Integrins mediate adhesive interactions between cells and the extracellular matrix, and play a role in cell migration, proliferation, differentiation, cytoskeletal organization, and signal transduction. We have identified an interaction between the \( \beta_1 \) integrin and the 16-kDa subunit of vacuolar H\(^{+}\)-ATPase (16K). This interaction was first isolated in a yeast two-hybrid screen and confirmed by coimmunoprecipitation and in \textit{in vitro} binding assays using bacterially expressed proteins. Immunofluorescent studies performed in L6 myoblasts expressing both native and epitope-tagged 16K demonstrate co-localization with \( \beta_1 \) integrin in focal adhesions. Deletion of the fourth of four transmembrane helices in 16K results in loss of interaction with \( \beta_1 \) integrin \textit{in vitro} and in the two-hybrid system, and less prominent staining in focal adhesions. This helix is also required for ligand-independent activation of platelet-derived growth factor-\( \beta \) receptor signaling by the human papillomavirus E5 oncoprotein. Overexpression of 16K or expression of 16K lacking this helix alters the morphology of myoblasts and fibroblasts, suggesting that the interaction of 16K with integrins could be important for cell growth control. We also discuss the possible role 16K might play in integrin movement.

Integrins comprise the major family of cell surface receptor proteins that interact with the extracellular matrix (ECM) \(^*\) or with counter receptors on adjacent cells. Integrin-ECM interactions (reviewed in Refs. 1–3) control the adhesion and migration of cells, as well as the transduction of signals regulating such processes as cell growth and differentiation. Integrin receptors involved in ECM interactions cluster at the points where stable binding to the matrix occurs, producing “focal adhesions” to which cytoskeletal components and signaling molecules co-localize (4, 5). Integrin \( \alpha/\beta \) heterodimers can be divided into subfamilies on the basis of their common \( \alpha \) subunit. Those of the \( \beta_1 \) subfamily include at least nine different heterodimers that function primarily as receptors for collagen, laminins, and fibronectin.

Much of the literature on integrins deals with their extracellular interactions with ECM ligands and how cytoplasmic domains tweak signaling pathways and influence cytoskeleton structure. However, little is known about the mechanisms whereby they redistribute in the cell membrane during focal adhesion formation; how they shuttle through the cell during synthesis, endocytosis, and transcytosis; and how they become targeted for degradation or recycling. It is believed that interactions within the plasma and organellar membranes are critical to these processes (6). Several types of integral plasma membrane proteins have been demonstrated by co-immunoprecipitation to interact with integrin heterodimers. These are the integrin-associated proteins, which interact with certain \( \alpha \) integrin heterodimers (7), EMMPRIN (8), and members of the four transmembrane domain (TM4) protein family: CD9, CD53, CD63, CD81, CD82, and NAG-2, which have been shown to complex with the \( \alpha_\beta \) integrin (9–11). Little is known about the functional consequences of interactions with these proteins, although integrin-mediated Ca\(^{2+}\) influx has been shown to be regulated by integrin-associated proteins (12).

Signaling from integrins and from transmembrane receptor tyrosine kinases can converge in the focal adhesion (13). For example, binding of growth factor PDGF-B causes dimerization of the \( \beta \) form of its receptor (PDGF-\( \beta \) receptor), leading to the activation of the receptor’s kinase activity and autophosphorylation. PDGF-B stimulation mimics many signaling responses triggered by integrin adhesion, such as phosphorylation of focal adhesion kinase, paxillin, and mitogen-activated protein kinases ERK-1 and ERK-2 (14, 15). The convergence of integrin and PDGF-\( \beta \) receptor functions is supported by data showing that fibronectin, collagen, or anti-integrin IgG binding to fibroblasts leads to phosphorylation of the receptor in the absence of PDGF (16).

The vacuolar H\(^{+}\)-ATPase (V-ATPase) is a large multisubunit enzyme present in intracellular membrane compartments such as endosomes, lysosomes, clathrin-coated vesicles, and the Golgi complex, where it plays a vital role in the maintenance of endocytic and exocytic pathways (17–21). One of its subunits, a 16-kDa protein (16K) also known as ductin, has been found in the plasma membrane of renal and kidney epithelial cells, macrophages, and some tumor cell lines (22–25). 16K is composed of four hydrophobic regions thought to form transmembrane \( \alpha \)-helices, and plays a key role in the assembly and function of the V-ATPase pump (26). In addition, it comprises structures independently of the V-ATPase, including gap junctional complexes and mediators, which are involved in the release of neurotransmitters (27–30). 16K also forms a complex with the E5 oncprotein of papillomaviruses (31, 32) and has been proposed to mediate the ability of E5 to cause ligand-independent activation of the PDGF-\( \beta \) receptor (33).

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We report here the identification of an interaction between the transmembrane domains of β1 integrin and 16K. The interaction was detected using a yeast two-hybrid screen, and confirmed by various in vitro and in vivo binding assays. We find the distribution of 16K within cells to be ECM-dependent, thereby minimizing artifacts in binding assays. The TRIC fragments were coupled to glutathione-agarose beads, and the protein A-tagged β1 moiety released by cleavage with thrombin. ERGA was added to 5 μl, and the beads with GST removed by centrifugation. The protein A-tagged integrin fragments were cross-linked to the immobilized protein A beads using glutaraldehyde. Immunoprecipitations were performed as described above, and competed with GST-tagged GST-16K upstream and downstream primers to PCR-amplify the full-length cDNA, GST-TM1 was isolated as a BamHI-EcoRI fragment from the two-hybrid isolate in pVP6, and GST-TM4 was amplified from the full-length cDNA using the 16K upstream primer and an internal downstream primer (5-CGGAATCTTACAGCAAGCTGAGG-3' TM downstream). For in vitro binding assays, 2 μg of GST-16K, GST-TM1, or GST-TM4 protein coupled to beads were incubated at 4 °C with 2 μg of soluble protA-TR, protA-TR only, or protA-IC only, for 1 h with gentle shaking in 40 μl Maxples, pH 7.9, 100 μM KCl, 5 μM MgCl2, 1 mM dithiothreitol, 0.5% Nonidet P-40, 1% milk powder. The beads were removed by centrifugation and washed five times in binding buffer without milk milk. Beads were then suspended in sample buffer, heated, and the samples loaded onto 10% SDS-polyacrylamide gels. The proteins were transferred to nitrocellulose and protein A conjugated rabbit anti-mouse IgG secondary antibody was from Sigma. anti-rabbit IgG antibody (Molecular Probes). Alkaline phosphatase conjugated rabbit anti-mouse alkaline phosphatase-conjugated antibody.

**Interaction of β1 Integrin with 16-KDa Subunit of V-ATPase**

**EXPERIMENTAL PROCEDURES**

*Two-hybrid Plasmids—β1 integrin bait plasmids were made by amplifying from the full-length cDNA of the bovine β1 subunit (35) sequences encoding amino acids 691–773 (transmembrane and intracellular domains, TR). These domains are 100% homologous to mouse and human proteins. The TR primers were 5'-TGAATTCCTGTTACCCTGAG-3' (TR upstream) and 5'-TATGCGACCTGGCAATTCCTCTTGTCG-3' (TR downstream), the TRIC primers were the TR upstream primer and 5'-TATGCGACCTACATGGAGGATTTAGTCACTATTCTCAGG-3' (TRIC upstream) and the IC downstream primer 5'-CAGCTTTGGG-3' (IC downstream). The PCR products were directly cloned into pBluescriptII (Stratagene) to generate vectors for use as bait plasmids.*

**Epitope Tagging and Transfections—** To raise an antibody against 16K, we cloned the 5′ and 3′ UTR upstream) and 5′-CTAGAATTCCCTAATCATCTGAG-3' and 5′-CGGATCCCTAATCATCTGAGG-3'. The 116-amino acid protein A tag (14 kDa) provides solubility and a uniformly sized larger carrier for the small protein domains that vary in size from 3 to 10 kDa and have different charges and hydrophobicities, thereby minimizing artifacts in binding assays. The TRIC fragment was amplified using 5'-TCCGAATCCGCAATTCTGAGG-3' and 5'-GTGGTTGAGGAGG-3'; 16K upstream and downstream, respectively) tagged with BglII and SacII sites to PCR-amplify the full-length cDNA, GST-TM1 was isolated as a BamHI-EcoRI fragment from the two-hybrid isolate in pVP6, and GST-TM4 was amplified from the full-length cDNA using the 16K upstream primer and an internal downstream primer (5-CGGAATCTTACAGCAAGCTGAGG-3'; TM downstream). For in vitro binding assays, 2 μg of GST-16K, GST-TM1, or GST-TM4 protein coupled to beads were incubated at 4 °C with 2 μg of soluble protA-TR, protA-TR only, or protA-IC only, for 1 h with gentle shaking in 40 μl Maxples, pH 7.9, 100 μM KCl, 5 μM MgCl2, 1 mM dithiothreitol, 0.5% Nonidet P-40, 1% milk powder. The beads were removed by centrifugation and washed five times in binding buffer without milk milk. Beads were then suspended in sample buffer, heated, and the samples loaded onto 10% SDS-polyacrylamide gels. The proteins were transferred to nitrocellulose and protein A conjugated rabbit anti-mouse alkaline phosphatase-conjugated antibody.

**Immunoprecipitations—** Cells grown as monolayers were chilled to 4 °C, washed with cold PBS and lysed in cold RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.5) for 30 min. They were then scraped from the dishes with a rubber policeman, transferred to a microcentrifuge tube, and spun for 10 min at 4 °C. The soluble protein was divided into 25-μg aliquots to which anti-16K-M, rabbit control serum, or HM-81-1 was added. After 1 h at 4 °C, protein A-Sepharose (20 μl of 50% slurry/ml) was added and the incubation continued for 30 min. Complexes recovered by centrifugation, washed three times with PBS, and run on 10% SDS-polyacrylamide gels. Western blot analysis was performed using HM-81-1 primary antibody, mouse anti-hamster secondary antibody, and alkaline phosphatase-conjugated rabbit anti-mouse antibody for detection.

**Cell Lines and Culture—** The rat myogenic cell line, L6 (American Tissue Culture Collection) and 5 integrin bait plasmid was made by amplifying from the bovine 16K cDNA (35) sequences encoding amino acids 341–385, which includes the transmembrane domain, using primers 5'-TGAATTCCTGCAAGCTTCTTCTT-3' (TR upstream) and 5'-TATGCGACCTATGGAGGATTTAGTCACTATTCTCAGG-3' (IC downstream). The PCR products were directionally cloned into pBluescriptII, creating the LexA fusion bait plasmids b and c.

**EXPERIMENTAL PROCEDURES**

Western Blot Analysis of Protein Expression in Yeast—Cultured myotubes were frozen at −20 °C, and the samples loaded onto 10% SDS-polyacrylamide gels. The proteins were transferred to nitrocellulose and protein A conjugated rabbit anti-mouse alkaline phosphatase-conjugated antibody.

**Antibodies—** Rabbit polyclonal anti-16K antibody (Y-11) was purchased from Santa Cruz Biotechnology. Anti-16K-M and anti-16K-N are rabbit polyclonal antibodies raised against the 70-kDa subunit of V-ATPase (45). monoclonal Armegian hamster anti-mouse β1 integrin (HM-81-1) was obtained from PharMingen. For double labeling experiments, primary antibodies were visualized using fluorescent isothiocyanate-conjugated mouse anti-hamster IgG (PharMingen), followed by fluorescent isothiocyanate-conjugated goat anti-mouse IgG (Sigma), or Texas red-conjugated goat anti-rabbit IgG antibody (Molecular Probes).

**Epitope Tagging and Transfections—** The HA epitope (YPDVPDYA) from the influenza virus hemagglutinin protein HA1 (46) was added, along with a SacI restriction site, to the N terminus of both 16K and TM4 by PCR amplification using an upstream primer encoding the epitope, 5′-TCCCCGCGGATCTACATGTGGAACACCCGCGCGCAC-3′, and the downstream primers used to make GST-16K and GST-TM4. Initially, SacI-EcoRI fragments from these PCR products were cloned into pEXK and expressed in E.coli (CLONTECH). In later experiments we switched to using the pJX40-KK0 vector, which enables consistent high levels of expression from an SV40 promoter, by amplifying the tagged full-length or TM4 fragments with terminal EcoRI and BamHI sites to allow insertion into this vector. The effects of 16K and TM4 on cell morphology were seen with both pEXK and pJX40-KK0 systems, but the latter proved to be a more reliable vector. Stable cell lines used to make GST-16K and GST-TM4 were grown as monolayers in 35-mm dishes, media was removed and cells were washed twice with PBS. Cells were fixed with 4% paraformaldehyde in PBS, washed three
times with PBS, and permeabilized by incubation with 0.1% Triton X-100 for 30 min. They were again washed three times in PBS, then blocked in 10% rabbit serum in PBS. Primary antibody was diluted according to suppliers recommendations in 2% rabbit serum and incubated with cells for 1 h, followed by one wash with PBS, and incubation with secondary antibody for 30 min at room temperature. For double-labeling experiments this procedure was then repeated for the other primary and secondary antibodies, after which the cells received three 10-min washes in PBS. They were then covered with mounting medium, affixed to glass slides, and viewed using a Bio-Rad MRC-600 laser scanning confocal microscope equipped with a krypton-argon mixed gas laser. Cells treated with preimmune rabbit serum instead of primary antibodies, or untransfected control cells treated with anti-HA antibody, served as negative controls. Single-labeling experiments in which the secondary antibodies were mismatched with the primary antibodies demonstrated that there was no cross-reactivity between antibodies in the double-labeling experiments.

**Bead Binding Assays—** Polylysine latex beads (Sigma) were coated with fibronectin, polylysine, and bovine serum albumin as described by Grinnell and Geiger (47). Approximately $3 \times 10^6$ polylysine latex beads (11.9 µm diameter) were incubated for 1 h at 37°C in 0.5 ml of α-MEM containing 25 µg of human plasma fibronectin (Sigma), 50 µg of polylysine (Sigma), or 50 µg of bovine serum albumin. Bead binding assays were performed according to the procedure of Burbelo et al. (48). Cells, with 25 µg/ml cycloheximide (Sigma) in fibronectin-depleted serum. After 2 h cells were washed twice with PBS, then detached with 0.05% trypsin and 25 µg/ml cycloheximide. Cells ($1 \times 10^5$) were subsequently plated on coverslips for 60 min at 37°C in α-MEM containing 25 µg/ml cycloheximide. Cells 1 h later were then fixed and stained with anti-16K antibody as described above. Controls in which primary antibody was replaced with preimmune serum showed no immunofluorescence.

**RESULTS**

β1 Integrin Interacts with 16K—The TRIC bait for the two hybrid screen comprised the transmembrane domain of β1 integrin, flanked by 16 amino acids on the extracellular side and the full intracellular cytoplasmic domain (Fig. 1A). This was done to avoid using only a short highly hydrophobic bait. Nine his-lacZ′ clones that did not react with the control lexA-lamin bait or with the empty BTM116 plasmid were obtained from screening $2 \times 10^6$ transformants, all encoding proteins that interacted with this bait and the β1-TR bait, but not the β1-IC bait consisting of the intracellular domain flanked by only four transmembrane residues. In addition, none of these proteins interacted with a bait containing the transmembrane domain of the α5 integrin subunit. Expression of both the α5 and β1 transmembrane (TR) bait proteins in yeast was similar, as detected by an anti-LeX antibody (Fig. 1A), but none of the proteins interacted with the α5 bait. Thus, the lack of an interaction between 16K and α5-TR was not simply due to poor expression of that bait protein, and the interaction with the β1 transmembrane domain was not due solely to a nonspecific interaction of hydrophobic helices. Three of the cDNA clones recovered in this two hybrid screen encoded overlapping segments of the 16-kDa (16K) subunit of V-ATPase (Fig. 1C), two contained identical regions of an uncharacterized human cDNA, KIAA0025, previously isolated from a human immature myeloid cell line (49), one encoded a fragment of a sugar transport protein (50), and three did not share homology with any database entries. All contained hydrophobic regions.

The clones identified, 16K has several known functions that were intriguing with respect to potential involvement with integrins in V-ATPase-driven acidification of vesicles, which determines whether secretory-like complexes are degraded or recycled (19, 21). Additionally, the 16K subunit can independently of the ATPase form gap junctions (30).

All of the two hybrid isolates of 16K were missing the first transmembrane domain, proteins we refer to as TM1. A representative filter of the two hybrid assay showing the interaction of one of the three TM1 isolates with the β1-TR and β1-TRIC baits is shown in Fig. 1B (center). To confirm that the absence of the first transmembrane helix or some part of the 3′-nontranslated region in these clones was not creating an artifact in the screen, we expressed the full-length 16K coding region in the prey plasmid pVP16 and found it to also interact specifically with the β1-TRIC and β1-TR baits, and not the β1-IC bait (Fig. 1B, left). Anderson et al. (33) reported that the last helix of 16K, TM4, was required for its interaction with the E5 protein of bovine papillomavirus. To determine whether the interaction with β1 integrin also required this helix, we constructed a TM4 deletion mutant of 16K in pVP16, and found that it was no longer able to interact with any of the baits in a two-hybrid assay (Fig. 1B, right). When we tested the full-length 16K, TM1, and TM4 proteins with the α5-TR bait, none were found to interact, and filters with no colonies were obtained, identical to that seen when TM4 was tested.

**In Vitro Binding Assays—** GST fusion proteins of full-length 16K, or the mutants lacking TM1 or TM4, were produced in bacteria, coupled to glutathione-agarose beads and assessed for their ability to bind fragments of β1 integrin. The original TRIC bait, the transmembrane domain alone (TR only), or the cytoplasmic domain alone (IC only) were synthesized as trimeric fusions with GST and a fragment of protein A, using the pGEX2T vector (Fig. 2). Cleavage of these fusions with thrombin released soluble protein A-tagged proteins (protA-TRIC, protA-TR only, protA-IC only). The protein A moiety allows for direct detection with antibody conjugates, thus eliminating the need for different primary antibodies specific for each integrin fragment, and thereby enabling quantitative comparison of the abilities of the different fragments to bind to the protein on beads. The protein A-tagged molecules were incubated with the beads to which equivalent amounts (2 µg), based on Coomassie staining (lanes 11–13), of GST-TM1, full-length GST-16K, or GST-TM4 were coupled, and complexes recovered by centrifugation. Western blot analysis of these complexes with an antibody conjugate showed that the TRIC fragment used in the initial two hybrid screen was able to bind full-length 16K (GST-16K) and 16K lacking TM1 (GST-TM1) with similar affinities (Fig. 2, lanes 3 and 4), in both cases retaining approximately 5% of the input protA-TRIC molecule (one quarter of the input is shown in lane 1), even after five rounds of washing the beads. The removal of TM4 prevented binding (lane 2). Various negative controls for the protA-TRIC binding were used, including beads carrying no fusion protein, beads loaded with only GST, and beads loaded with a DNA-binding protein, the transcription factor TEF-1. None of these bound the input molecule (data not shown). Since GST-TM1 bound as efficiently as GST-16K, and was easier to purify from bacterial cells, it was used for subsequent experiments. The β1 integrin also required this helix, we constructed a TM4 deletion mutant of 16K in pVP16, and found it to also interact specifically with the prey plasmid pVP16 and found it to also interact specifically with the β1 integrin and 16K interact in vivo was obtained from co-immunoprecipitation analyses, using extracts from rat L6 myoblasts. These cells express β1 integrin and produce visible focal adhesions (Ref. 51 and our own observations). Immunoprecipitation of RIPA lysates with anti-16K recovered a protein that was reactive to anti-β1 antibody, and identical in size to immunoprecipitated β1 (Fig. 3, lanes 1 and 3). We also metabolically labeled cells with [35S]methionine, and immunoprecipitated with anti-16K an identically
sized labeled product, which comigrated with one of the proteins present in complexes pulled down by anti-b₁ (Fig. 3, lanes 5 and 6). With immunoprecipitations of non-labeled extracts, we detected several molecular mass forms of b₁ interacting with 16K (lane 3). The larger of these (~160–170 kDa) was less efficiently pulled down with our anti-b₁ antibody (lane 1), which recovered primarily the ~125-kDa b₁ species also seen in the radiolabeled extracts.

Double fluorescence labeling was used to localize b₁ integrin and 16K in L6 cells and in an unrelated human pancreatic tumor cell line (Capan 2), chosen because of reports that 16K is up-regulated in human metastatic pancreatic cancer (42). Considering that 16K can be present both as part of the V-ATPase complex and on its own in gap junctions, it was anticipated as well that b₁ would likely not co-localize to all sites where 16K is found. In agreement with this, in L6 cells 16K was found predominantly in the cytoplasm (Fig. 4A). However, a portion localized to focal adhesions, where much of the b₁ integrin (Fig. 4B) was located. The pancreatic cell line Capan 2 also showed co-localization of b₁ with 16K in the plasma membrane, both in isolated cells (Figs. 5, B and E) and in clusters of compacted cells that typify the growth form of this cell line in culture (Fig. 5, A and D). The co-localization of 16K with b₁ was largely independent of the V-ATPase, since an antibody to the en-
Interaction of β₁ Integrin with 16-kDa Subunit of V-ATPase

FIG. 2. In vitro binding of β₁ and 16K. At the top is shown the C-terminal TRIC segment of β₁ integrin used in the two-hybrid screen, as well as two additional fragments consisting of only the transmembrane or intracellular regions. The lower part of the figure shows Western blot assays used to detect whether protein A-tagged integrin fragments were retained on glutathione beads to which 2 µg of different GST fusions of 16K were coupled. Lanes 2–4 show binding of the 25-kDa protA-TRIC molecule to full-length 16K (GST-16K) and to 16K lacking TM1 (GST-TM1), but not to a mutant lacking TM4 (GST-TM4). Lanes 6 and 7 show binding of the 17-kDa “protA-TR only” molecule to beads carrying GST-TM1 but not to beads alone (-ve). Lanes 9 and 10 show that the 20-kDa “protA-IC only” molecule is not able to bind to GST-TM1. Lanes 1, 5, and 8 contain one-fourth (0.5 µg) of the soluble protA-tagged molecule used in each of the three sets of experiments. Lanes 11, 12, and 13 show Coomassie-stained aliquots of the GST-TM1, GST-16K, and GST-TM4 proteins used in the assays, confirming that the amount of each protein that was used in the binding assays was equivalent.

FIG. 3. Co-immunoprecipitation of β₁ integrin with 16K in L6 myoblast cells. L6 myoblasts were lysed at subconfluence, to avoid differentiated myotubes, and 25 µg of total cellular protein were used in immunoprecipitations with either anti-β₁ (lane 1), nonspecific rabbit serum (lane 2), or anti-16K (lane 3). Complexes recovered on protein A-Sepharose were analyzed by Western blot using anti-16K antibody. Metabolically labeled cells were also used in immunoprecipitations with either control rabbit serum (lane 4), anti-16K (lane 5), or anti-β₁ (lane 6). On reducing gels, β₁ integrin migrates with a molecular mass of approximately 125 kDa (arrow in lane 1).

zyme’s 70-kDa subunit (70K) showed that it was largely absent from focal adhesions (Fig. 4, C and D). Capan 2 cells showed a striking dissimilarity in 70K staining versus that of either 16K or β₁. In clumps of cells (Fig. 4C) and single cells (Fig. 4F), the 70K staining was predominantly in the cytoplasm and absent from plasma membranes and surfaces where cell-cell contact was occurring.

Epitope Tagging of 16K—The inability of the TM4 mutant to bind β₁ raised the possibility that this truncated protein’s distribution in cells might be altered. To permit distinction of a TM4 mutant from native 16K, we generated stable L6 cell lines expressing either HA-epitope-tagged full-length or TM4 mutant proteins. Cells expressing the TM4 mutant showed variations from typical myoblast structure, and the HA-TM4 protein (Fig. 4G) had a more perinuclear distribution than that of epitope-tagged full-length 16K (Fig. 4E). The HA-tagged full-length protein, but not tagged TM4 protein, showed a distribution that mirrored that of β₁ integrin, similar to native un-tagged 16K (panel A). β₁ staining revealed that focal adhesions in the TM4-expressing cells (panel H) were less punctate than in untransfected control cells (panels B and D). Nevertheless, these cells expressing TM4, when stained with anti-16K and anti-β₁, had endogenous 16K that colocalized with β₁ to these structures (data not shown). These experiments showed that
the fourth transmembrane helix of 16K is important for the cellular localization of the protein in vivo, and plays a role in defining focal adhesions.

**Fibronectin-dependent Redistribution of 16K**—Although the mechanisms whereby integrins become redistributed in the membrane are not known, the presence of integrin ligands can lead to formation of focal adhesions (52). We used fibronectin-coated beads to cluster integrins in L6 cells, and looked for a concurrent redistribution of 16K. Cells were first trypsinized and incubated with cycloheximide in fibronectin-depleted serum to obtain an initial random dispersion of fibronectin receptors. The binding of cells to fibronectin-coated beads then can then be used to induce redistribution of receptors to the points of contact (53). When this experiment was done, 16K redistributed to a pool at those contact points (Fig. 6A). Beads coated with polylysine, a positively charged nonspecific adhesion promoting polypeptide, bound to cells, but 16K did not localize to the contact points (panel C). These results suggest that insoluble fibronectin initiates redistribution of 16K by clustering and immobilizing β1 integrin.

**Altered Morphology of L6 Myoblasts and REF52 Cells**—The above experiments predicted that perturbation of 16K within cells could potentially lead to a change in cell-ECM interactions, and in this regard it had been previously reported that the expression of a TM4 mutant of 16K in 3T3 fibroblasts led to anchorage-independent growth as assessed by formation of colonies in soft agar (33). We used L6 and rat embryonic fibroblast (REF52) cells to inquire whether 16K might be involved in the regulation of cell morphology and growth (Fig. 7). Stable transfectants overexpressing full-length 16K, or expressing the TM4 mutant, showed a change in morphology in both cell types. L6 cells with 16K became more spindle-shaped, and defined edges, and to show reduced effects of contact with other cells. REF52 cells overexpressing full-length 16K became strikingly elongated and underwent a conversion to a flattened morphology, while maintaining long tenuous end-to-end connections. REF52 cells expressing the TM4 mutant became shorter and broader than control cells, with less well defined edges. The full-length REF-16K transfectants had a doubling time approximately twice that of control cells or REF-TM4 transfectants, a possible consequence of their highly extended shape. Thus, with both a myoblast and a fibroblast cell line, the overexpression of wild type 16K led to an elongation of the cells, while the TM4 mutant led to a smaller, more distorted appearance. The conclusions from these experiments were based on three independently isolated stable cell lines for both full-length and TM4 transfectants in both cell types, all of which exhibited the described phenotypes.

**DISCUSSION**

Integrins, like many membrane-bound receptors, are directed after synthesis to the cell membrane in vesicles that bud off from the endoplasmic reticulum and cycle through the Golgi complex (54). These vesicles emerge from the trans-Golgi and fuse with the cell membrane, extruding the amino termini of integrin chains to the extracellular environment. Integrins are not thought to be in this way directed to focal adhesions, but rather to redistribute within the membrane to those points in response to binding to an ECM ligand (4). This response has been shown, at least with β1 and β2 integrins, to involve the cytoplasmic domain, perhaps through interaction with a cytoskeletal component, with ligand binding overcoming a block to directed movement (55–57).

The finding of an interaction between the 16-kDa subunit of V-ATPase and the transmembrane domain of β1 integrin raises a number of new possibilities for how integrins move. Specifically, 16K could be an important mediator of integrin movement via vesicles that shuttle receptor-ligand complexes through the cell. These complexes are engulfed in coated and non-clathrin-coated vesicles en route to endosomes, where 16K is abundant and where the V-ATPases play a key role in regulating acidification that leads to complex dissociation (58–61).
Our observation that fibronectin-coated beads caused 16K, and as seen in Fig. 6B, associated vesicles, to pool at the site where contact between the cell and the bead was made is consistent with the hypothesis that at those points there is active engulfment and cycling of receptors. Experiments using fibronectin-coated beads and anti-β₁ integrin-coated beads showed a strikingly similar accumulation of β₁ integrin, α-actinin, actin, talin (53), and rho (48) in the cytoplasm adjacent to the site of attachment to beads. It is a reasonable hypothesis that 16K is helping to direct and stabilize β₁ transmembrane interactions during the recycling process.

Vesicles derived when clathrin is removed can also shuttle complexes through the process of transcytosis, which β₁ integrin has been observed to undergo in fibroblasts, hepatic epithelial cells, and Chinese hamster ovary cells (62, 63). In light of this, it is possible that focal adhesions form when integrins that are being continuously endocytosed and recycled back to the surface of the cell become retained only at points where they contact the ECM. The redistribution need not involve movement through the membrane, but rather a repetitive recycling that “finds” the points where the ECM would allow for formation of a focal adhesion. Once integrins contact a ligand, their half-life at that point is extended (64), and subsequent cytoskeletal proteins of the focal adhesion aggregate. The ligand-dependent requirement of the cytoplasmic domain of integrins for their relocation to focal adhesions can be considered in light of this hypothesis of integrin cycling. The need for ligand binding could simply reflect the need to get integrins into mobile vesicles through internalization of receptor-ligand complexes. Once internalized only the cytoplasmic domain would protrude from these vesicles, and through its interactions with talin, vinculin, paxillin, α-actinin, and other cytoskeletal associated proteins direct vesicle movement along the cytoskeletal network that organizes in response to focal adhesions.

The importance of transcytosis and integrin recycling also has relevance to migratory cells, where it has been proposed that integrins holding the cell to the matrix move rearwards as the cell advances, thereby polarizing within the cell the formation and dissolution of integrin-ligand complexes (65). Further attachments at the cell’s leading edge would require replenishments of matrix receptors to move the cell forward. Consistent with this, it has been shown that neutrophil migration involves endocytosis of integrins from the rear of the cell, followed by transport through the cell and exocytosis at the leading edge (66).

The perturbation of cell morphology observed in our full-length and TM4 transfectants suggests a role for 16K in regulating cell-ECM interactions. The TM4 mutant, although reportedly defective in ATPase activity (67) and β₁ binding (our data), is nevertheless able to interact with 16K (33). One interpretation is that the presence of the TM4 mutant proteins in the ATPase interferes with the normal acidification of endosomes and leads to greater recycling of intact receptors to the cell membrane, thereby promoting migration and leading to cells with shorter extensions. Bretscher (65) predicted, based on his analysis of fibronectin receptor cycling (62), that fibroblasts would be longer if receptor cycling were impaired. Overexpression of native 16K led to elongation of REF52 cells, possibly because the additional molecules caused β₁ to be aberrantly retained in the cell membrane. An impairment of endosome acidification by the TM4 mutant is reminiscent of the observation that the E5 transmembrane oncoprotein of papillomaviruses, which binds 16K (32), also prevents this acidification, and leads to enhanced receptor signaling (68).

The involvement of 16K with integrins also provides an interesting link with the observation that adhesion of fibroblasts to fibronectin leads to alkalinization of the cytoplasm (69). The V-ATPases do not need a counter-ion when they pump protons across membranes (70), and they are one of the principle enzymes that can affect cellular pH. The movement of H⁺ ions from the cytoplasm into acidic vesicles could be enhanced when contact with a particular matrix component leads to reorganization of the repertoire of receptor-ligand complexes, a process that would necessarily involve endosome processing. Within the V-ATPase enzyme, the membrane bound hexamer of 16K molecules, known as the V0 subunit of the enzyme, provides a docking site for the V1 subunit that includes the 70K protein, which leads to ATPase activity (67, 71). Immunofluo-
Hynes, R. O. (1993) Cell 99, 11–25

Clark, E. A., and Brugge, J. S. (1995) Curr. Opin. Cell Biol. 7, 191–197

Stewart, M. A., Brown, E. J., and Fazeli, B. (1993) J. Biol. Chem. 268, 1991–1994.

Plopper, G. E., McNamara, H. P., Dike, L. E., Bojanowski, K., and ingber, D. E. (1995) Mol. Biol. Cell 6, 1349–1365.

Rankin, S., and Rosengurt, E. (1994) J. Biol. Chem. 269, 704–710.

Claesson-Welsh, L. (1994) J. Biol. Chem. 269, 32023–32026.

Sundberg, C., and Rubin, K. (1996) J. Cell Biol. 132, 741–752.

Furgo, M. (1992) Exp. Cell Res. 19, 155–169.

Nelson, N. (1992) J. Exp. Biol. 172, 149–153.

Johnson, L. S., Dunn, K. W., Pytowski, B., and McGraw, T. E. (1993) Mol. Biol. Cell 4, 1251–1266.

Rees, B., and Bunting, G. (1994) FEBBS Lett. 345, 61–66.

Clague, M. J., Urbé, S., Aniento, F., and Gruenberg, J. (1994) J. Biol. Chem. 269, 21–24.

Swallow, C. J., Grinstein, S., and Botstein, D. O. (1990) J. Biol. Chem. 265, 7645–7654.

Márquez-Sterling, N., Herman, I. M., Peacreta, T., Arai, H., Terres, G., and Furgo, M. (1991) Eur. J. Cell Biol. 56, 19–33.

Grück, S. L., Nelson, K. D., Lee, B. S., Wang, Z. Q., Guo, X. L., Fu, J. Y., and Zhang, K. (1992) J. Exp. Biol. 172, 219–229.

Martínez-Zaguirre, R., Lynch, R. M., Martinez, G. M., and Gillies, R. J. (1993) Am. J. Physiol. 265, C1015–C1029.

Hanan, H., Hasebe, M., Moriyama, Y., Maeda, M., and Futai, M. (1991) Biochem. Biophys. Res. Commun. 176, 1062–1067.

Birman, S., Menon, P.-M., Lesbats, B., Le Caer, J.-P., Rossier, J., and Israel, M. (1990) FEBBS Lett. 253, 303–308.

Leitch, B., and Fimbres, M. E. (1990) Exp. Cell Res. 190, 218–226.

Fimbres, M. E., Goodwin, S. F., Meagher, L., Lane, N. J., Keen, J., Findlay, A. B., and Kaiser, R. (1994) J. Cell Sci. 107, 1817–1824.

Fimbres, M. E., Harrison, M., and Jones, P. (1995) BioEssays 17, 247–255.

Goldstein, D. J., Fimbres, M. E., Andressson, T., McLean, P., Smith, K., Bubb, L., and Schlegel, R. (1991) Nature 352, 347–349.

Goldstein, D. J., Andressson, T., Sparkowski, J. J., and Schlegel, R. (1992) EMBO J. 11, 4851–4859.

Andressson, T., Sparkowski, J. K., and Schlegel, D. J. (1995) J. Biol. Chem. 270, 6383–6387.

Solowska, J., Edelman, J. M., Albeida, S., and Bick, C. A. (1991) J. Cell Biol. 114, 1079–1088.

Maclaren, L. A., and Wildeman, A. G. (1995) Biochim. Biophys. Acta 122, 237–242.

Hollenberg, S. M., Sternlanz, R., Cheng, P. F., and Weintraub, H. (1995) Mol. Cell Biol. 15, 3813–3822.

Vijayakrishnan, A. B., Hollenberg, S. M., and Cooper, J. A. (1993) Cell 74, 205–214.

Goldstein, D. J., Jean, A., Woods R. A., and Schlessel, R. H. (1992) Nucleic Acids Res. 20, 1425.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) J. Mol. Biol. 215, 403–410.

Yaffe, D. (1968) Proc. Natl. Acad. Sci. U. S. A. 61, 477–483.

Nexu, J.-I., Motojima, K., Tamura, H.-O., and Ohkuma, S. (1992) J. Biochem. (Tokyo) 112, 212–219.

Ohtani, T., Numa, M., Yagishita, H., Futagami, T., Tsukuioka, Y., Kitigawa, M., Kayahara, M., Nakagawa, T., Miyazaki, I., Yamamoto, M., Iseki, S., and Ohkuma, S. (1996) Br. J. Cancer 73, 1511–1517.

Lee, B. S., Holliday, L. S., Okutaka, B., Krits, I., and Gluck, S. L. (1996) Am. J. Physiol. 270, C382–C388.

Smith, D. B., Berger, L. C., and Wildeman, A. G. (1993) Nucleic Acids Res. 21, 359–360.

Berger, L. C., Smith, B. D., Davidson, L. H., Hwang, J.-J., Fanning, E., and Wildeman, A. G. (1996) J. Virol. 70, 1203–1212.

Wilson, L. A., Niman, H. L., Houghten, R. A., Chenerson, A. R., Connolly, M. L., and Lerner, R. A. (1994) J. Virol. 68, 767–769.

Grinnell, F., and Geiger, B. (1986) Exp. Cell Res. 162, 449–461.

Burbero, P. D., Miyamoto, S., Utani, A., Birl, S., Yamada, K. M., Hall, A., and Yamada, Y. (1995) J. Biol. Chem. 270, 30919–30926.

Nomura, N., Miyajima, N., Saruca, T., Tanaka, A., Kawarabayasi, Y., Sato, S., Nagase, T., Seki, N., Ishikawa, K.-I., and Tabata, S. (1994) DNA Res. 1, 47–56.

Nakae, K., Kawamoto, S., Matsubara, K., and Okubo, K. (1997) Biochem. Biophys. Res. Commun. 238, 126–129.

Bergman, M., Joukov, V., Virtanen, I., and Alttilo, K. (1995) Mol. Cell. Biol. 15, 1004–1011.

Fath, K. R., Edgell, C. J., and Burridge, K. (1998) J. Cell Sci. 99, 67–75.

Mueller, S. C., Kelly, T., Dai, M. Z., Dai, H. N., and Chen, W. T. (1989) J. Cell Biol. 109, 3455–3464.

Costa, J. P., and McFarland, R. P. (1989) J. Biol. Chem. 264, 12596–12603.

Laflamme, S. R., Akaiyama, S. K., and Yamada, K. M. (1992) J. Cell Biol. 117, 457–474.

Yamaoka, J., Chen, Y., O’Toole, T. E., Lofiis, J. C., Takada, Y., and Ginsberg, M. H. (1993) J. Cell Biol. 122, 223–233.

Felsenfeld, D. P., Choquet, D., and Sheetz, M. P. (1996) Nature 383, 438–440.

Raub, T. J., and Kuentzel, S. L. (1998) Exp. Cell Res. 184, 407–426.

LeVeit, B., Stoughton, M. C., Gauthier, M., and Schmitt, D. (1991) J. Dermatol. Sci. 4, 77–83.

Bretscher, M. S. (1992) EMBO J. 11, 405–410.

Johnson, K. S., and Anderson, R. G. (1996) Exp. Cell Res. 224, 237–242.

Bretscher, M. S. (1989) EMBO J. 8, 1341–1348.

Pol, A., Ortega, D., and Enrich, C. (1997) Biochem. J. 323, 435–443.

Dutton, S. L., Scharf, E., Brisewitz, R., Marcontarino, E. A., and Assoun, R. K. (1995) Mol. Biol. Cell 6, 1781–1791.
Interaction of β1 Integrin with 16-kDa Subunit of V-ATPase

U. S. A. 88, 1938–1942

68. Straight, S. W., Herman, B., and McCance, D. J. (1995) J. Virol. 69, 3185–3192
69. Ingber, D. E., Prusty, D., Frangioni, J. V., Cragoe, E. J., Jr., Lechene, C., and Schwart, M. A. (1990) J. Cell Biol. 110, 1803–1811
70. Harvey, W. R. (1992) J. Exp. Biol. 172, 1–17
71. Merzendorfer, H., Graf, R., Huss, M., Harvey, W. R., and Wieczorek, H. (1997) J. Exp. Biol. 200, 225–235
72. Petti, L., Nilson, L. A., and DiMaio, D. (1991) EMBO J. 4, 845–855
73. Cohen, B. D., Goldstein, D. J., Rutledge, L., Vass, W. C., Lowy, D. R., Schlegel, R., and Schiller, J. T. (1993) J. Virol. 67, 5303–5311
74. Hannigan, G. E., Leung-Hagesteijn, C., Fitz-Gibbon, L., Coppolino, M. G., Radeva, G., Filmus, J., Bell, J. C., and Dedhar, S. (1996) Nature 379, 91–96
75. Radeva, G., Petrocelli, T., Behrend, E., Leung-Hagesteijn, C., Filmus, J., Slingerland, J., and Dedhar, S. (1997) J. Biol. Chem. 272, 13837–13844
76. Ohkuma, S., Shimizu, S., Noto, M., Sai, Y., Kinoshita, K., and Tamura, H.-O. (1993) In Vitro Cell Dev. Biol. Anim. 29A, 862–866
77. Manabe, T., Yoshimori, T., Henomatsu, N., and Tashiro, Y. (1993) J. Cell. Physiol. 157, 445–452