Integrin receptors can mediate transmembrane signaling in response to ligand binding. To further examine the role of the integrin α subunit in these signaling functions, we assessed the contribution of the α6 cytoplasmic domain variants to the signaling properties of the α6β1 integrin using P388D1 cells that had been transfected with either the α6A or the α6B cDNA. The α6Aβ1 and α6Bβ1 receptors induced marked quantitative differences in the tyrosine phosphorylation of several proteins after binding to laminin. Specifically, the α6A cytoplasmic domain was more effective than the α6B cytoplasmic domain in inducing the tyrosine phosphorylation of three major proteins (molecular mass, 120, 110, and 76 kDa). In addition to these proteins, we also observed that the tyrosine phosphorylation of the cytoskeletal protein paxillin was increased significantly more by α6Aβ1 integrin-mediated adhesion to laminin than by that of α6Bβ1. This differential pattern of tyrosine phosphorylation induction does not appear to be a secondary event initiated by cell shape changes. Also, differences in tyrosine phosphorylation in the α6 subunits were not evident in response to attachment to other substrates. These findings provide biochemical evidence for functional differences between α subunit cytoplasmic domain variants of the same integrin.

The ability of integrins to function as transmembrane signaling receptors has been well-established (reviewed in Refs. 1-3). This function is based on the findings that integrin-mediated adhesion of cells to extracellular matrix ligands or clustering of integrins with antibodies increases the tyrosine phosphorylation of cytoplasmic proteins and alters intracellular pH, [Ca2+]i, and gene expression (1-3). Although integrin cytoplasmic domains do not contain intrinsic kinase motifs (4), it is assumed that these domains associate either directly or indirectly with other proteins to affect signaling functions. In this connection, a role for β subunit cytoplasmic domains in the activation of tyrosine kinase signaling has been reported. Specifically, chimeric proteins containing the β1, β3, and β5 cytoplasmic domains induced the tyrosine phosphorylation of pp125 Fak when clustered with antibodies (5, 6). The ability of some but not all β integrin heterodimers to induce pp125 Fak tyrosine phosphorylation after antibody clustering suggests that the α subunit can influence the signaling capabilities of the β subunit cytoplasmic domain (7). However, there are no data at present that focus specifically on the role of the α subunit cytoplasmic domain in the regulation of integrin-mediated tyrosine phosphorylation.

In this study, we addressed the question of whether the cytoplasmic domain of the α6 subunit contributes to tyrosine kinase-mediated signaling by the α6β1 integrin. This possibility was supported by our previous functional data that demonstrated that the α6Aβ1 and α6Bβ1 integrin receptors, which are identical except for the sequence of the α6 cytoplasmic domain (8, 9), differed in their ability to promote cell migration on EHS1 laminin. Cells transfected with the α6A subunit extended numerous pseudopodia and were more motile on EHS laminin than cells transfected with the α6B subunit (10). We hypothesized that these differences could be attributed to differential activation of signaling pathways by these receptor variants. We report here that the α6Aβ1 and α6Bβ1 receptors induce marked quantitative differences in the tyrosine phosphorylation of several proteins after binding to EHS laminin. Specifically, the α6A cytoplasmic domain was much more effective in inducing tyrosine phosphorylation of specific proteins than the α6B cytoplasmic domain. One of these proteins has been identified as the cytoskeletal protein paxillin (11). Our findings are significant because they are the first to attribute differences in the regulation of integrin-dependent tyrosine phosphorylation to α subunit cytoplasmic domain variants.

MATERIALS AND METHODS

Cells—The P388D1 mouse macrophage cell line was obtained from the American Type Tissue Collection (Rockville, MD). Cells were transfected with either the human α6A or α6B cDNAs as described previously (12). Cells were maintained in RPMI containing 25 mm Heps, 15% certified fetal bovine serum (Life Technologies, Inc.), and 300 μg/ml G418 (Life Technologies, Inc.). Transfected cells expressing equivalent levels of α6 surface expression were obtained by fluorescence-activated cell sorting (FACS) using the α6 specific antibody, 2B7 (12).

Adhesion to Substrates—Tissue culture plates (60 mm; Costar) were coated overnight with 4 ml of PBS containing murine EHS laminin (30 μg/ml). EHS laminin was purified from the Englebreth-Holm-Swarm sarcoma as described elsewhere (13). Plates were washed three times with PBS before the addition of cells to remove nonadsorbed laminin. P388D1 cells were removed from tissue culture plates by scraping and maintained in suspension for 30 min at room temperature in either RPMI culture medium containing 25 mm Heps or Puck’s saline A containing 25 mm Heps and 500 μM MnCl2. After this incubation, the cells were either maintained in suspension or added to either EHS laminin-coated or uncoated tissue culture plastic plates and allowed to adhere for 30 min at 37 °C. The plates were then washed two times with

**Recipient of an American Cancer Society Faculty Research Award.**

1 The abbreviations used are: EHS, Englebreth-Holm-Swarm; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody.
Cell Extraction and Immunoprecipitation—After washing, the cells were solubilized at 4°C for 15 min in a 50 mM Tris buffer, pH 7.5, containing 0.15 M NaCl, 0.1% NaN₃, 2 mM EDTA, 0.1 mM sodium orthovanadate, 0.1% Triton X-100, 0.1% sodium deoxycholate, 2 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin and pepstatin, and 50 μg/ml leupeptin. Nuclei were removed by centrifugation at 12,000 x g for 10 min. The protein concentration of the cell extracts was determined using the Bio-Rad microprotein assay. Cell extracts were normalized for protein concentration, and aliquots containing 60–200 μg of protein were incubated overnight at 4°C with the paxillin-specific monoclonal antibody 165 (14). Immune complexes were recovered with a goat anti-mouse IgG antibody conjugated to agarose beads (Sigma). 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% Triton X-100, 0.15 M NaCl, and 0.1 mM sodium orthovanadate, two times with the same buffer containing 0.5 M NaCl, and one time with 0.05 M Tris, pH 6.8 containing 0.1 mM sodium orthovanadate. Laemmli sample buffer containing 5% β-mercaptoethanol and 0.1 M sodium orthovanadate was added to the samples, which were then incubated for 4 min at 100°C. Immunoprecipitates were resolved by SDS-PAGE (8%) and transferred to nitrocellulose filters. In addition, aliquots of total cell extracts (5–10 μg of protein) from each condition were resolved in a similar manner.

Immunoblotting—To analyze the phosphotyrosine content of the immunoprecipitates, the nitrocellulose filters were blocked for 1 h at room temperature in PBS containing 3% (w/v) Carnation dry milk. The filters were then, incubated for 90 min at room temperature in the same buffer containing the phosphotyrosine-specific monoclonal antibody, 4G10 (1 μg/ml; Upstate Biotechnology Incorporated, Lake Placid, NY). The filters were washed three times for 10 min in PBS containing 0.05% Tween 20 (TBST) and 5% (w/v) Carnation milk. The filters were then incubated for 1 h at room temperature in PBST containing 3% (w/v) Carnation dry milk and an anti-mouse IgG antibody conjugated to horseradish peroxidase (0.2 μg/ml; Kirkegaard and Perry Laboratories, Inc.). After four 10-min washes in PBST, protein was detected by enhanced chemiluminescence (Amersham Corp.).

For immunoblot analysis of total paxillin, filters were blocked for either 1 h at room temperature or overnight at 4°C in a 50 mM Tris buffer, pH 7.5, containing 0.15 M NaCl and 0.05% Tween 20 (TBST). The filters were then incubated for 1 h at room temperature in PBST containing 3% (w/v) Carnation dry milk and an anti-mouse IgG antibody conjugated to horseradish peroxidase (0.2 μg/ml; Kirkegaard and Perry). The filters were washed as before and proteins were detected by enhanced chemiluminescence (Amersham Corp.).

RESULTS

The αvβ6 integrin subunit exists as two structural variants, αv6A and αv6B, that differ only in the sequence of their cytoplasmic domains (8, 9). Expression of these structural variants differentially influence the behavior of P388D1 cells upon α6β1-mediated adhesion to an EHS laminin substratum (10). In this report, we investigated the possibility that the α6Aβ1 and α6Bβ1 receptors induce either qualitative or quantitative differences in tyrosine phosphorylation in response to laminin attachment and that such differences contribute to the distinct functional properties of these receptors. To compare the ability of the α6Aβ1 and α6Bβ1 integrin receptors to activate tyrosine kinase signaling pathways subsequent to ligand binding, we used P388D1 cells, an αv-deficient mouse macrophage cell line, that had been transfected with either the human α6A or α6B cDNAs (12). Populations of transfected cells that expressed equivalent levels of α6β1 on the cell surface were obtained by FACS. Expression levels were examined frequently by flow cytometry, and only cells that had comparable levels of surface expression were used for experiments (Fig. 1).

The transfected cells were either maintained in suspension or allowed to adhere for 30 min to an EHS laminin substratum. Cell extracts containing equivalent amounts of total protein were analyzed for their phosphotyrosine content by immunoblotting with the phosphotyrosine-specific mAb 4G10. As shown in Fig. 2A, identical patterns of basal phosphorylation were observed for the α6A-P388D1 and α6B-P388D1 cells when they were maintained in suspension. Interestingly, after adhesion of these cells to an EHS laminin substratum, the phosphotyrosine content of proteins of approximately 120, 110, and 76 kDa increased markedly more in the α6A-P388D1 cells than in the α6B-P388D1 cells (Fig. 2A). These quantitative differences in tyrosine phosphorylation were αv6β1-specific because similar patterns of tyrosine phosphorylation were obtained in the α6A-P388D1 and α6B-P388D1 cells after adhesion to tissue culture plastic (Fig. 2B).

To investigate further the differences in the tyrosine kinase signaling pathways that are activated by the α6Aβ1 and α6Bβ1 integrins, we sought to identify other proteins that are phosphorylated on tyrosine in response to EHS laminin attachment. The cytoskeletal protein paxillin is phosphorylated on tyrosine in response to extracellular matrix adhesion and by growth factor stimulation in many cell types (15–21). Moreover, paxillin phosphorylation has been correlated with cell spreading (16). In light of the fact that the α6A-P388D1 and α6B-P388D1 transfectants adhere to EHS laminin to the same extent but exhibit differences in their morphology (10), we hypothesized that this difference in cell “spreading” might be a result of differential tyrosine phosphorylation of paxillin by the α6Aβ1 and α6Bβ1 receptors. This possibility was also supported by the presence of a diffuse band in the phosphotyrosine blot of total cellular protein (Fig. 2A) that approximated the size of paxillin (molecular mass, 68 kDa). To examine this possibility, aliquots of cell extracts containing equivalent amounts of total protein were immunoprecipitated with the paxillin-specific monoclonal antibody 165 (14) and then immunoblotted with 4G10. Very little tyrosine phosphorylation of paxillin was detected when the cells were maintained in suspension (Fig. 3A). However, there was a substantial increase in the phosphorylation of paxillin on tyrosine residues when the α6A-P388D1 transfectants adhered to an EHS laminin substratum. In contrast, the increase in paxillin phosphorylation observed for the α6Bβ1 cells was considerably less than that seen in the α6Aβ1
cells. Aliquots of total protein were also immunoblotted with the paxillin-specific antibody to confirm that equal amounts of paxillin protein were present in all of the cell extracts (Fig. 3B).

The tyrosine phosphorylation of paxillin in neutrophils is dependent upon expression of p2 integrins (16, 20). Neutrophils that do not express p2 integrins fail to induce the phosphorylation of paxillin in response to adhesion or growth factor stimulation. To demonstrate that the low level of paxillin phosphorylation in the α6B-P388D1 transfectants was not the result of differences in p2 expression, we compared the α6A-P388D1 and α6B-P388D1 transfectants for their ability to induce tyrosine phosphorylation of paxillin in response to adhesion to another substrate. As shown in Fig. 4A, paxillin phosphorylation was induced in both the α6A-P388D1 and α6B-P388D1 transfectants after adhesion to tissue culture plastic and, more importantly, the level of paxillin tyrosine phosphorylation was the same for both populations. In contrast, markedly increased levels of paxillin tyrosine phosphorylation after adhesion to EHS laminin were observed only for the α6A-P388D1 transfectants. Therefore, the α6B-P388D1 transfectants are capable of phosphorylating paxillin to equivalent levels as the α6A-P388D1 transfectants in response to substrates other than EHS laminin. Similar amounts of total paxillin protein were present in all of the cell extracts (Fig. 4B).

One question that arises from the results obtained is whether the differences in morphology of the α6A-P388D1 and α6B-P388D1 transfectants on laminin are responsible for the observed differences in protein tyrosine phosphorylation. This possibility derives from the fact that the α6 transfectants exhibit quite different morphologies on laminin in normal culture medium (10). To resolve this issue, the α6A- and α6B-transfectants were plated on laminin in medium containing the divalent cation Mn2+. Previously, we had observed that both populations of transfectants exhibit maximal attachment to laminin in the presence of this cation (10). As shown in Fig. 5, they also exhibit a similar morphological appearance and degree of pseudopod extension in medium containing Mn2+. However, quantitative differences in the pattern of tyrosine phosphorylation induced by laminin attachment were similar to those seen in normal culture medium (Fig. 6). Specifically, the phosphorylation content of proteins of approximately 120, 110, and 76 kDa increased noticeably more in the α6A-P388D1 cells than in the α6B-P388D1 cells. Importantly, the difference in paxillin phosphorylation between these two cell populations was also evident in response to Mn2+-induced laminin attachment (Fig. 7A), even though these cells expressed equivalent amounts of paxillin protein (Fig. 7B).

**DISCUSSION**

Our data demonstrate that integrin α subunit cytoplasmic domain variants can differentially influence the activation of tyrosine kinase signaling pathways by an integrin receptor. Previous studies had demonstrated the importance of the β subunit in regulating tyrosine phosphorylation. For example, chimeric molecules comprised of the extracellular and transmembrane domains of the interleukin-2 receptor and β subunit cytoplasmic domains were capable of phosphorylating pp125Fak after clustering with antibodies (5, 6). This finding suggested that these domains contain the information that is necessary for linking signaling proteins to integrin receptors. A role for integrin α subunits in influencing integrin-mediated signaling of tyrosine phosphorylation was suggested by a report that antibody clustering of the α3β1 integrin but not the α2β1, α5β1, and α6β1 integrins resulted in tyrosine phosphorylation of pp125Fak (7). However, no studies to date have addressed the specific role of the α subunit cytoplasmic domain in this regulation. In this regard, several functional studies have provided data that indicate that the α subunit cytoplasmic domain is important for influencing integrin signaling (10, 22). The data presented here provide biochemical evidence for such an involvement. Moreover, a recent finding that the α6β1 and α6β1 integrins exhibit different properties on size fractionation columns supports the possibility that these receptors associate with distinct complexes of proteins (23).

More emphasis should be placed on elucidating the mechanism by which α subunits influence tyrosine kinase activity. The α6 cytoplasmic domains could interact directly with tyro-
Differential Signaling by the α6β1 and α6β3 Integrins

been shown to be dependent upon the β subunit cytoplasmic domain. For example, the cytoplasmic domain of the β subunit is required for receptor localization to cell substratum attachment sites, but the α subunit cytoplasmic domain can determine the type of adhesive structure that is formed (i.e. focal adhesion, point contact, or podosome) (24).

The identification of paxillin as one of the substrates of the differential tyrosine phosphorylation induced by the αδ structural variants is intriguing in light of the known properties of this protein. Paxillin is a cytoskeletal protein that is localized to sites of cell substratum attachment such as focal adhesions (14). In in vitro binding experiments, paxillin associates with the cytoskeletal protein vinculin (14, 25). More recently, it has been shown that tyrosine-phosphorylated paxillin can also interact with components of the cellular signaling machinery such as pp125Fak, p47Gag-Crk, Csk, and pp60Src, presumably by binding to the SH2 and SH3 domains of these proteins (25–28). These interactions implicate a role for paxillin in organizing the downstream signaling complexes that are required for integrin signaling. One issue that arises in this context is whether quantitative differences in paxillin phosphorylation could contribute to the functional differences observed between the α6Aβ1 and α6β3 transfectants such as migration. Migration is a complex and dynamic process that most likely requires cycles of phosphorylation and dephosphorylation of cellular components (29). The increased tyrosine phosphorylation of paxillin in the cells that express the α6Aβ3 integrin could enhance the recruitment of signaling molecules necessary for regulating such dynamic events.

Although the α6A- and α6B-transfectants exhibit markedly different morphologies on laminin (10), the results obtained in this study indicate that the observed differences in the induction of tyrosine phosphorylation between these cells are not dependent upon cell shape. When these cells were allowed to adhere to laminin in the presence of Mn2+, they exhibited similar morphologies, but the α6A cells still yielded significantly more phosphorylation of paxillin and other proteins than the α6B cells. We conclude from these data that these two integrin isoforms differ in their intrinsic ability to activate tyrosine kinase signaling and that this induction of tyrosine phosphorylation is not a secondary event initiated by cell shape changes.

The identity of the three major proteins that are phosphorylated in response to α6Aβ1-mediated adhesion to laminin is being investigated. Although the size of the 120-kDa protein is indicative of pp125Fak, we have shown that these cells express

![Fig. 5. Mn2+-induced laminin attachment: Morphology.](image)

![Fig. 6. Mn2+-induced laminin attachment: Total tyrosine phosphorylation.](image)

![Fig. 7. Mn2+-induced laminin attachment: Paxillin phosphorylation.](image)
very low levels of this kinase. This finding agrees with the previous report that monocytes and macrophages express little if any pp125\(^{\text{AK}}\) (30). For this reason, it is unlikely that pp125\(^{\text{AK}}\) is the sole kinase responsible for the increased tyrosine phosphorylation induced by the \(\alpha 6\beta 1\) integrin. However, additional pp125\(^{\text{AK}}\) family members have been identified in hematopoietic cells, and these related kinases could contribute to integrin signaling in P388D1 cells (30, 31). The 76-kDa protein may be the same protein that was recently reported to be the major protein that is phosphorylated on tyrosine residues in response to monocyte attachment to tissue culture plastic and several extracellular matrix substrates (32).

The results obtained in this study for the \(\alpha 6\) subunit may be relevant for other integrin \(\alpha\) subunits that have cytoplasmic domain variants (4). Although the existence of alternately spliced forms of integrin subunits has been known for some time, their functional significance is just beginning to be understood. Subsequent work should focus on the mechanisms by which the cytoplasmic domains of these variants exert differences in the activation of specific integrin-mediated signaling pathways.

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