

The Integrin $\beta 1$ 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 $\beta 1$ subunit, this amino acid was replaced with leucine, and the mutated $\beta 1$ subunit ($\beta 1A^{K756L}$) was stably expressed in $\beta 1$ -deficient GD25 cells. The extracellular domain of $\beta 1A^{K756L}$ 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 9EG7 epitope. However, the spreading of GD25- $\beta 1A^{K756L}$ cells on fibronectin and laminin-1 was impaired, and the rate of migration of GD25- $\beta 1A^{K756L}$ cells on fibronectin was reduced compared with GD25- $\beta 1A$ cells. Phosphorylation of tyrosines in focal adhesion kinase (FAK) and the Y416 in c-Src in response to $\beta 1A^{K756L}$ -mediated adhesion was similar to that induced by wild-type $\beta 1$. The tyrosine phosphorylation level of paxillin, a downstream target of FAK/Src, was unaffected by the $\beta 1$ 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 $\beta 1$ subunit was found to interfere with $\beta 1$ -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 $\beta 1$ 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 α and β 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 the 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 β 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

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Abbreviations used: BSA, bovine serum albumin; CAS, Crk-associated substrate; ECM, extracellular matrix; FAK, focal adhesion kinase; FN, fibronectin; GST, glutathione S-transferase; LN, laminin; PI3K, phosphatidylinositol 3-kinase; SH, Src homology; VN, vitronectin.

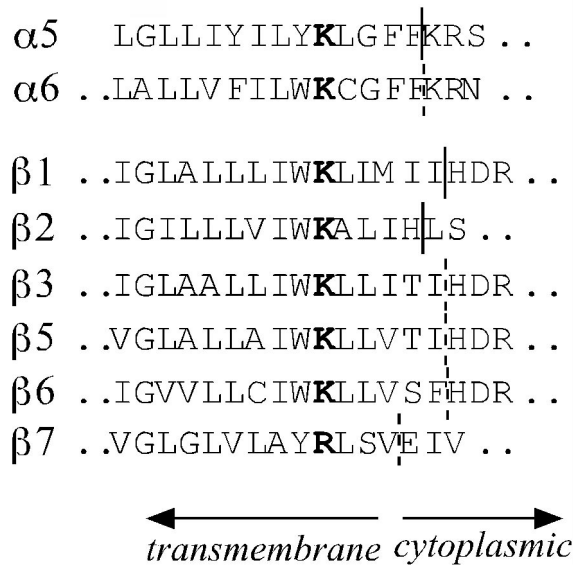


Figure 1. Alignment of the amino acid sequences at the membrane/cytoplasm interface of the selected integrin chains. The conserved transmembrane lysine is in bold. The border between the transmembrane and cytoplasmic domains of integrin $\alpha 5$, $\beta 1$, and $\beta 2$ has been determined experimentally (Armulik *et al.*, 1999) and was extrapolated for the other subunits.

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 $\beta 1$ 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 $\beta 1$ subunit has been extensively studied, only a few studies have addressed the role of the transmembrane domain in integrin function (Hayashi *et al.*, 1990; Briesewitz *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 α and β 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 $\beta 1$) is positioned in the plasma membrane in absence of interacting proteins (Armulik *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 $\beta 1$ subunit in $\beta 1$ -deficient GD25 cells. The mutation interfered with cell spreading and migration and caused strongly reduced phosphorylation of CAS and the PI3K effector PKB/Akt. These results indicate the presence of an uncharacterized PI3K-dependent signaling pathway triggered by $\beta 1$ 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. Fällman (Umeå University, Umeå, Sweden). Invasin binds selectively to a subset of $\beta 1$ integrins and efficiently promotes cell spreading and assembly of focal adhesions (Isberg 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 (Yatohgo *et al.*, 1988; Smilenov *et al.*, 1992). Mouse EHS laminin was from Invitrogen (Carlsbad, CA). The GRGDS peptide was obtained from Bachem Feinchemikalien AG. Protein A-Sepharose CL-4B and glutathione-Sepharose CL were purchased from Amersham Biosciences (Piscataway, NJ). The PI3K inhibitors LY294002 and wortmannin were from Calbiochem (San Diego, CA) and Sigma-Aldrich (St. Louis, MO), respectively.

Antibodies

The rabbit anti-rat $\beta 1$ serum was prepared in our laboratory and has been described previously (Bottger *et al.*, 1989). The monoclonal antibody (mAb) GoH3 against integrin subunit $\alpha 6$ was generously provided by Dr. A. Sonnenberg (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Monoclonal antibodies against the following proteins were purchased: mouse $\beta 1$ (clone HM $\beta 1$ -1), rat $\beta 1$ (clones Ha2/5 and 9EG7), mouse $\alpha 5$ (clone MFR5) (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, Santa Cruz, CA) and human vinculin (clone hVIN-1) (Sigma-Aldrich). Polyclonal rabbit antibodies against the following proteins/epitopes were used: human Akt1/2 (H-136) (Santa Cruz Biotechnology), FAK phosphotyrosine-397 and FAK phosphotyrosine-576 (BioSource International, Camarillo, CA), and Akt phosphoserine-473 and Src family phosphotyrosine 416 (Cell Signaling Technology, Beverly, MA). Rabbit anti-mouse IgG (H+L) and fluorochrome-labeled (fluorescein isothiocyanate and Cy3) secondary goat antibodies (anti-mouse, anti-rat, and anti-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 $\beta 1A$

The mutation K756L was introduced into the integrin $\beta 1A$ subunit by using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Briefly, the gel-purified *Bgl*III-*Xba*I fragment from pUHD10-3 expression vector (BD Biosciences Clontech, Palo Alto, CA) containing a doxycycline-regulated cytomegalovirus promoter upstream of a cDNA for mouse $\beta 1A$ (pTet $\beta 1A$) was cloned into pSP70 (Promega). The pSP70 $\beta 1A$ (*Bgl*III-*Xba*I) was used as a template for polymerase chain reaction by using the following primers: K756L:1 (5'-CCCTGCTGCTGATTGGT-TACTTTTAATGATAATTC-3'), and K756L:2 (5'-GAATTATCATTTAAAG-TAACCAATCAGCAGCAAGG-3'). Mutated base pairs are in bold. The generation of the mutation was verified by DNA sequencing. The *Bgl*III-*Xba*I fragment of $\beta 1A$ carrying the mutation was cloned into the *Bgl*III-*Xba*I site of pTet $\beta 1A$ and sequenced.

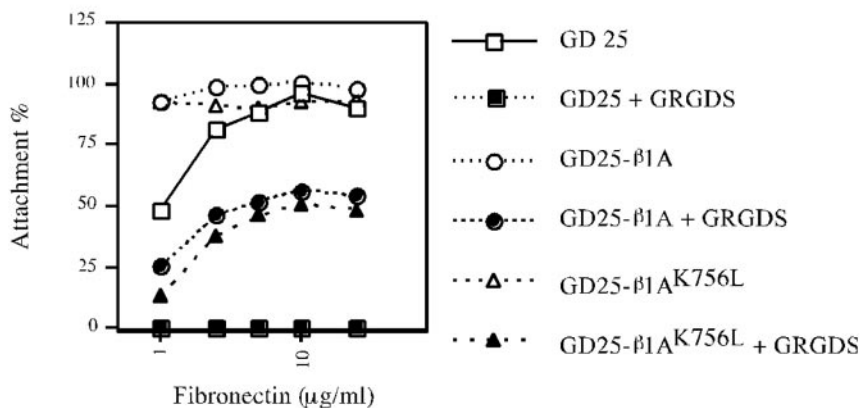
Cells

The integrin $\beta 1$ -deficient GD25 cell line, its subclone GD25T, which was established by stable transfection with the Tet repressor-encoding vector pUHD15-1hyg, and GD25 cells expressing wild-type $\beta 1A$ and $\beta 1A^{Y783,795FF}$ have been described previously (Svineng and Johansson, 1999; Wennerberg *et al.*, 2000). The GD25T cells were transfected with linearized pTet $\beta 1A^{K756L}$ and pPGKpuro vectors by using Superfect (QIAGEN, Valencia, CA) according to manufacturer's recommendations. Culture medium containing 10 μ g/ml puromycin was added to the transfected cells 48 h posttransfection. Surviving clones were tested for expression of $\beta 1$ by flow cytometry, and clones expressing high surface levels of $\beta 1$ were expanded. The GD25 cells were continuously cultured in DMEM containing 10% fetal calf serum, 2 mM L-glutamine, and hygromycin B (100 μ g/ml). The GD25 cells expressing the integrin $\beta 1$ subunit were cultured in the same medium with the addition of puromycin (20 μ g/ml). Experiments were performed using clones expressing similar levels of mutated $\beta 1A$ or wild-type $\beta 1A$ on the cell surface. The expression of $\beta 1A$ on the cell surface was verified by flow cytometry throughout the time course of these studies.

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% Na₃. Before adding a fluorescein-labeled secondary antibody the cells were washed twice with PBS. Alternatively, the cells were harvested and resuspended in Tris buffer (25 mM Tris-HCl, pH 7.5, 137 mM NaCl, 2.7 mM KCl, 5% bovine serum albumin [BSA]) containing 2 mM EDTA and incubated at 37°C for 10 min. Subsequently, the cells were washed twice with Tris-buffer 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. Finally, after washing, the cells were incubated with fluorescein-labeled secondary antibodies and analyzed (10,000/sample) in a FACScan (BD Biosciences).

A.



B.

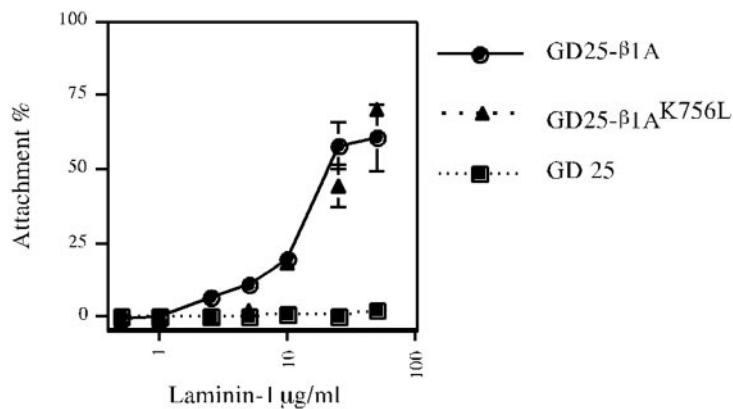


Figure 2. Attachment of GD25, GD25-β1A, and GD25-β1A^{K756L} to FN (A) and LN-1 (B). Cell attachment is expressed as percentage of the number of cells bound to VN. The GRGDS peptide (0.5 mg/ml) was added to block cell attachment to FN via αvβ3.

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 (1×10^5 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 (1×10^5) 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 quantified 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 Na₄P₂O₇, 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 preclearing 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

Table 1. Cell surface expression of integrins on untransfected and transfected GD25 cells

Cell line/mAb	anti- $\beta 1$ (Ha2/5)			mAb 9EG7	mAb 9EG7 + Mn^{2+}
	anti- $\alpha 5$	anti- $\alpha 6$			
GD25	3.2	3.1	8.1	3.4	4.8
GD25- $\beta 1A$	32.8	25.0	18.1	43.3	48.3
GD25- $\beta 1A^{K756L}$	34.9	20.0	15.7	43.7	39.9

plated on the chamber slides in serum-free DMEM. In some experiments, the GRGDS 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 (Molecular Probes Europe, Leiden, The Netherlands) and examined for fluorescein, rhodamine, and Cy3 staining.

RESULTS

Expression of the $\beta 1A^{K756L}$ Mutant in GD25 Cells

Lysine 756 in the transmembrane domain of mouse integrin subunit $\beta 1$ 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 $\beta 1$, and associated $\alpha 5$ and $\alpha 6$ subunits, was verified by flow cytometry (Table 1). The expression of the $\beta 1A^{K756L}$ in GD25 cells resulted in the appearance of $\alpha 5$, and up-regulation of $\alpha 6$, on the cell surface as described previously for wild-type $\beta 1$ (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 $\beta 1$ integrins (Lenter *et al.*, 1993; Bazzoni *et al.*, 1998; Wennerberg *et al.*, 1998; Armulik *et al.*, 2000). GD25- $\beta 1A$ and GD25- $\beta 1A^{K756L}$ cells were incubated with the reference antibody mAb Ha2/5 and with mAb 9EG7 in the absence or in the presence of Mn^{2+} and analyzed by flow cytometry. The binding of antibodies was similar for both cell lines and was not notably increased after Mn^{2+} treatment (Table 1). These data show that the K756 mutation in $\beta 1$ integrin did not alter the exposure of the 9EG7 epitope in integrin extracellular domain.

Attachment of GD25- $\beta 1A^{K756L}$ Cells to ECM Proteins

The competence of $\beta 1A^{K756L}$ integrins to mediate cell adhesion to laminin-1 (LN-1) via $\alpha 6\beta 1$ and to FN via $\alpha 5\beta 1$ was tested. When cells were plated on FN, the GRGDS peptide was added to block adhesion via endogenous $\alpha v\beta 3$ on GD25 cells (Wennerberg *et al.*, 1998). The GD25- $\beta 1A^{K756L}$ cells attached to LN-1 and to FN as efficiently as GD25 cells expressing wild-type $\beta 1$ (Figure 2). Thus, the extracellular domain of $\beta 1A^{K756L}$ integrins possesses a conformation that is competent for ligand binding.

Spreading and Migration

Although the GD25- $\beta 1A^{K756L}$ cells adhered equally well as GD25- $\beta 1A$ cells to LN-1 and FN, they spread poorly (Figure 3A). Almost all GD25- $\beta 1A$ cells had spread within 1 h after

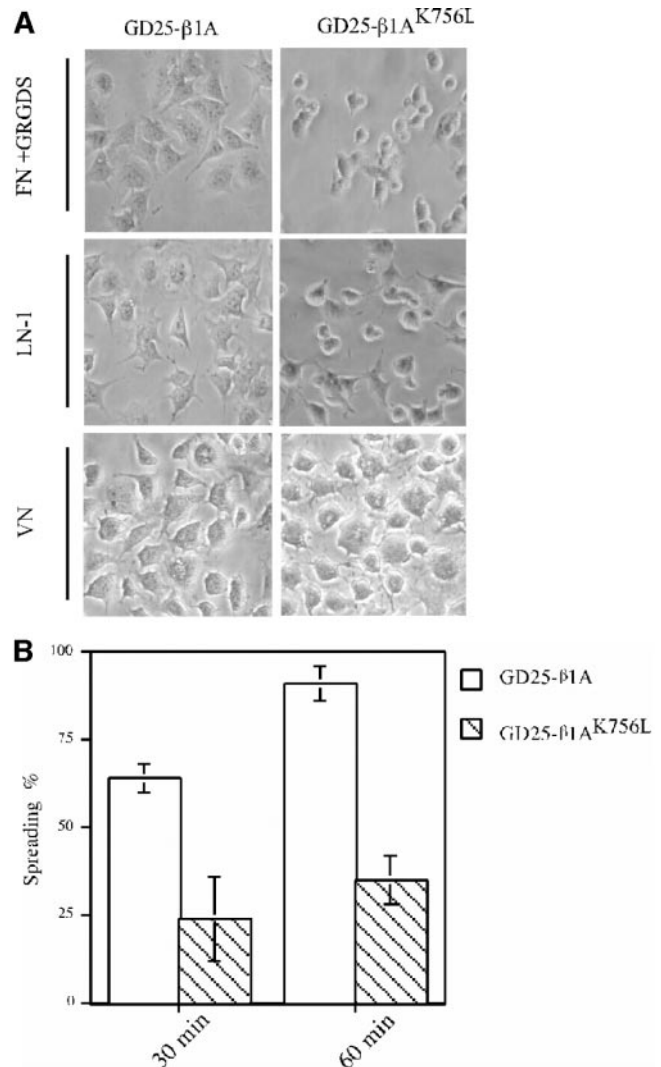


Figure 3. Consequences of the K756 mutation in integrin $\beta 1$ subunit on cell spreading and morphology. (A) Phase-contrast images of GD25- $\beta 1A$ and GD25- $\beta 1A^{K756L}$ cells spread on FN in the presence of the GRGDS peptide (0.2 mg/ml), LN-1, and VN for 2 h. (B) Diagram depicting the percentage of spread GD25- $\beta 1A$ (white bars) and GD25- $\beta 1A^{K756L}$ (striped bars) cells on LN-1 (25 μ g/ml) (see MATERIALS AND METHODS for details).

plating to LN-1, whereas >50% of GD25- $\beta 1A^{K756L}$ remained unspread (Figure 3B). No obvious differences in morphology were observed between the GD25- $\beta 1A$ and GD25- $\beta 1A^{K756L}$ cells when cells were spread on vitronectin (VN) via $\alpha v\beta 3$ (Figure 3A). However, the GD25- $\beta 1A^{K756L}$ cells plated on LN-1 and FN acquired a rounded shape, whereas the GD25- $\beta 1A$ 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 $\beta 1A^{K756L}$ to contribute to cell migration, a transfilter migration assay was performed. Migration of the GD25- $\beta 1A^{K756L}$ cells was dramatically reduced and those cells exhibited only a minimal migration on FN when $\alpha v\beta 3$ was blocked (Figure 4). Thus, K756 in the $\beta 1$ subunit is necessary for efficient $\beta 1$ -mediated cell spreading and migration.

GD25- $\beta 1A$ and GD25- $\beta 1A^{K756L}$ cells were stained for $\beta 1$ integrins and focal adhesion proteins after spreading on FN.

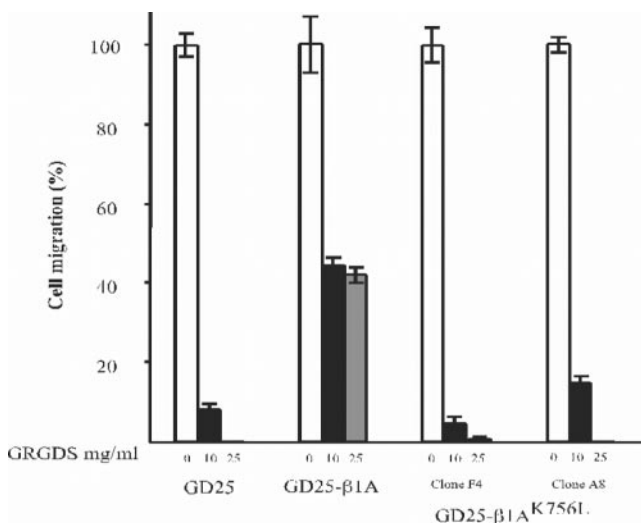


Figure 4. Cell migration through FN-coated filters in response to serum. The cells were allowed to migrate for 12 h as described in MATERIALS AND METHODS. Migration of two different clones expressing $\beta 1A^{K756L}$ is shown.

Both wild-type and K756L $\beta 1$ 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- $\beta 1A^{K756L}$ cells. Notably, the clusters of $\beta 1$ integrins and focal adhesion proteins at the periphery of GD25- $\beta 1A^{K756L}$ cells were small compared with focal adhesions formed in GD25- $\beta 1A$ cells. The possibility that the mutation K756L could cause ligand-independent localization to focal adhesion was tested. However, no $\beta 1A^{K756L}$ was found at focal contacts when the GD25- $\beta 1A^{K756L}$ cells were plated on VN (our unpublished data).

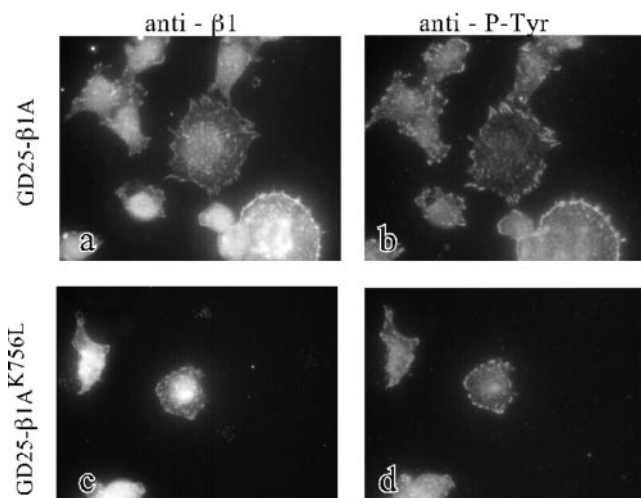


Figure 5. Immunofluorescent detection of colocalization of $\beta 1$ -integrins with phosphotyrosine-containing clusters in GD25- $\beta 1A$ and GD25- $\beta 1A^{K756L}$ cells. Double stainings for $\beta 1$ (a and c) and phosphotyrosine (b and d) are shown for GD25- $\beta 1A$ (a and b) and GD25- $\beta 1A^{K756L}$ (c and d) cells. The cells were plated on FN coated (25 μ g/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 $\beta 1$ and phosphotyrosine.

Activation of FAK/Src in GD25- $\beta 1A^{K756L}$ 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- $\beta 1A^{K756L}$ cells after $\beta 1$ 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- $\beta 1$ 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- $\beta 1A^{K756L}$ cells after $\beta 1$ -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 $\beta 1$ -subunit (Figure 7), showing that the activation of FAK and Src was not significantly disturbed in the GD25- $\beta 1A^{K756L}$ cells.

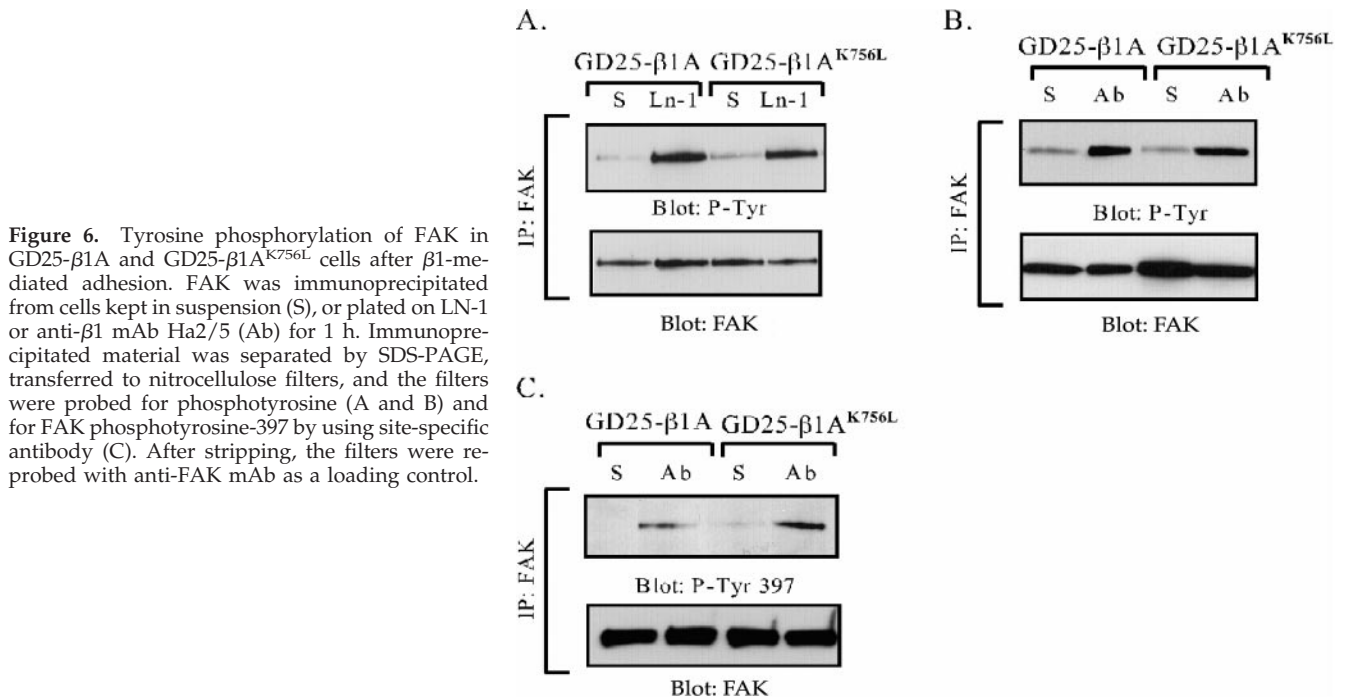
Tyrosine Phosphorylation of CAS

CAS is a docking protein that becomes tyrosine phosphorylated in response to cell adhesion and is required for integrin-mediated cell migration (Burrage *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- $\beta 1A^{K756L}$ cells, the phosphorylation state of CAS was examined in immunoprecipitates from GD25- $\beta 1A$ and GD25- $\beta 1A^{K756L}$ cells. As shown in Figure 8A, a peak of tyrosine phosphorylation of CAS was seen 60 min after plating the cells onto anti- $\beta 1$ integrin antibody in both cell lines. However, CAS tyrosine phosphorylation was strongly reduced in the mutant cells compared with GD25- $\beta 1A$ cells. In contrast to CAS, tyrosine phosphorylation of paxillin, another major docking protein multiply tyrosine phosphorylated upon cell adhesion (Burrage *et al.*, 1992), was not affected by the $\beta 1A^{K756L}$ 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).

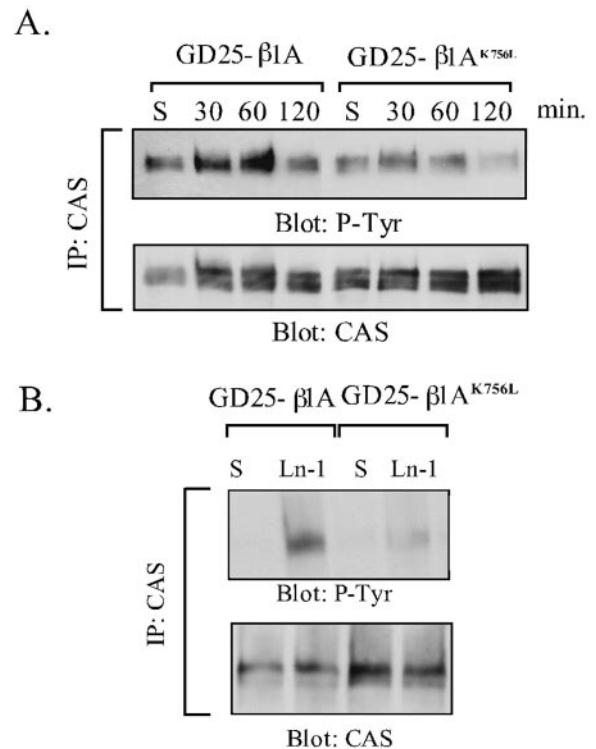
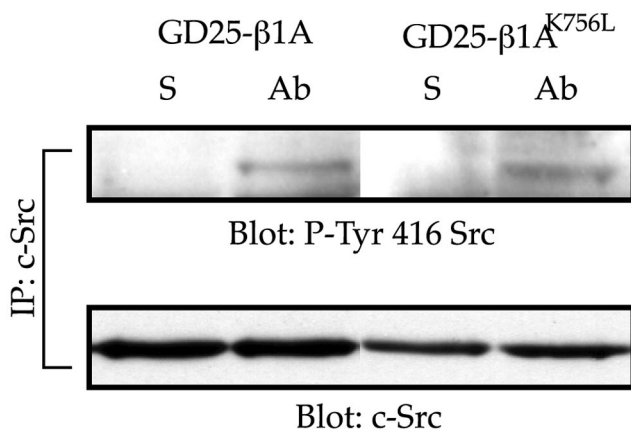
Thus, although FAK is activated after $\beta 1A^{K756L}$ -mediated adhesion to levels comparable to those of the wild-type $\beta 1A$, the phosphorylation of CAS is considerably reduced. These data support the conclusion (Wennerberg *et al.*, 2000; Gustavsson *et al.*, 2002) that both FAK-dependent and -independent signals contribute to tyrosine phosphorylation of CAS after $\beta 1$ -mediated adhesion and identify the lysine in the membrane proximal region of $\beta 1$ 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 $\beta 1A$ 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- $\beta 1A^{K756L}$ and GD25- $\beta 1A^{Y783,795FF}$ 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- $\beta 1$ 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- $\beta 1A$ cells in the presence of either inhibitor in cells seeded both on anti- $\beta 1$ antibody (Figure 9, A and B) or GST-invasin (Figure 9C). Although LY294002 attenuated tyrosine phosphorylation of CAS in GD25- $\beta 1A^{YY783,795FF}$ cells, it caused little or no effect in GD25- $\beta 1A^{K756L}$ 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



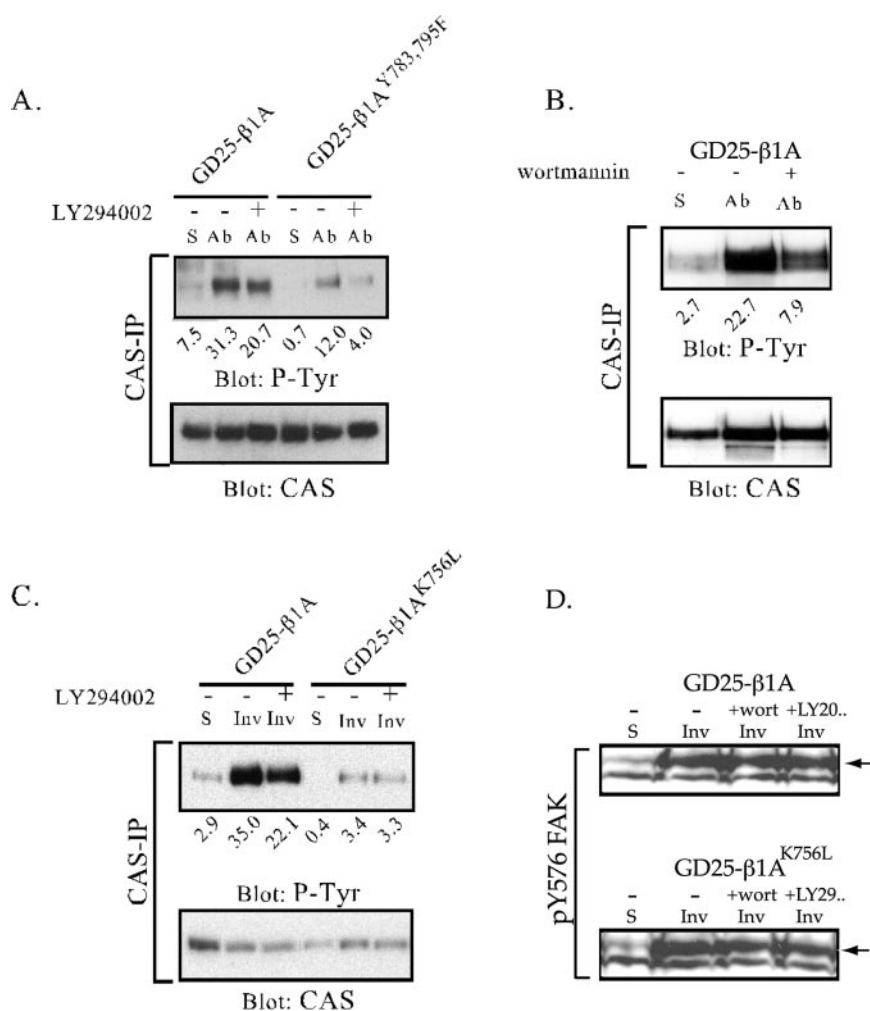


Figure 9. Tyrosine phosphorylation of CAS and FAK after $\beta 1$ -mediated adhesion in the presence of PI3K inhibitors. CAS was immunoprecipitated from cell lysates prepared from suspended cells (S) or from cells adhering to anti- $\beta 1$ mAb (Ab) or to invasin (Inv) for 1 h. For the analysis of FAK phosphorylation the cells were lysed directly in SDS sample buffer. Before plating, the cells were treated with LY294002 (20 μ M; A and C) or wortmannin (100 nM; B) (+), or with the diluent (dimethyl sulfoxide) as a control (-). Precipitated material was subjected to SDS-PAGE, transferred to nitrocellulose filters, and sequentially blotted for phosphotyrosine and CAS protein. The relative intensities of the bands versus the gel background are represented by arbitrary units under each lane. In D, equal amounts of cell lysate were blotted for pY576 FAK. Arrows point at bands corresponding to FAK.

suggest that the FAK/Src-independent pathway to CAS tyrosine phosphorylation involves activation of PI3K by the K756-containing part of the $\beta 1$ 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 $\beta 1$ -integrins to anti- $\beta 1$ antibodies or to invasin. In contrast, only minimal phosphorylation of serine-473 was induced by $\beta 1A^{K756L}$ 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 $\beta 1$ (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 $\beta 1$ 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 $\beta 1A^{K756L}$ 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 $\beta 1A^{K756L}$.

Diminished CAS phosphotyrosine levels in GD25- $\beta 1A^{K756L}$ 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- $\beta 1A^{K756L}$ 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

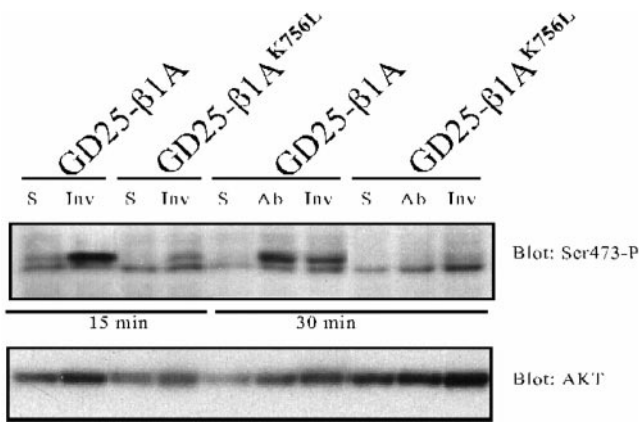


Figure 10. Phosphorylation of PKB/Akt on serine-473 after $\beta 1$ -mediated adhesion. Cell lysates were prepared from suspended cells (S) or from cells adhering to anti- $\beta 1$ mAb (Ab) or to invasin (Inv) for the indicated times. The lysates were subjected to SDS-PAGE, transferred to nitrocellulose 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.

integrin-mediated tyrosine phosphorylation of CAS in cells lacking Src-family kinases (Klinghoffer *et al.*, 1999). Nevertheless, several studies have found that tyrosine phosphorylation of CAS did not correlate closely with FAK activity (Jucker *et al.*, 1997; Eisenmann *et al.*, 1999; Kira *et al.*, 2002; Konrad *et al.*, 2003; Kwong *et al.*, 2003) and that other tyrosine kinases, such as Fyn/Yes (Klinghoffer *et al.*, 1999), Abl (Feller *et al.*, 1994; Riggins *et al.*, 2003), and Etk (Abassi *et al.*, 2003) can phosphorylate CAS. Interestingly, tyrosine phosphorylation of CAS in platelets occurs before FAK activation and seems to be dependent on phosphoinositide turnover (Ohmori *et al.*, 2000). PI3K activity was also necessary for epidermal growth factor induced tyrosine phosphorylation of CAS in Rat-1 cells (Ojaniemi *et al.*, 1997). These reports are in line with our data, which indicate that PI3K activity promotes tyrosine phosphorylation of CAS after $\beta 1$ -mediated adhesion.

Our previous work with GD25 cells transfected with the $\beta 1B$ splice variant, or the $\beta 1A$ variant with mutated tyrosines in the two NPXY motifs, showed that activation of FAK upon clustering of $\beta 1$ integrins was attenuated in both cell lines. The $\beta 1$ integrin-mediated CAS tyrosine phosphorylation still occurred in these cells but was strongly reduced (Wennerberg *et al.*, 2000; Gustavsson *et al.*, 2002). In the present report, we show that mutation of lysine in the transmembrane domain of the $\beta 1$ subunit caused reduced ty-

rosine phosphorylation of CAS, although FAK activity was unaffected. By applying PI3K inhibitors, and monitoring the levels of Akt phosphoserine 473 as a readout of PI3K activity, we demonstrated that mutation of K756 affected integrin-mediated activation of PI3K, which in turn was required for full tyrosine phosphorylation of CAS. Figure 11 depicts a summary of these findings and suggests the existence of two pathways leading to tyrosine phosphorylation of CAS: tyrosines in the cytoplasmic NPXY motifs of $\beta 1$ are required for FAK activation and thereby for tyrosine phosphorylation of CAS by the FAK/Src complex, whereas the lysine in the transmembrane domain is required for PI3K-dependent tyrosine phosphorylation of CAS. The identity of the proposed integrin-regulated PI3K-dependent tyrosine kinase is presently not known. Based on the published data and our observations using protein kinase inhibitors, several candidate kinases can be considered, including the Src-family kinases Fyn and Yes (Wary *et al.*, 1998; Klinghoffer *et al.*, 1999), growth factor receptors (Sundberg and Rubin, 1996; Moro *et al.*, 2002), Abl (Feller *et al.*, 1994; Riggins *et al.*, 2003), and the Tec family kinases (Chen *et al.*, 2001; Smith *et al.*, 2001; Abassi *et al.*, 2003).

Tyrosine phosphorylation has been shown to be important for assembly and turnover of focal adhesions and for integrin-mediated signaling (Kornberg *et al.*, 1991; Burridge *et al.*, 1992; Crowley and Horwitz, 1995; Defilippi *et al.*, 1995). FAK is not required for formation of focal adhesions, but it clearly has a key role in dissociating focal adhesions (Ilic *et al.*, 1995; Wennerberg *et al.*, 2000). The cellular phenotype resulting from the K756L mutation in $\beta 1$ suggests that the affected tyrosine kinase pathway is involved in earlier responses of integrin ligation, which lead to cell spreading and actin reorganization. The conserved lysine is located in the segment of β subunits, often referred to as "membrane proximal," which has been suggested to regulate transmembrane conformational changes during activation ("inside-out" signaling) of integrin $\alpha IIb\beta 3$ (Li *et al.*, 2002; Liddington and Ginsberg, 2002; Vinogradova *et al.*, 2002). However, this process was apparently not affected by the K756L mutation because the mutant integrin maintained ligand binding competence. Thus, in addition to proposing the existence of a new signaling pathway from $\beta 1$ integrins to PI3K to CAS, the present study demonstrates that 1) the membrane-proximal segment of integrins mediates also outside-in signals, and 2) this mechanism is distinct from that used during integrin activation. Further studies are required to determine the exact role of the membrane proximal basic residue in integrin signaling, for example, its possible participation in integrin oligomerization or association of integrins with other plasma membrane-anchored or cytoplasmic proteins (Li *et al.*, 2001; Stipp *et al.*, 2003).

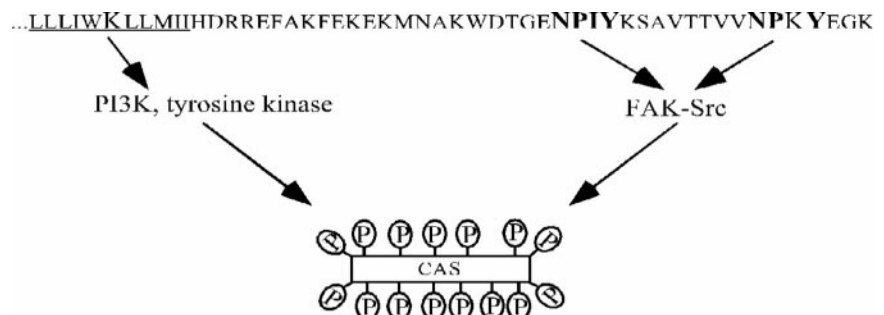


Figure 11. Proposed model for $\beta 1$ integrin-induced tyrosine phosphorylation of CAS. The NPXY motifs in the cytoplasmic tail of the $\beta 1$ -subunit are required for activation of FAK/Src, whereas the lysine in the transmembrane domain is involved in the activation of a putative PI3K-dependent tyrosine kinase leading to the tyrosine phosphorylation of CAS.

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