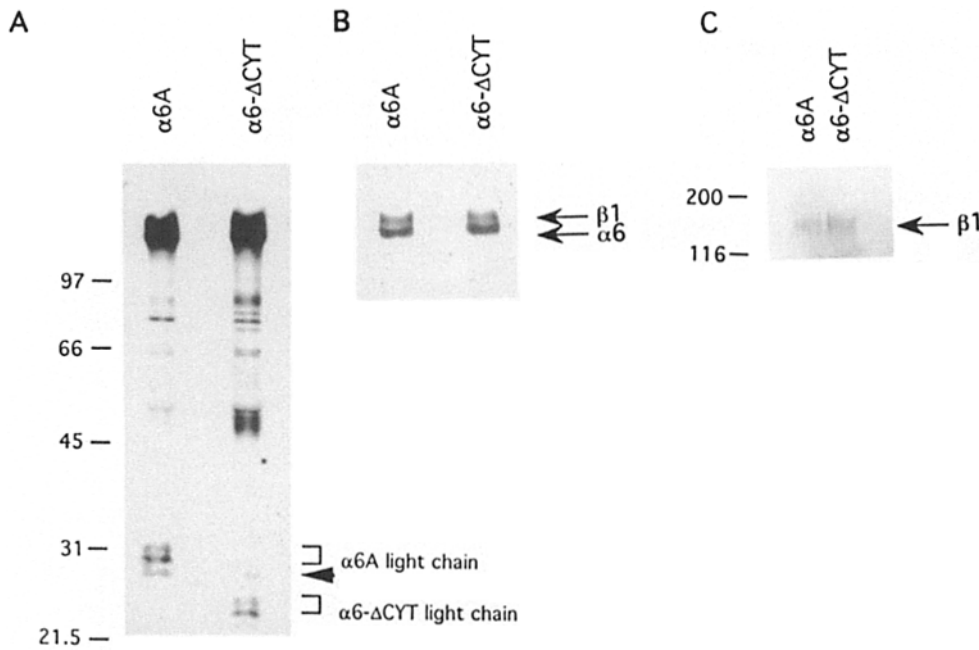
The  $\alpha 6A$  and  $\alpha 6$ - $\Delta$ CYT integrin transfectants were examined for their ability to adhere to a laminin substratum. As shown in Fig. 5, the  $\alpha 6A$  transfectants exhibited a low level of laminin adhesion that was significantly increased in response to PMA stimulation. However, the  $\alpha 6$ - $\Delta$ CYT transfectants did not adhere to laminin, even after stimulation with PMA (Fig. 5). The slight increase in absorbance observed for the  $\alpha 6$ - $\Delta$ CYT cells after PMA stimulation is equal to that observed for cells transfected with vector alone (neo) and is not significant. Moreover, this result does not reflect a non-specific adhesion defect because these cells adhered normally to a fibronectin substratum (Fig. 5). The inability of the  $\alpha 6$ - $\Delta$ CYT cDNA to restore the ability of P388D<sub>1</sub> macrophages to adhere to laminin demonstrates that sequences within the  $\alpha 6$  cytoplasmic domain are critical for the  $\alpha 6\beta 1$  integrin to function as a laminin receptor.

Mouse macrophages do not adhere to laminin in normal cell culture medium that contains physiological concentrations of  $Ca^{++}$  and  $Mg^{++}$ , unless activated by PMA or cytokines (Mercurio and Shaw, 1988; Shaw and Mercurio, 1989). Based on several reports which indicate that divalent cations can influence integrin ligand binding (Sonnenberg et al., 1988; Kirchhofer et al., 1991; Dransfield et al., 1992), we examined the possibility that altering the cation composition of the medium would promote macrophage adhesion to laminin in the absence of PMA activation. We found that TG-elicited macrophages adhered to laminin without PMA activation if  $Ca^{++}$  and  $Mg^{++}$  were replaced with  $150 \mu M Mn^{++}$  in the culture medium (Fig. 6 A). In contrast, macrophage adhesion to both fibronectin and tissue culture plastic was not dependent on the presence of specific divalent cations (data not shown). These observations suggest that at least two distinct mechanisms will promote macrophage adhesion to laminin: inside-out signaling through integrin cytoplasmic domains and divalent cation interactions with extracellular domains.

The divalent cation data prompted us to examine whether extracellular  $Mn^{++}$  could promote laminin adhesion of the  $\alpha 6$ - $\Delta$ CYT transfectants. For the wild-type  $\alpha 6A$  transfectants, the presence of  $150 \mu M Mn^{++}$ , in the absence of  $Ca^{++}/Mg^{++}$ , resulted in a level of laminin adhesion that was equivalent to that observed after PMA activation (Fig. 6 B). Interestingly, the  $\alpha 6$ - $\Delta$ CYT transfectants also adhered to laminin in the presence of  $Mn^{++}$  to the same extent as the wild-type transfectants and this adhesion was not influenced by PMA activation (Fig. 6 B). P388D<sub>1</sub> cells transfected with the pRc/CMV plasmid alone did not adhere to laminin under any of the conditions tested (Fig. 6 B). Taken together with the results shown in Fig. 5, these data indicate that the  $\alpha 6$  cytoplasmic domain is essential for physiological regulation of  $\alpha 6\beta 1$  laminin receptor function. However, the divalent cation data provide evidence that the extracellular domain of  $\alpha 6\beta 1$  can be regulated independently of the  $\alpha 6$  cytoplasmic domain.

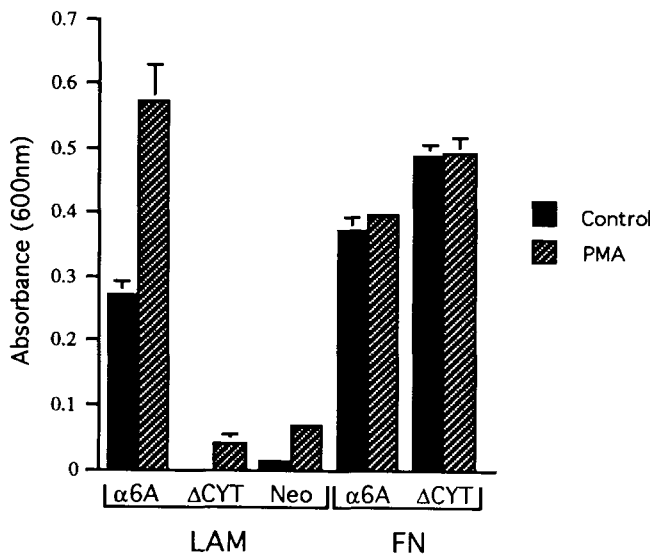
#### Analysis of the $\alpha 6A$ Serine Mutants

Populations of P388D<sub>1</sub> cells were obtained that expressed



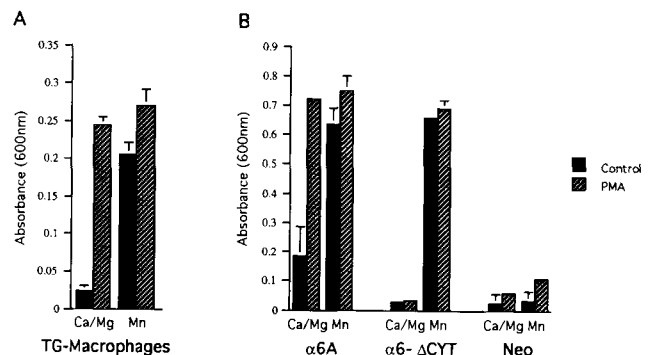
**Figure 4.** Surface expression of the human/mouse  $\alpha 6 A \beta 1$  and  $\alpha 6 - \Delta C Y T \beta 1$  integrin chimeras in P388D<sub>1</sub> cells. The transfected cells shown in Fig. 2 were surface labeled with biotin, and aliquots of detergent extracts from equal numbers of cells were 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  $\alpha 6 A$  and  $\alpha 6 - \Delta C Y T$  subunits are shown in the right margin. Both are doublets. The light chain of the  $\alpha 6 - \Delta C Y T$  subunit migrates faster than the wild-type  $\alpha 6 A$  light chain due to the deletion of  $\sim 3$  kD. 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  $\alpha 6$  and  $\beta 1$  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 antisera specific for the  $\beta 1$  subunit.

(A) The migration positions of the light chains of the wild-type  $\alpha 6 A$  and  $\alpha 6 - \Delta C Y T$  subunits are shown in the right margin. Both are doublets. The light chain of the  $\alpha 6 - \Delta C Y T$  subunit migrates faster than the wild-type  $\alpha 6 A$  light chain due to the deletion of  $\sim 3$  kD. 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  $\alpha 6$  and  $\beta 1$  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 antisera specific for the  $\beta 1$  subunit.

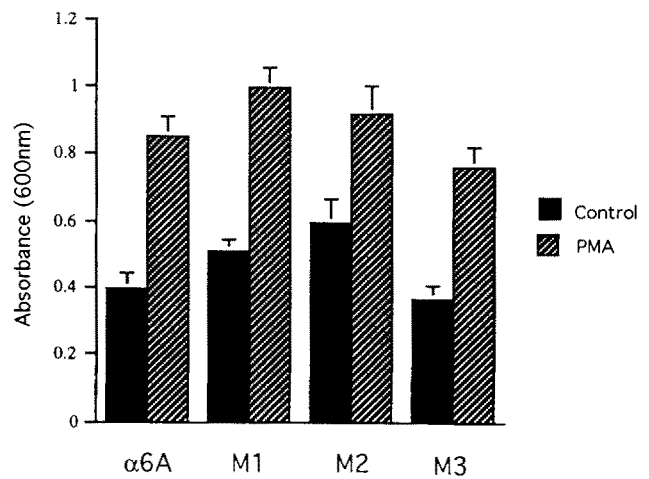
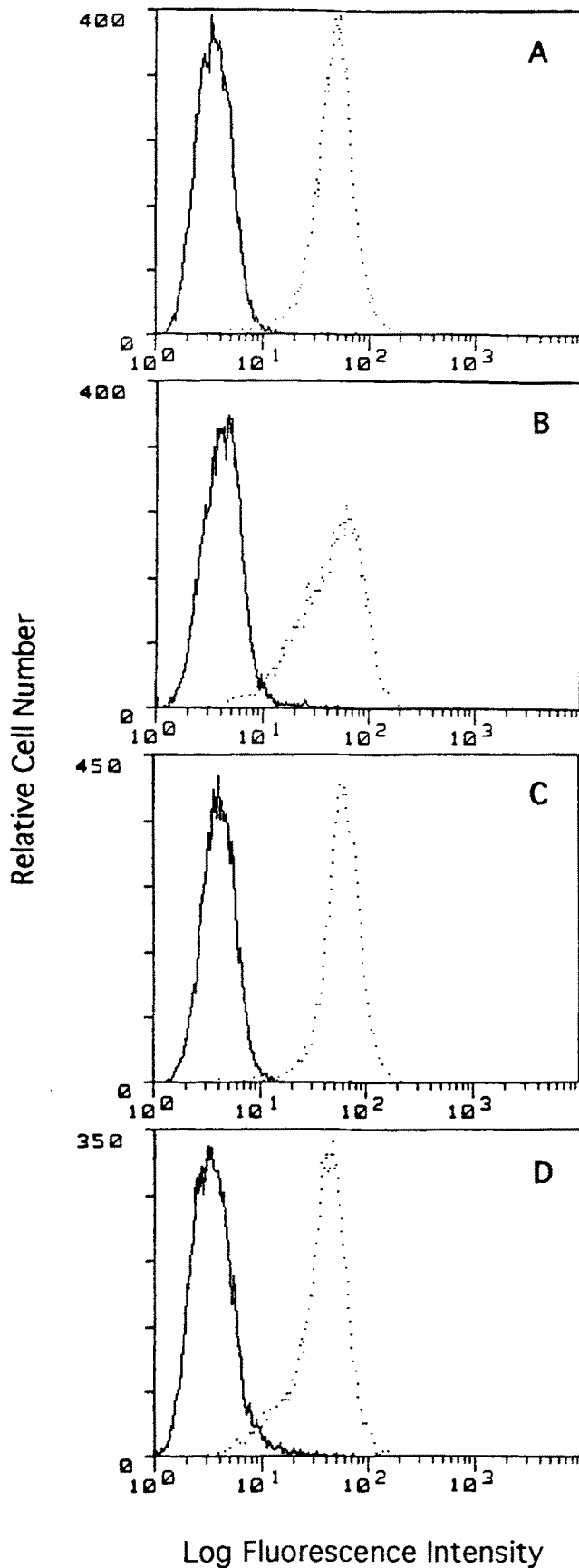


**Figure 5.** Adhesive properties of P388D<sub>1</sub> cells transfected with the  $\alpha 6 A$  and  $\alpha 6 - \Delta C Y T$  cDNAs. Transfected cells were enriched for  $\alpha 6 A \beta 1$  and  $\alpha 6 - \Delta C Y T \beta 1$  integrin surface expression by FACS using 2B7, and then assayed for their ability to adhere to laminin and fibronectin substrata. Tissue culture wells were coated with either EHS laminin (20  $\mu$ g/ml) or human fibronectin (20  $\mu$ g/ml). Transfected cells ( $1.5 \times 10^5$ ) 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 ( $\pm$ SD) from a representative experiment done in triplicate. (Solid bars) control transfectants; (hatched bars) PMA-stimulated transfectants; (Neo) P388D<sub>1</sub> cells transfected with the vector alone.

levels of the  $\alpha 6 A$  serine mutant subunits on the cell surface comparable to those obtained after transfection of the wild-type  $\alpha 6 A$  cDNA (Fig. 7). To examine the importance of serine phosphorylation in adhesion to laminin, the sorted  $\alpha 6 A$ ,  $\alpha 6 A - M 1$ ,  $\alpha 6 A - M 2$ , and  $\alpha 6 A - M 3$  integrin transfectants were examined for their ability to adhere to a laminin substratum. As shown in Fig. 8, the serine mutant transfectants exhibited some constitutive adhesion to laminin. Most importantly, all



**Figure 6.** 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  $\mu$ M  $Mn^{++}$ , and added to laminin-coated wells at a concentration of  $1.5 \times 10^5$  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 ( $\pm$ SD) from a representative experiment done in triplicate. (A) TG-elicited macrophages; (B) Human  $\alpha 6 A$ ,  $\alpha 6 - \Delta C Y T$ , and Neo P388D<sub>1</sub> transfectants.



**Figure 8.** Adhesive properties of P388D<sub>1</sub> 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 ( $1.5 \times 10^5$ ) 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 ( $\pm$ 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 P388D<sub>1</sub> 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

**Figure 7.** Surface expression of the α6A serine mutants in P388D<sub>1</sub> cells. Populations of transfected P388D<sub>1</sub> cells expressing either: (A) the wild-type α6A; (B) α6A-M1; (C) α6A-M2; or (D) α6A-M3 cDNAs were isolated by sequential FACS using the 2B7 mAb, and then analyzed by flow cytometry using 2B7 as shown in this figure. (Solid line) Secondary mAb alone; (Dotted line) 2B7 mAb.

was unable to mediate laminin adhesion under normal conditions, i.e., in medium that contained physiological concentrations of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ , 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  $\alpha\text{IIb}\beta 3$  (O'Toole et al., 1991) and  $\alpha\text{L}\beta 2$  (Hibbs et al., 1991a). Because the  $\alpha 6\text{A}$  cytoplasmic domain is phosphorylated on serine residues in response to stimuli that activate the laminin receptor function of  $\alpha 6\text{A}\beta 1$  (Shaw et al., 1990; Hogervorst et al., 1993), we also examined the role of the two serine residues in this cytoplasmic domain by site-directed mutagenesis and subsequent expression in P388D<sub>1</sub> 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  $\alpha\text{IIb}\beta 3$  and  $\alpha\text{L}\beta 2$  integrins. Deletion of the  $\alpha\text{IIb}$  cytoplasmic domain resulted in the generation of a constitutively active  $\alpha\text{IIb}\beta 3$  receptor (O'Toole et al., 1991), but deletion of the  $\alpha\text{L}$  cytoplasmic domain had no effect on  $\alpha\text{L}\beta 2$  receptor function (Hibbs et al., 1991a). In contrast, deletion of the  $\beta 3$  cytoplasmic domain had no effect on  $\alpha\text{IIb}\beta 3$  receptor function (O'Toole et al., 1991), but deletion of the  $\beta 2$  cytoplasmic domain generated an inactive  $\alpha\text{L}\beta 2$  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  $\alpha\text{IIb}\beta 3$ ,  $\alpha\text{L}\beta 2$ ,  $\alpha 1\beta 1$ , 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  $\alpha\text{L}\beta 2$  integrin that contained a deleted  $\beta 2$  cytoplasmic domain were induced to mediate adhesion to ICAM-1 by a mAb, NKI-L16, that stimulates  $\alpha\text{L}\beta 2$  function in the absence of PMA stimulation (Hibbs et al., 1991a). This result is similar to our finding that P388D<sub>1</sub> cells expressing the truncated  $\alpha 6\text{-}\Delta\text{CYT}\beta 1$  integrin adhered to laminin if  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  in the culture medium were replaced with  $\text{Mn}^{++}$ . 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 dem-

onstrate a change in conformation of the  $\alpha\text{IIb}\beta 3$  integrin heterodimer upon activation using resonance energy transfer. mAbs that promote adhesion have also been described and these antibodies are presumed to induce a conformational change in the integrin heterodimers that increases their ligand-binding affinities (O'Toole et al., 1990; van Kooyk et al., 1991; Neugebauer and Reichardt, 1991; Faull et al., 1993; Arroyo et al., 1993). Although these activating mAbs 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 (Detmers 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  $\alpha 6A$  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  $\alpha 6A\beta 1$  dependent laminin adhesion. In addition, it has been reported recently that the  $\alpha 6B$  integrin is not phosphorylated in response to PMA stimulation (Hogervorst et al., 1993), even though  $\alpha 6B\beta 1$  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  $\alpha 6A\beta 1$  or  $\alpha 6B\beta 1$  to function as a laminin receptor. This conclusion is in agreement with related studies that have been done on  $\alpha L\beta 2$  (Hibbs et al., 1991b). However, it would be premature to exclude any role for phosphorylation in  $\alpha 6A\beta 1$  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  $\alpha$  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  $\alpha 6A\beta 1$  and  $\alpha 6B\beta 1$  would be fruitful because  $\alpha 6B\beta 1$  is not phosphorylated (Hogervorst et al., 1993).

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