The Integrin \( \beta1 \) Subunit Transmembrane Domain Regulates Phosphatidylinositol 3-Kinase-dependent Tyrosine Phosphorylation of Crk-associated Substrate

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Our previous studies on the transmembrane domain of human integrin subunits have shown that a conserved basic amino acid in both subunits of integrin heterodimers is positioned in the plasma membrane in the absence of interacting proteins. To investigate the possible functional role of the lipid-embedded lysine in the mouse integrin \( \beta1 \) subunit, this amino acid was replaced with leucine, and the mutated \( \beta1 \) subunit (\( \beta1A^{K756L} \)) was stably expressed in \( \beta1 \)-deficient GD25 cells. The extracellular domain of \( \beta1A^{K756L} \) integrins possesses a competent conformation for ligand binding as determined by the ability to mediate cell adhesion, and by the presence of the monoclonal antibody 9E67 epitope. However, the spreading of GD25-\( \beta1A^{K756L} \) cells on fibronectin and laminin-1 was impaired, and the rate of migration of GD25-\( \beta1A^{K756L} \) cells on fibronectin was reduced compared with GD25-\( \beta1A \) cells. Phosphorylation of tyrosines in focal adhesion kinase (FAK) and the Y416 in c-Src in response to \( \beta1A^{K756L} \)-mediated adhesion was similar to that induced by wild-type \( \beta1 \). The tyrosine phosphorylation level of paxillin, a downstream target of FAK/Src, was unaffected by the \( \beta1 \) mutation, whereas tyrosine phosphorylation of CAS was strongly reduced. The results demonstrate that CAS is a target for phosphorylation both by FAK-dependent and -independent pathways after integrin ligation. The latter pathway was inhibited by wortmannin and LY294002, implicating that it required an active phosphatidylinositol 3-kinase. Furthermore, the K756L mutation in the \( \beta1 \) subunit was found to interfere with \( \beta1 \)-induced activation of Akt. The results from this study identify phosphatidylinositol 3-kinase as an early component of a FAK-independent integrin signaling pathway triggered by the membrane proximal part of the \( \beta1 \) subunit.

INTRODUCTION

Signals from the extracellular matrix (ECM) that regulate the proliferation, differentiation, migration, and survival of adherent cells are mainly mediated by integrins. The integrin family consists of a large number of receptors composed of transmembrane \( \alpha \) and \( \beta \) subunits (Hynes, 1992). The short cytoplasmic tails of integrins connect ECM to the actin cytoskeleton and to cell signaling machinery (Hibbs et al., 1991; LaFlamme et al., 1994; O’Toole et al., 1994; Leong et al., 1995). Depending on the composition of the ECM, assembly of complexes of signaling proteins and specific integrins leads to activation of a variety of signaling pathways, often coordinately regulated by growth factors (Giancotti and Ruoslahti, 1999). ECM engagement by most integrins leads to activation of a variety of signaling pathways, often coordinately regulated by growth factors (Giancotti and Ruoslahti, 1999). ECM engagement by most integrins leads to activation of focal adhesion kinase (FAK) (Cary and Guan, 1999). In addition to the activation of FAK, other less characterized tyrosine kinases have been reported to become activated after integrin-mediated adhesion (Lewis et al., 1996; Yan and Berton, 1998; Yang et al., 1999; Obergfell et al., 2002). The molecular events that lead to FAK activation (autophosphorylation) after integrin ligand binding are not understood, but they are known to require integrin clustering, the NPXY motifs in the cytoplasmic domain of integrin \( \beta \) subunits, and a functional actin filament system (Burridge et al., 1992; Miyamoto et al., 1995a,b; Wennerberg et al., 2000). After integrin clustering, the autophosphorylation of Tyr-397 in FAK creates a binding site for the Src homology 2 domain of Src family kinases that promotes further phosphorylation of FAK and focal adhesion components such as paxillin and Crk-associated substrate (CAS) (Eide et al., 1995; Schlaepfer and Hunter, 1996; Richardson et al., 1997; Tachibana et al., 1997). FAK, its downstream target CAS, and phosphatidylinositol 3-kinase (PI3K) have been shown to be key mediators of integrin-mediated cell migration (Cary et al., 1996, 1998; Klemke et al., 1998; Reiske et al., 1999; Bakin et al., 2000). Tyrosine phosphorylation of CAS results in recruitment of CrkII and DOCK180, the subsequent activation of the small GTPase Rac1, and a number of downstream responses such as activation of the c-Jun NH2-terminal kinase cascade, remodeling of actin cytoskeleton and promotion of cell motility (Vuori et al., 1996; Dolfi et al., 1998; Kiyokawa et al., 1998a,b). The mechanism by which integrins transfer signals across the plasma membrane involves ligand-induced conformational changes as well as receptor clustering, in which the cytoplasmic and transmembrane domains of integrins have...
central roles (Miyamoto et al., 1995a,b; Takagi et al., 2001; Takagi et al., 2002; Li et al., 2003). Consistent with this view, the amino acid sequence of the β1 subunit from sponge to human is particularly well conserved in the transmembrane and cytoplasmic domains (Brower et al., 1997). Although the functional importance of the cytoplasmic domain of the β1 subunit has been extensively studied, only a few studies have addressed the role of the transmembrane domain in integrin function (Hayashi et al., 1990; Breisewitz et al., 1996; Li et al., 2003). A striking feature of transmembrane domains of integrin subunits is the presence of a strictly conserved basic amino acid. In all known integrin subunits, the transmembrane domain contains a lysine (or arginine in two cases) after a long stretch of hydrophobic amino acids, followed again by five to six hydrophobic residues (Figure 1). Our previous work showed that the lysine (e.g., K756 in mouse β1) is positioned in the plasma membrane in absence of interacting proteins (Armulk et al., 1999).

To investigate the functional role of this conserved unusual arrangement, we replaced the lysine in the transmembrane domain with leucine and expressed the mutated β1 subunit in β1-deficient GD25 cells. The mutation interfered with cell spreading and migration and caused strongly reduced phosphorylation of CAS and the PISK effector PKB/Akt. These results indicate the presence of an uncharacterized PISK-dependent signaling pathway triggered by β1 integrins.

**MATERIALS AND METHODS**

**Proteins and Reagents**

DNA restriction and modifying enzymes were purchased from Promega (Madison, WI). A cDNA construct coding for glutathione S-transferase (GST) fused with an 80-kDa integrin-binding fragment of invasin was kindly provided by Dr. M. Fallman (Umeå University, Umeå, Sweden). Invasin binds selectively to a subset of β1 integrins and efficiently promotes cell spreading and assembly of focal adhesions (Ibsberg and Leong, 1990; Gustavsson et al., 2002). The GST-fusion protein was purified from Escherichia coli cultures as described previously (Klint et al., 1995). Fibronectin (FN) and vitronectin were purified from human plasma as described previously (Yatabe et al., 1988; Snilenov et al., 1992). Mouse EHS laminin was from Invitrogen (Carlsbad, CA). The GRGD5 peptide was obtained from Bachem Finechemicalen AG.

Protein A-Sepharose CL-4B and glutathione-Sepharose CL-4B were purchased from Amersham Biosciences (Piscataway, NJ). The PI3K inhibitor LY294002 and wortmannin were from Calbiochem (San Diego, CA) and Sigma-Aldrich (St. Louis, MO), respectively.

**Antibodies**

The rabbit anti-rat β1 serum was prepared in our laboratory and has been described previously (Bottger et al., 1989). The monoclonal antibody (mAb) GoD3 against integrin subunit αv6 was generously provided by Dr. A. Sonnenberg (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Monoclonal antibodies against the following proteins were purchased: mouse mAb 1A (clone HMβ1-1), rat β1 (clones Ha2/5 and 9EG7), mouse α5 (clone ME10) (BD Biosciences, San Jose, CA), chicken FAK (clone 77), chicken paxillin (clone 399), rat CAS (clone 21), phosphotyrosine (PY-99) and horseradish peroxidase-conjugated RC-20 (BD Transduction Laboratories, Lexington, KY), mouse c-Src (clone H-12) (Santa Cruz Biotechnology), rabbit IgG) were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Horseradish peroxidase conjugated sheep anti-mouse IgG and anti-rabbit IgG were purchased from Amersham Biosciences.

**Mutation of β1A**

The mutation K756L was introduced into the integrin β1A subunit by using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. Briefly, the gel-purified BglII-Xhol fragment from pUHD10-3 expression vector (BD Biosciences Clontech, Palo Alto, CA) containing a doxycycline-regulated cytoplasmic domain of sheep Cas (Miyamoto et al., 1995a,b) was cloned into pSP70 (Promega). The pSP70(β1A/BglII-Xhol) was used as a template for polymerase chain reaction by using the following primers: K756L (5'-CCCTGCGTCTGATGTTGTTACTTTTAAATGATAAAC-3') and K756L (5'-GAAATATGACAGGTAACCAAATCACAGGAGCG-3'). Mutated base pairs are in bold. The generation of the mutation was verified by DNA sequencing. The BglII-Xhol fragment of β1A carrying the mutation was cloned into the BglII-Xhol site of pTetB1A and sequenced.

**Cells**

The integrin β1A-deficient GD25 cell line, its subclone GD25T, which was established by stable transfection with the Tet repressor-encoding vector pUHD15-1, and GD25 cells expressing wild-type β1A and β1AY783,793FF have been described previously (Svineng and Johansson, 1996; Wennerberg et al., 1997). The GD25 cells were transfected with linearized pSP70(β1A/BglII-Xhol) vectors by using Superfect (Qiagen, Valencia, CA) according to manufacturer’s recommendations. Culture medium containing 10 μg/ml puromycin was added to the transfected cells 48 h posttransfection. Surviving clones were tested for expression of β1 by flow cytometry, and clones expressing high surface levels of β1 were expanded. The GD25 cells were continuously cultured in DMEM containing 10% fetal calf serum, 2 mM l-glutamine, and hygromycin B (100 μg/ml). The GD25 cells expressing the integrin β1A subunit were cultured in the same medium with the addition of puromycin (20 μg/ml). Experiments were performed using clones expressing similar levels of mutated β1A or wild-type β1A on the cell surface. The expression of β1A on the cell surface was verified by flow cytometry throughout the entire time course of the experiment.

**Flow Cytometry**

The cells were harvested, washed with phosphate-buffered saline (PBS), and sequentially incubated with appropriate primary and secondary antibodies. Antibodies were diluted in 10% goat serum in PBS containing 0.01% NaN3. Before adding a fluorescent-labeled secondary antibody the cells were washed twice with PBS and incubated in the same buffer containing 0.3 mM MnCl2 at 37°C for 10 min, followed by incubation with mAbs Ha2/5 or 9EG7 on ice for 1 h. After washing, the cells were incubated with fluorescein-labeled secondary antibody and analyzed (10,000/sample) in a FACScan (BD Biosciences).
Cell Attachment and Cell Spreading Assays

The cell attachment assay was carried out in 96-well microtiter plates (Nalge Nunc, Naperville, IL) as described previously (Wennerberg et al., 1996). Briefly, cells (1 x 10^5 in 100 μl) were added to each well and allowed to attach to ECM proteins for 1 h at 37°C in a humidified atmosphere of 5% CO₂. All samples were assayed in triplicate, and the background attachment to BSA was subtracted from all measurements. To quantify cell spreading, cells were plated on eight-well chamber slides (Falcon Plastics, Oxnard, CA) precoated with extracellular matrix proteins and allowed to attach for 30 and 60 min at 37°C. The wells were washed with PBS, and the cells were fixed in 96% ethanol and stained with 0.1% crystal violet. The samples were photographed, and the percentage of spread cells in three microscopic fields was calculated.

Transmembrane Migration Assay

Cells were starved for 24 h in serum-free DMEM and detached by trypsin-EDTA treatment. A polycarbonate membrane (Neuro Probe, Inc., Gaithersburg, MD) was coated on both sides with 50 μg/ml FN in PBS, blocked by incubation in 1% heat-treated BSA, and subsequently rinsed with PBS before mounting in a 96-well migration chamber (Neuro Probe). In lower chambers, 115 μl of serum-free DMEM or DMEM containing 10% fetal calf serum was added; upper chambers contained cells (1 x 10^5) in serum-free DMEM and, where indicated, the GRGDS peptide (10 or 25 μg/ml). The cells were allowed to migrate for 12 h at 37°C, and cells remaining on the upper side of the membrane were removed by scraping. Subsequently, the membrane was fixed in 96% ethanol for 10 min, stained with 1% crystal violet in water for 40 min, and washed with water. The amount of stained cells at the lower side of the membrane was quantitated by scanning the filter using the Molecular Analyst 2.1 software (Bio-Rad, Hercules, CA).

Immunoprecipitations and Western Blotting

Cells were trypsinized, washed once with serum-free DMEM, plated on cell culture dishes coated with anti-β1 mAb HA2/5, GST-invasin, or with ECM proteins and incubated at 37°C for 1 h, unless indicated otherwise. As a negative control, cells were kept in suspension. Where indicated, cells were preincubated with LY294002 (20 μM), wortmannin (100 nM), or with dimethyl sulfoxide at room temperature (RT) for 30 min before plating on substrates. For immunoprecipitations, cells were lysed on ice for 10 min in 20 mM Tris-HCl buffer, pH 7.4, containing 1% NP-40, 1% deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM NaF, 30 mM Na₃P₂O₇, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 2 mM N-ethylmaleimide, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 200 μM Na₃VO₄, and insoluble material was removed by centrifugation. After pre-clearing the cell lysates with protein A-Sepharose, the primary antibodies were added to the samples and incubated overnight at 4°C. Subsequently, the bridging antibody (rabbit anti-mouse IgG) was added to each sample and incubated for 30 min at 4°C, and for an additional 30-min period after addition of protein A-Sepharose. The protein A-Sepharose was collected by centrifugation, and the pellet was washed three times with lysis buffer. Alternatively, the cells were lysed directly in SDS sample buffer containing 40 μM dithiothreitol. The samples were subjected to SDS-PAGE followed by wet electrophoretic transfer to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Filters were incubated with primary antibody and secondary antibody conjugated with horseradish peroxidase. Protein bands were detected using enhanced chemiluminescence (Amersham Biosciences), followed by exposure to Fuji Super RX film. Where indicated, filters were stripped in 62.5 mM Tris-HCl, pH 6.7, 2% SDS, and 100 mM β-mercaptoethanol for 30 min at 50°C and reprobed with relevant antibodies. Protein bands were scanned and selected bands were quantified using the Molecular Analyst 2.1 software (Bio-Rad). Results presented in figures in each case originate from one gel. In some cases, the lanes not adding any essential information were removed.

Immunocytochemistry

Eight-well chamber slides (Falcon Plastics) were coated with ECM proteins overnight at 4°C and blocked with 1% BSA (heat-treated) in PBS for 2 h at 37°C. The cells were trypsinized, washed once with serum-free DMEM, and

Figure 2. Attachment of GD25, GD25-β1A, and GD25-β1A/K756L to FN (A) and LN-1 (B). Cell attachment is expressed as percentage of the number of cells bound to VN. The GRGDS peptide (0.5 mg/ml) was added to block cell attachment to FN via αvβ3.
plated on the chamber slides in serum-free DMEM. In some experiments, the GRGDSP peptide was added (final concentration 0.2 mg/ml) to the medium. The cells were incubated at 37°C for 1–2 h, fixed with 2 or 4% paraformaldehyde at RT for 10 min, permeabilized with 0.5% Triton X-100 in PBS for 30 min at RT, and blocked with 10% goat serum in PBS overnight at 4°C. The samples were subsequently incubated with primary and secondary antibodies diluted in 10% goat serum in PBS. Actin cytoskeleton was visualized using tetramethylrhodamine B isothiocyanate conjugated phalloidin according to the manufacturer’s protocol. The samples were mounted in ProLong Antifade in 10% goat serum in PBS.}

**RESULTS**

**Expression of the β1A<sub>K756L</sub> Mutant in GD25 Cells**

Lysine 756 in the transmembrane domain of mouse integrin subunit β1 was mutated to leucine using the QuikChange mutagenesis kit (Stratagene), the full-length cDNA was transfected into GD25 cells, and stably expressing clones were established. At least two different clones were used in experiments with comparable results. In Figure 4, two clones designated F4 and A8 are included, otherwise the results obtained with clone A8 are shown. The cell surface expression of β1, and associated α5 and α6 subunits, was verified by flow cytometry (Table 1). The expression of the β1A<sub>K756L</sub> in GD25 cells resulted in the appearance of α5, and up-regulation of α6, on the cell surface as described previously for wild-type β1 (Wennerberg et al., 1996). The mAb 9EG7 recognizes an extracellular conformation-sensitive epitope, whose exposure often, but not always, correlates with active or ligand-bound state of β1 integrins (Lenter et al., 1993; Bazzoni et al., 1998; Wennerberg et al., 1998; Armulik et al., 2000). GD25-β1A and GD25-β1A<sub>K756L</sub> cells were incubated with the reference antibody mAb Ha2/5 and with mAb 9EG7 in the absence or in the presence of Mn<sup>2+</sup> and analyzed by flow cytometry. The binding of antibodies was similar for both cell lines and was not notably increased after Mn<sup>2+</sup> treatment (Table 1). These data show that the K756 mutation in β1 integrin did not alter the exposure of the 9EG7 epitope in integrin extracellular domain.

**Attachment of GD25-β1A<sub>K756L</sub> Cells to ECM Proteins**

The competence of β1A<sub>K756L</sub> integrins to mediate cell adhesion to laminin-1 (LN-1) via α6β1 and to FN via α5β1 was tested. When cells were plated on FN, the GRGDSP peptide was added to block adhesion via endogenous αβ3 on GD25 cells (Wennerberg et al., 1998). The GD25-β1A<sub>K756L</sub> cells attached to LN-1 and to FN as efficiently as GD25 cells expressing wild-type β1 (Figure 2). Thus, the extracellular domain of β1A<sub>K756L</sub> integrins possesses a conformation that is competent for ligand binding.

**Spreading and Migration**

Although the GD25-β1A<sub>K756L</sub> cells adhered equally well as GD25-β1A cells to LN-1 and FN, they spread poorly (Figure 3A). Almost all GD25-β1A cells had spread within 1 h after plating to LN-1, whereas >50% of GD25-β1A<sub>K756L</sub> remained unspread (Figure 3B). No obvious differences in morphology were observed between the GD25-β1A and GD25-β1A<sub>K756L</sub> cells when cells were spread on vitronectin (VN) via αβ3 (Figure 3A). However, the GD25-β1A<sub>K756L</sub> cells plated on LN-1 and FN acquired a rounded shape, whereas the GD25-β1A cells were flattened and exhibited membrane extensions, ruffles, and organized stress fibers, as revealed by staining the actin cytoskeleton (our unpublished data).

To test the capability of β1A<sub>K756L</sub> to contribute to cell migration, a transfilter migration assay was performed. Migration of the GD25-β1A<sub>K756L</sub> cells was dramatically reduced and those cells exhibited only a minimal migration on FN when αβ3 was blocked (Figure 4). Thus, K756 in the β1 subunit is necessary for efficient β1-mediated cell spreading and migration.

| Table 1. Cell surface expression of integrins on untransfected and transfected GD25 cells |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Cell line/mAb | anti-β1 (Ha2/5) | anti-α5 | anti-α6 | mAb 9EG7 | mAb 9EG7 + Mn<sup>2+</sup> |
|GD25          | 3.2             | 3.1     | 8.1     | 3.4          | 4.8               |
|GD25-β1A      | 32.8            | 25.0    | 18.1    | 43.3         | 48.3              |
|GD25-β1A<sub>K756L</sub> | 34.9            | 20.0    | 15.7    | 43.7         | 39.9              |

**Figure 3.** Consequences of the K756 mutation in integrin β1 subunit on cell spreading and morphology. (A) Phase-contrast images of GD25-β1A and GD25-β1A<sub>K756L</sub> cells spread on FN in the presence of the GRGDSP peptide (0.2 mg/ml), LN-1, and VN for 2 h. (B) Diagram depicting the percentage of spread GD25-β1A (white bars) and GD25-β1A<sub>K756L</sub> (striped bars) cells on LN-1 (25 μg/ml) (see MATERIALS AND METHODS for details).
Both wild-type and K756L β1 integrins colocalized with phosphotyrosine (Figure 5) as well as with paxillin, vinculin, and FAK (our unpublished data). Photographs were taken only from relatively well spread GD25-β1A<sup>K756L</sup> cells. Notably, the clusters of β1 integrins and focal adhesion proteins at the periphery of GD25-β1A<sup>K756L</sup> cells were small compared with focal adhesions formed in GD25-β1A cells. The possibility that the mutation K756L could cause ligand-independent localization to focal adhesion was tested. However, no β1A<sup>K756L</sup> was found at focal contacts when the GD25-β1A<sup>K756L</sup> cells were plated on VN (our unpublished data).

**Figure 4.** Cell migration through FN-coated filters in response to serum. The cells were allowed to migrate for 12 h as described in MATERIALS AND METHODS. Migration of two different clones expressing β1A<sup>K756L</sup> is shown.

**Figure 5.** Immunofluorescent detection of colocalization of β1-integrins with phosphotyrosine-containing clusters in GD25-β1A and GD25-β1A<sup>K756L</sup> cells. Double stainings for β1 (a and c) and phosphotyrosine (b and d) are shown for GD25-β1A (a and b) and GD25-β1A<sup>K756L</sup> (c and d) cells. The cells were plated on FN coated (25 μg/ml) chamber slides in the presence of the GRGDS peptide (0.2 mg/ml) and incubated at 37°C for 2 h, fixed with paraformaldehyde, and permeabilized before incubation with antibodies against β1 and phosphotyrosine.

**Activation of FAK/Src in GD25-β1A<sup>K756L</sup> Cells**

Because the cytosolic protein tyrosine kinase FAK has been implicated in integrin-mediated cell spreading and migration (Ilic et al., 1995), we tested whether FAK was activated in the GD25-β1A<sup>K756L</sup> cells after β1 clustering. No difference in total tyrosine phosphorylation of FAK was seen between wild-type and mutant cells in response to adhesion to either LN-1 or anti-β1 IgG (Figure 6, A and B) or to FN + GRGDS peptide (our unpublished data) when analyzed with a generic anti-phosphotyrosine antibody. Western blot with the site-specific phosphotyrosine-397 and -576 antibodies showed that FAK was phosphorylated at these sites also in the GD25-β1A<sup>K756L</sup> cells after β1-mediated adhesion (Figures 6C and 9D). According to current models, Tyr-397 is auto- or transphosphorylated by FAK as a response to unknown activation signals after integrin ligation (Parsons, 2003). Src can be activated by binding to the pTyr-397 site in FAK as well as by other mechanisms. We found that the phosphorylation of c-Src on the Tyr-416 in the activation loop was not affected by the K756L mutation in the β1-subunit (Figure 7), showing that the activation of FAK and Src was not significantly disturbed in the GD25-β1A<sup>K756L</sup> cells.

**Tyrosine Phosphorylation of CAS**

CAS is a docking protein that becomes tyrosine phosphorylated in response to cell adhesion and is required for integrin-mediated cell migration (Burridge et al., 1992; Vuori and Ruoslahti, 1995; Abassi et al., 2003). Because the altered phosphorylation of CAS could cause the observed defects in spreading and migration of the GD25-β1A<sup>K756L</sup> cells, the phosphorylation state of CAS was examined in immunoprecipitates from GD25-β1A and GD25-β1A<sup>K756L</sup> cells. As shown in Figure 8A, a peak of tyrosine phosphorylation of CAS was seen 60 min after plating the cells onto anti-β1 integrin antibody in both cell lines. However, CAS tyrosine phosphorylation was strongly reduced in the mutant cells compared with GD25-β1A cells. In contrast to CAS, tyrosine phosphorylation of paxillin, another major docking protein multiply tyrosine phosphorylated upon cell adhesion (Burridge et al., 1992), was not affected by the β1A<sup>K756L</sup> mutation (our unpublished data). Similar results were obtained when the cells were seeded onto the natural ligands LN-1 (Figure 8B), FN (in the presence of the GRGDS peptide; our unpublished data), or invasin (Figure 9).

Thus, although FAK is activated after β1A<sup>K756L</sup>-mediated adhesion to levels comparable to those of the wild-type β1A, the phosphorylation of CAS is considerably reduced. These data support the conclusion (Wennerberg et al., 2000; Gustavsson et al., 2002) that both FAK-dependent and -independent signals contribute to tyrosine phosphorylation of CAS after β1-mediated adhesion and identify the lysine in the membrane proximal region of β1 as an essential residue for regulation of the FAK-independent pathway. Furthermore, no difference in tyrosine phosphorylation of FAK and CAS was observed whether cells were adhering to the anti-integrin mAb or to natural ligands.

**The K756L Mutation in β1A Subunit Affects PI3K-dependent Tyrosine Phosphorylation of CAS**

CAS is known to become tyrosine phosphorylated also after growth factor stimulation (e.g., epidermal growth factor), and this event is dependent on PI3K (Ojaniemi et al., 1997). We therefore investigated whether PI3K is involved in any of the two integrin-mediated pathways of CAS phosphorylation. For this analysis, the cell lines GD25-β1A<sup>K756L</sup> and GD25-β1A<sup>YY783,795FF</sup> were used, in which the FAK-indepen-
dent and FAK-dependent pathway, respectively, are blocked (Figure 9; Wennerberg et al., 2000). Before plating cells on dishes coated with anti-
/H92521 mAbs or with GST-invasin, the cells were preincubated with the PI3K inhibitors LY294002 or wortmannin, or left untreated. As shown in Figure 9, a significant reduction was seen in GD25-
/H92521A cells in the presence of either inhibitor in cells seeded both on anti-
antibody (Figure 9, A and B) or GST-invasin (Figure 9C). Although LY294002 attenuated tyrosine phosphorylation of CAS in GD25-
/H92521AYY783,795FF cells, it caused little or no effect in GD25-
/H92521AK756L cells (Figure 9). Neither of the inhibitors had any detectable effect on phosphorylation of FAK (Figure 9D) or Src family kinases (our unpublished data). These data

Figure 6. Tyrosine phosphorylation of FAK in GD25-
/H92521A and GD25-
/H92521A
K756L, cells after 
mediated adhesion. FAK was immunoprecipitated from cells kept in suspension (S), or plated on LN-1 or anti-
/H92521 mAb Ha2/5 (Ab) for 1 h. Immunoprecipitated material was separated by SDS-PAGE, transferred to nitrocellulose filters, and the filters were probed for phosphotyrosine (A and B) and for FAK phosphotyrosine-397 by using site-specific antibody (C). After stripping, the filters were re-probed with anti-FAK mAb as a loading control.

Figure 7. Detection of active c-Src in GD25-
/H92521A and GD25-
/H92521A
K756L cells adhering to anti-
/H92521 mAb. c-Src was immunoprecipitated from cell lysates prepared from suspended cells (S) or from cells adhering to anti-
/H92521 mAb for 1 h. The immunoprecipitates were resolved by SDS-PAGE and transferred to nitrocellulose filters. The membranes were first blotted for pY-416 Src and, after stripping, for total c-Src.

Figure 8. Tyrosine phosphorylation of CAS in GD25-
/H92521A and GD25-
/H92521A
K756L, cells. CAS was immunoprecipitated from lysates of suspended cells (S), or cells adhering to anti-
/H92521 mAb (A) or LN-1 (B). (A) Time curve of CAS phosphorylation after 
mediated cell adhesion. (B) Phosphorylation of CAS in cells plated on LN-1 for 1 h. The immunoprecipitates were resolved by SDS-PAGE and transferred to nitrocellulose filters, which were blotted for phosphotyrosine (mAb RC-20) and, after stripping, for CAS protein.
suggest that the FAK/Src-independent pathway to CAS tyrosine phosphorylation involves activation of PI3K by the K756-containing part of the /H9252 subunit.

Thus, the primary effect of the K756L mutation could be on the activation of PI3K or on a tyrosine kinase downstream of PI3K. The former possibility was investigated by monitoring phosphorylation of serine-473 on Akt, a well-documented PI3K-dependent event. Akt was rapidly phosphorylated at this site in response to adhesion via wild-type /H9252-integrins to anti-/H9252 antibodies or to invasin. In contrast, only minimal phosphorylation of serine-473 was induced by /H9252AK756L integrins (Figure 10). The phosphorylation of Ser-473 on Akt was completely blocked by preincubation of the cells with LY294002 or wortmannin (our unpublished data). We therefore conclude that the mutation affects PI3K activity, which in turn results in reduced tyrosine phosphorylation of CAS.

**DISCUSSION**

Our previous work has shown that lysine 752 in human / (756 in mouse) and the corresponding lysine in other integrin α and β chains is buried in the membrane (Armulik et al., 1999). Because this basic residue is conserved in the 18 α and eight β subunits, which represent two separate gene families, it could be important for integrin function. In this study, we show that this indeed is the case. Replacement of the lysine with leucine, which essentially corresponds to removal of an amino group, strongly affected /H9252 integrin-mediated cell spreading and migration. To make an initial characterization of the defect underlying the mutant integrin phenotype we looked at the activity of signaling proteins involved in cell migration.

Activation of FAK and Src after /H9252AK756L adhesion occurred normally, as judged by the presence of specific phosphotyrosine residues in FAK and Src. Furthermore, paxillin, the downstream target of the FAK/Src complex, was phosphorylated to an extent similar to that induced by the wild-type integrin. In contrast, tyrosine phosphorylation of CAS was significantly reduced. Thus, defective CAS phosphorylation may be the cause of, or at least contribute to, the reduced spreading and migration mediated by /H9252AK756L.

Diminished CAS phosphotyrosine levels in GD25-/H9252AK756L cells could result from reduced kinase activity or increased tyrosine phosphatase activity. However, the latter alternative seems less likely because incubation of cells with the tyrosine phosphatase inhibitor vanadate did not normalize the phosphorylation of CAS in GD25-/H9252AK756L cells in response to integrin ligation (our unpublished data).

CAS is generally considered to become tyrosine phosphorylated by the FAK/Src complex in response to cell adhesion. This conclusion is supported by the absence of
Integrin β1 Subunit Transmembrane Domain

Figure 10. Phosphorylation of PKB/Akt on serine-473 after β1-mediated adhesion. Cell lysates were prepared from suspended cells (S) or from cells adhering to anti-β1 mAb (Ab) or to invasin (Inv) for the indicated times. The lysates were subjected to SDS-PAGE, transferred to nitrocellulose filter, and the filter was blotted with an antibody specific for PKB/Akt phospho-serine-473. After stripping, the filter was blotted for Akt1/2 protein. The lower band occurring in phospho-serine-473 blot is unspecific.

Figure 11. Proposed model for β1 integrin-induced tyrosine phosphorylation of CAS. The NPXY motifs in the cytoplasmic tail of the β1-subunit are required for activation of FAK/Src, whereas the lysine in the transmembrane domain is involved in the activation of a putative PI3K-dependent tyrosine kinase leading to the tyrosine phosphorylation of CAS.
Integrin β1 Subunit Transmembrane Domain


