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Differential Role of β_{1C} and β_{1A} Integrin Cytoplasmic Variants in Modulating Focal Adhesion Kinase, Protein Kinase B/AKT, and Ras/Mitogen-activated Protein Kinase Pathways

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The integrin cytoplasmic domain modulates cell proliferation, adhesion, migration, and intracellular signaling. The β_1 integrin subunits, β_{1C} and β_{1A}, that contain variant cytoplasmic domains differentially affect cell proliferation; β_{1C} inhibits proliferation, whereas β_{1A} promotes it. We investigated the ability of β_{1C} and β_{1A} to modulate integrin-mediated signaling events that affect cell proliferation and survival in Chinese hamster ovary stable cell lines expressing either human β_{1C} or human β_{1A}. The different cytodomains of either β_{1C} or β_{1A} did not affect either association with the endogenous α_2, α_V, and α_5 subunits or cell adhesion to fibronectin or TS2/16, a mAb to human β_1. Upon engagement of endogenous and exogenous integrins by fibronectin, cells expressing β_{1C} showed significantly inhibited extracellular signal–regulated kinase (ERK) 2 activation compared with β_{1A} stable cell lines. In contrast, focal adhesion kinase phosphorylation and Protein Kinase B/AKT activity were not affected. Selective engagement of the exogenously expressed β_{1C} by TS2/16 led to stimulation of Protein Kinase B/AKT phosphorylation but not of ERK2 activation; in contrast, β_{1A} engagement induced activation of both proteins. We show that Ras activation was strongly reduced in β_{1C} stable cell lines in response to fibronectin adhesion and that expression of constitutively active Ras, Ras 61 (L), rescued β_{1C}-mediated down-regulation of ERK2 activation. Inhibition of cell proliferation in β_{1C} stable cell lines was attributable to an inhibitory effect of β_{1C} on the Ras/MAP kinase pathway because expression of activated MAPK kinase rescued β_{1C} antiproliferative effect. These findings show that the β_{1C} variant, by means of a unique signaling mechanism, selectively inhibits the MAP kinase pathway by preventing Ras activation without affecting either survival signals stimulated by integrins or cellular interactions with the extracellular matrix. These findings highlight a role for β_1-specific cytodomain sequences in maintaining an intracellular balance of proliferation and survival signals.

INTRODUCTION

Integrins are a large family of heterodimeric transmembrane receptors composed of α and β subunits (Hynes, 1992). In addition to their role as adhesion receptors, integrins have been shown to regulate intracellular signaling pathways and cellular functions such as cell migration, proliferation, and survival (Schwartz *et al.*, 1995; Bottazzi and Assoian, 1997; Frisch and Ruoslahti, 1997).

It is well established that the cytoplasmic domain of the β subunit is required for integrins to modulate many cellular functions and to trigger signaling events that result in protein phosphorylation (Hemler *et al.*, 1995; Fornaro and Languino, 1997; Wei *et al.*, 1998) and interactions with intracellular proteins (Hemler, 1998). Thus, mutations or deletions in the β_{1A} subunit cytodomain have been shown to alter the ability of this integrin to trigger focal adhesion kinase (FAK) phosphorylation (Guan *et al.*, 1991) and to interact with cytoskeletal proteins such as talin and filamin (Chen *et al.*, 1995; Lewis and Schwartz, 1995; Pfaff *et al.*, 1998).

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Abbreviations used: AKT, Protein Kinase B/AKT; CHO, Chinese hamster ovary; ERK, extracellular signal–regulated kinase; FAK, focal adhesion kinase; ILK, integrin-linked kinase; MEK, MAPK kinase; MEK EE, constitutively active MEK; MEK WT, wild-type MEK; PI 3-kinase, phosphatidylinositol 3-kinase.

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The identification and characterization of a number of spliced variants of the integrin cytoplasmic domain in the β and α subgroups (Fornaro and Languino, 1997) have added a new level of complexity to integrin functions. Four different β_1 isoforms have been identified (β_{1A}, β_{1B}, β_{1C}, and β_{1D}) and have been shown to differentially affect receptor localization, cell proliferation, cell adhesion and migration, interactions with intracellular proteins, and ultimately phosphorylation and activation of signaling molecules (Belkin *et al.*, 1997; Fornaro and Languino, 1997; Belkin and Retta, 1998; Pfaff *et al.*, 1998; Retta *et al.*, 1998; Meredith *et al.*, 1999).

The β_{1C} integrin is an alternatively spliced variant of the β_1 subfamily that contains a unique 48-amino acid sequence in its cytoplasmic domain (Languino and Ruoslahti, 1992). We and others have shown that either full-length β_{1C} or its cytoplasmic domain inhibits prostate cancer epithelial cell (Fornaro *et al.*, 1998; Meredith *et al.*, 1999), endothelial cell (Meredith *et al.*, 1999), and fibroblast (Fornaro *et al.*, 1995; Meredith *et al.*, 1995, 1999) proliferation. In vivo, β_{1C} is expressed in nonproliferative, differentiated epithelium and is selectively down-regulated in prostatic adenocarcinoma, and its expression inversely correlates with markers of cell proliferation in breast carcinoma (Fornaro *et al.*, 1996, 1998, 1999; Manzotti *et al.*, 2000). However, the signaling pathways affected by β_{1C} are still unknown.

FAK is a nonreceptor protein tyrosine kinase that has been shown to colocalize with integrins at focal contact sites (Guan *et al.*, 1991). FAK becomes tyrosine phosphorylated in response to integrin engagement (Guan *et al.*, 1991; Kornberg *et al.*, 1991) and has been shown to prevent apoptosis (Frisch *et al.*, 1996; Hungerford *et al.*, 1996; Xu *et al.*, 1996; Illic *et al.*, 1998; Cary and Guan, 1999). Two recent reports have highlighted a new role for FAK in the modulation of cell cycle progression and in the inhibition of integrin-stimulated signaling events during mitosis (Zhao *et al.*, 1998; Yamakita *et al.*, 1999). The first study showed that FAK overexpression accelerates the G1/S phase transition, increases cyclin D1 levels, and decreases $p21^{waf1}$ expression (Zhao *et al.*, 1998). The second study demonstrated that FAK undergoes mitosis-specific serine phosphorylation accompanied by tyrosine dephosphorylation, which results in FAK/Cas/c-Src complex dissociation and inhibition of signal transduction pathways involving integrins (Yamakita *et al.*, 1999).

In addition to stimulating FAK, integrins can also activate the phosphatidylinositol 3-kinase (PI 3-kinase) pathway (Keely *et al.*, 1998). PI 3-kinases are a family of lipid kinases activated by a wide variety of extracellular stimuli. The lipid products of PI 3-kinases, specifically phosphatidylinositol (3,4)biphosphate and phosphatidylinositol(3,4,5)triphosphate, affect cell proliferation, survival, differentiation, and migration by targeting specific signaling molecules such as the serine/threonine protein kinase B, also known as AKT, and PKC (Jiang *et al.*, 1999; Rameh and Cantley, 1999). Integrin-mediated adhesion to the extracellular matrix stimulates the production of phosphatidylinositol(3,4)biphosphate and phosphatidylinositol(3,4,5)triphosphate (Khwaja *et al.*, 1997; King *et al.*, 1997), the association of the p85 PI 3-kinase subunit with FAK (Chen and Guan, 1994), and AKT activation (Khwaja *et al.*, 1997; King *et al.*, 1997). AKT plays an important role in transducing survival signals in response to several growth factors and integrin engagement (Khwaja *et al.*, 1997; Downward, 1998).

The small GTPase Ras is a critical component of signaling pathways that control cell proliferation, differentiation, and survival (Campbell *et al.*, 1998; Rebollo and Martinez-A, 1999). The Ras/extracellular signal–regulated kinase (ERK) 1 and 2/MAP kinase pathway plays a pivotal role in modulating gene expression and cell cycle progression in response to mitogens (Robinson and Cobb, 1997; Guadagno and Ferrell, 1998; Brunet *et al.*, 1999). Integrin clustering has been shown to stimulate Ras GTP loading (Clark and Hynes, 1996; Wary *et al.*, 1996; King *et al.*, 1997; Mainiero *et al.*, 1997; Miranti *et al.*, 1999) and to activate specific effectors of the Ras/MAP kinase signaling cascade such as Raf-1 and MAPK kinase (MEK) (Howe *et al.*, 1998; Schlaepfer and Hunter, 1998). In several studies, the dominant negative N17 mutant of Ras has been shown to block extracellular matrix–mediated ERK2 activation (Clark and Hynes, 1996; Wary *et al.*, 1996; King *et al.*, 1997; Mainiero *et al.*, 1997; Schlaepfer and Hunter, 1997; Wei *et al.*, 1998), whereas in one report it had no effect (Chen *et al.*, 1996b). The mechanisms of integrin-mediated activation of the MAP kinase cascade comprise three models (Howe *et al.*, 1998). Two models include Src family kinases and Ras as critical links between integrin-mediated adhesion and MAP kinase activation. In the first model, integrin ligation leads to Src and FAK activation, Grb2 binding to FAK, and membrane localization of the guanine nucleotide exchange factor Sos, which then promotes Ras activation (Schlaepfer *et al.*, 1994, 1998). In the second model, integrins activate the Ras/MAP kinase pathway via the tyrosine kinase Fyn and the adaptor protein Shc (Wary *et al.*, 1996, 1998). A recent report has indicated that fibronectin-induced PKC activation plays a role in ERK2 activation upstream of Shc (Miranti *et al.*, 1999). The third model proposes a Ras-independent activation of Raf and, thus, ERK2 by integrins (Chen *et al.*, 1996b; Lin *et al.*, 1997).

Using Chinese hamster ovary (CHO) stable cell lines expressing either human β_{1C} or human β_{1A}, we have analyzed the ability of β_{1C} and β_{1A} to modulate signaling pathways that control cell proliferation and survival. The β_{1C} variant associates with the same α subunits as β_{1A} and does not affect cell adhesion to β_1 ligands. We show that β_{1C} has an inhibitory effect on ERK2 activation mediated by fibronectin without affecting FAK phosphorylation and AKT activity. We also show that Ras activation stimulated by adhesion to fibronectin is inhibited in β_{1C} transfectants and that constitutively active Ras and MEK rescue β_{1C}-mediated down-regulation of ERK2 activation and inhibition of cell growth, respectively. This is the first description of a selective inhibitory role of the integrin cytoplasmic domain on the Ras/MAP kinase pathway. Moreover, AKT phosphorylation is observed in response to antibody-mediated engagement of human β_{1C} and β_{1A}, and ERK2 activation is supported by β_{1A} but not by β_{1C} ligation, indicating a different role for β_1 variants in the activation of AKT and MAP kinase pathways. We suggest that by expressing variant β_1 intracellular domains, cells may accomplish the delicate task of inhibiting proliferation without affecting either selective downstream sur-

vival signals (FAK and AKT) mediated by integrins or interactions with the extracellular environment.

MATERIALS AND METHODS

Reagents and Antibodies

Rabbit antibodies specific for the β_{1C} subunit cytoplasmic domain were affinity-purified as described previously (Fornaro *et al.*, 1996). The following antibodies were used: mouse mAbs P4C10 and TS2/16 to human β_1 integrin (Life Technologies, Gaithersburg, MD, and American Type Culture Collection, Rockville, MD, respectively), 7E2 to hamster β_1 integrin, PB1 to hamster $\alpha_5\beta_1$ (a kind gift of Dr. R.L. Juliano, University of North Carolina, Chapel Hill, NC), E10 to phospho-ERK1 and 2 (New England Biolabs, Beverly, MA), 12CA5 to hemagglutinin (Boehringer Mannheim, Indianapolis, IN), and to pan Ras (Transduction Laboratories, Lexington, KY); rabbit affinity-purified antibodies to FAK Y^{397} (Biosource International, Camarillo, CA), to AKT (New England Biolabs), and to FAK and ERK1 and 2 (Santa Cruz Biotechnology, Santa Cruz, CA). Rabbit antisera to α_5, α_v, or α_4 were provided by Dr. E. Ruoslahti (The Burnham Institute, La Jolla, CA), antiserum to α_2 was provided by Dr. M.E. Hemler (Dana-Farber Cancer Institute, Boston, MA), and antiserum to β_{1C} was described previously (Fornaro *et al.*, 1996). Human plasma fibronectin and human vitronectin were purified as described (Engvall and Ruoslahti, 1977; Yatohgo *et al.*, 1988). Poly-L-lysine and nonimmune rabbit and mouse immunoglobulin G were purchased from Sigma Chemical (St. Louis, MO).

Cells and Plasmids

To obtain stable cell lines expressing β_{1A} in a tetracycline-regulated system, *Cla*I–*Xba*I fragment encoding full-length human β_{1A} was isolated from Bluescript-β_{1A} and subcloned into *Cla*I–*Spe*I sites in the pTet-Splice plasmid (a kind gift of Dr. D. Schatz, Yale University, New Haven, CT) to generate the pTet-β_{1A} construct. The pTet-β_{1C} construct has been described previously (Fornaro *et al.*, 1999). CHO stable cell lines expressing either human β_{1C} (clones 16.4, 16.28, and 16.30) or human β_{1A} (clones 10.2, 10.18, and 10.23) integrins under the control of a tetracycline-regulated promoter were generated and maintained in growth medium containing 1 μg/ml tetracycline (Boehringer Mannheim) and 0.1 mg/ml G418 (Life Technologies) as described (Fornaro *et al.*, 1999).

pMLC-1 plasmids containing hemagglutinin-tagged wild-type MEK (MEK WT) and constitutively active MEK (MEK EE) have been described previously (Bennett and Tonks, 1997). The pGEX-RBD plasmid encodes amino acids 1–149 of cRaf-1 fused to GST (Taylor and Shalloway, 1996). The pMT3-Ras 61 (L) encodes a c-ras^H form containing a codon 61 mutation (Bennett *et al.*, 1996).

β_{1C}-CHO stable cell lines were transiently transfected by electroporation by using 10 μg of either MEK WT, MEK EE, Ras 61 (L), or vector alone as described (Fornaro *et al.*, 1999). Cells were incubated for 48 h at 37°C in growth medium either in the absence or in the presence of 1 μg/ml tetracycline and serum-starved during the last 24 h of the 48-h culture before analysis of either cell proliferation or ERK2 activity as described below.

Flow Cytometry

Surface expression of exogenous human β_{1C} and β_{1A} integrins was achieved by withdrawal of tetracycline from the growth medium; in both cell transfectants, maximal and comparable β_{1C} or β_{1A} expression were consistently obtained 48 h after tetracycline removal. For each experiment, exogenous human β_1 integrin expression was monitored by FACS with TS2/16 serum-free culture supernatant or 12CA5 as negative control antibody (Fornaro *et al.*, 1999). Endogenous hamster β_1 or $\alpha_5\beta_1$ integrin expression was analyzed with either 5 μg/ml 7E2 or 1 μg/ml PB1, respectively (Fornaro *et al.*, 1995).

Immunoprecipitation of β_{1C} and β_{1A} Integrins

CHO stable cell lines were cultured for 48 h in the absence of tetracycline to induce β_{1C} or β_{1A} integrin expression (Fornaro *et al.*, 1999). Cells were detached with 0.05% trypsin/0.53 mM EDTA (Life Technologies) and surface iodinated as described previously (Bartfeld *et al.*, 1993). Cells were lysed in 1% NP-40 (Calbiochem, La Jolla, CA), 0.5% sodium deoxycholate (Sigma), 0.1% SDS (American Bioanalytical, Natick, MA), 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM PMSF (Life Technologies), 10 μg/ml aprotinin (Sigma), 10 μg/ml leupeptin (Calbiochem), and 10 μg/ml pepstatin (Sigma) for 30 min at 4°C. β_{1C} and β_{1A} integrins were immunoprecipitated with P4C10 and protein A–Sepharose (Sigma) as described (Fornaro *et al.*, 1995). Immunocomplexes were dissociated with 20 mM Tris-HCl, pH 7.5, 2% SDS and boiled for 5 min. The dissociated material was then diluted 10-fold with lysis buffer and reprecipitated overnight at 4°C with 30 μl of rabbit serum specific for the β_{1C} subunit cytoplasmic domain. Immunoprecipitates were recovered with protein A–Sepharose, washed four times with lysis buffer, and resuspended in loading buffer (2% SDS, 50 mM Tris-HCl, pH 6.8, 100 mM DTT [American Bioanalytical], 10% glycerol, and 0.1% bromphenol blue [Bio-Rad, Hercules, CA]). Proteins were separated by SDS-PAGE (7.5%) and visualized by autoradiography.

Immunoprecipitations of α Subunits Associated with β_{1C}

CHO stable cell lines were cultured for 72 h in the absence of tetracycline; cells were then detached with 0.05% trypsin/0.53 mM EDTA and surface iodinated as described above. Cells were lysed in 1% Triton X-100 (American Bioanalytical), 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin for 30 min at 4°C. β_{1C} and β_{1A} integrins were immunoprecipitated with P4C10 as described above. Immunoprecipitates were washed five times with lysis buffer, resuspended in 10 mM Tris-HCl, pH 7.5, 0.5% SDS, and incubated for 10 min at 70°C. The eluted material was diluted threefold with lysis buffer and reprecipitated with rabbit antiserum to α_5, α_V, α_4, or α_2 overnight at 4°C. Immunoprecipitates were recovered with protein A–Sepharose, washed three times with lysis buffer, and resuspended in loading buffer. Proteins were separated by SDS-PAGE (10%) and visualized by autoradiography.

Cell Adhesion Assay to β_1 Ligands

Cell adhesion to fibronectin (10 μg/ml), 7E2 (1 μg/ml), TS2/16 (1:10 dilution of culture supernatant), mouse immunoglobulin G (1 μg/ml), and BSA (10 mg/ml; Sigma) was performed as described previously (Languino *et al.*, 1993) with 25,000 ^{51}Cr-labeled cells (^{51}Cr from DuPont–New England Nuclear, Wilmington, DE).

For analysis of FAK, AKT, and ERK2, CHO stable cell lines were cultured for 48 h either in the absence or in the presence of 1 μg/ml tetracycline, starved, and then detached as described above. Cells were held in suspension for 30–60 min at 37°C and either kept in suspension or plated on tissue culture plates coated with poly-L-lysine (5–10 μg/ml), fibronectin (10 μg/ml), TS2/16 (1:10 dilution of culture supernatant), or 7E2 (3 μg/ml) at 37°C for the indicated times. Where indicated, cells were incubated for 15 min at 37°C with 100 nM wortmannin (Calbiochem) before plating onto ligand-coated dishes. The cells were then washed twice with PBS (Life Technologies) and lysed in the appropriate ice-cold lysis buffer. The protein content in each lysate was quantitated with the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL).

In all instances, quantification of immunoreactive bands was performed by densitometric analysis; the values are given as fold increase on fibronectin, TS2/16, or 7E2 versus poly-L-lysine or suspension within each established cell line after normalization for protein loading. The values from several experiments are reported as means $\pm$ SEM.

FAK Analysis

CHO stable cell lines were lysed with 1% NP-40, 0.5% deoxycholate, 50 mM HEPES, pH 7.5, 150 mM NaCl, 100 mM sodium fluoride (Sigma), 1 mM sodium vanadate (Sigma), 5 mM $Na_4P_2O_7$ (J.T. Baker, Phillipsburg, NJ), 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin for 30 min at 4°C, and insoluble material was removed by centrifugation at 14,000 × *g* for 15 min at 4°C.

FAK was immunoprecipitated from 500 μg of total cell lysate with 0.5 μg of C-20, an affinity-purified antibody to FAK. Immunocomplexes were collected with protein A–Sepharose, washed five times with lysis buffer, and resuspended in loading buffer. Proteins were separated by 10% SDS-PAGE, and FAK phosphorylation on Tyr^{397} was analyzed by immunoblotting with a rabbit affinity-purified antibody that recognizes FAK only when phosphorylated on Y^{397}. FAK protein levels were analyzed by immunoblotting with C-20 rabbit affinity-purified antibody to FAK as described (Zheng *et al.*, 1999).

AKT Analysis

CHO stable cell lines were lysed with 1% NP-40, 50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM EDTA, 50 mM sodium fluoride, 1 mM sodium vanadate, 1 mM $Na_4P_2O_7$, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin for 30 min at 4°C. Analysis of AKT phosphorylation was performed by immunoblotting with phospho-specific antibody to Ser^{473} (New England Biolabs) according to the manufacturer's instructions.

AKT kinase activity was assayed according to Franke *et al.* (1995). Briefly, 50 μg of detergent cell extracts were cleared by centrifugation at 14,000 × *g* for 15 min at 4°C. AKT was immunoprecipitated with 0.1 μg of affinity-purified antibody to AKT. Immunocomplexes were collected with protein A–Sepharose and washed three times with lysis buffer, once with 20 mM HEPES, pH 7.5, and once with kinase buffer (20 mM HEPES, pH 7.5, 1 mM DTT, 10 mM $MnCl_2$, 10 mM $MgCl_2$). The AKT kinase activity was assayed with kinase buffer containing 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; Amersham Life Sciences, Arlington Heights, IL), 5 μM ATP (Boehringer Mannheim), and 100 μg/ml histone H2B (Boehringer Mannheim) as a substrate for 20 min at 30°C. The reactions were terminated with loading buffer. Phosphorylated histone H2B was viewed by autoradiography.

ERK2 Analysis

CHO stable cell lines were lysed as described for analysis of AKT activation. Analysis of ERK2 phosphorylation by immunoblotting was performed with 0.5 μg/ml E10, a mAb that recognizes ERK2 only when phosphorylated at Thr^{202}/Tyr^{204}, according to the manufacturer's instructions (New England Biolabs). ERK2 activation was analyzed by in vitro kinase assay with myelin basic protein as described (Fornaro *et al.*, 1999).

Assay for Detection of Activated Ras

Ras activation was analyzed as described previously (Taylor and Shalloway, 1996). Briefly, GST-RBD expression in transformed *Escherichia coli* DH5α was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (American Bioanalytical) for 2 h at 37°C. The cells were then washed once with ice-cold 20 mM HEPES, pH 7.5, 150 mM NaCl and lysed by sonication in the following buffer: 20 mM HEPES, pH 7.5, 120 mM NaCl, 10% glycerol, 2 mM EDTA, 100 mg/ml lysozyme (American Bioanalytical), 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin. The lysate was clarified by centrifugation and incubated with glutathione Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) for 30 min at 4°C. The Sepharose beads were then washed six times with lysis buffer containing 0.5% NP-40 and stored in the same buffer at 4°C.

For affinity precipitation, cells were washed twice with ice-cold 20 mM HEPES, pH 7.5, 150 mM NaCl and lysed with the following buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% deoxycholate, 10% glycerol, 10 mM $MgCl_2$, 25 mM sodium fluoride, 1 mM EDTA, 1 mM sodium vanadate, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin) for 30 min at 4°C. One milligram of whole cell lysate was incubated with GST-RBD bound to glutathione Sepharose for 30 min at 4°C. Bound proteins were washed three times with lysis buffer, eluted with loading buffer, and separated by SDS-PAGE (12%). Proteins were visualized by immunoblotting with 2 μg/ml anti-pan Ras mouse mAb according to the manufacturer's instructions (Transduction Laboratories).

Proliferation Assay

CHO stable cell lines were cultured for 48 h either in the absence or in the presence of 1 μg/ml tetracycline, starved during the last 24 h of the 48-h culture, and then detached with 0.05% trypsin/0.53 mM EDTA. Cells were resuspended in serum-free medium and plated (2,500–20,000 cells/well) on either 96- or 24-well plates coated with 1 μg/ml fibronectin for 1 h at 37°C. Attached cells were cultured for 72–96 h at 37°C in growth medium containing 5% FCS either in the absence or in the presence of 1 μg/ml tetracycline. Cells were washed, fixed with 3% paraformaldehyde, and stained overnight with 0.5% toluidine blue. Triplicate observations were performed. Two to 10 fields/well were randomly chosen and counted by microscopic examination. The results are expressed as number of cells per well. Group differences were compared with one-way analysis of variance.

RESULTS

Analysis of α Subunits Associated with β_{1C} and of β_{1C}-CHO Cell Adhesion

CHO stable cell lines expressing either human β_{1C} or human β_{1A} under the control of a tetracycline-regulated promoter were characterized for their ability to associate with α subunits and to adhere to integrin ligands. Exogenous expression of either β_{1C} or β_{1A} in CHO cells was analyzed by FACS with TS2/16 mAb to human β_1 integrin; comparable levels of surface expression of β_{1C} and β_{1A} were consistently obtained in all the experiments 48 h after tetracycline removal (Figure 1, A and B). In parallel, the levels of endogenous β_1 were evaluated in both β_{1C} and β_{1A} CHO stable cell lines by FACS with 7E2 mAb to hamster β_1 integrin (Figure 1, A and B). Exogenous expression of either β_{1C} or β_{1A} was completely prevented by tetracycline (Figure 1, C and D). The expression of human β_{1C} and β_{1A} was also analyzed by immunoprecipitation from detergent cell extracts of ^{125}I-labeled CHO cells. P4C10, a mAb to the human β_1 extracellular domain, immunoprecipitated surface-expressed integrin complexes containing either β_{1C} or β_{1A} (Figure 1E, lanes 2 and 4). P4C10 immunocomplexes were reprecipitated with rabbit serum to the β_{1C} cytodomain. These results confirm appropriate β_{1C} cell surface expression (Figure 1E, lane 6). To characterize the α subunits associated with β_{1C}, P4C10 immunocomplexes were reprecipitated with rabbit serum against α_2, α_V, or α_5, which are known to be associated predominantly with β_1 in CHO cells (Takada *et al.*, 1992; Balzac *et al.*, 1993). As shown in Figure 2, both exogenous β_{1C} and β_{1A} were associated with endogenous α_2, α_V, or α_5 integrin subunits in CHO stable cell lines. The β_{1C}- and β_{1A} CHO stable cell lines also attached in a comparable manner to increasing concentrations of fibronectin, 7E2, or TS2/16; no differences were observed in the number of attached cells at 30, 90, or 120 min (Figure 3; our unpublished results).

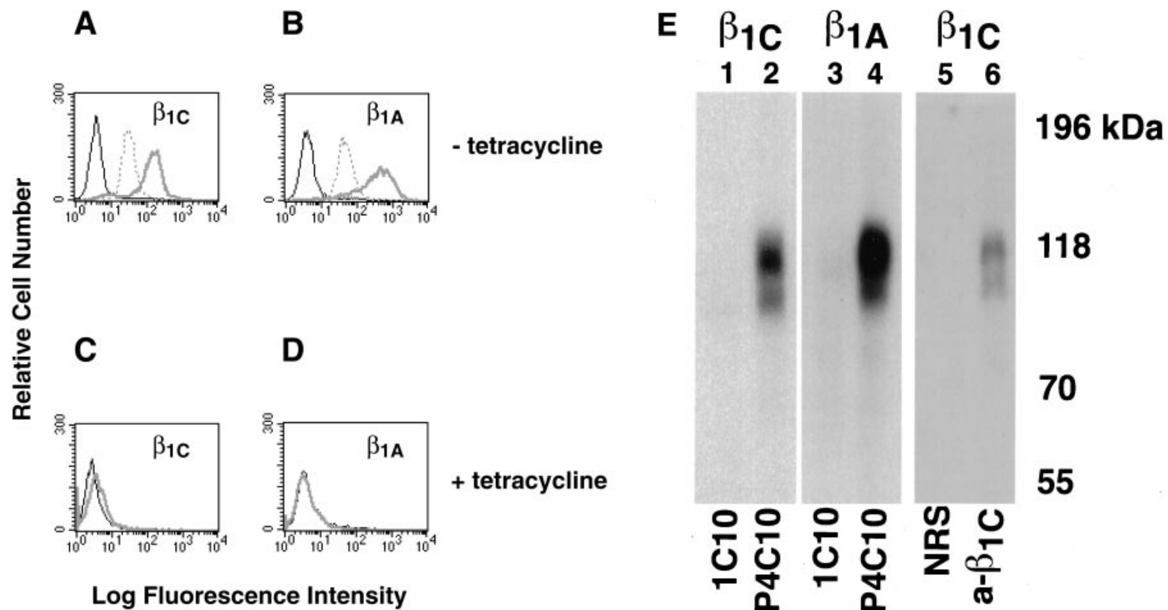


Figure 1. Surface expression of β_{1C} and β_{1A} in CHO cells. (A–D) β_{1C} or β_{1A} CHO stable cell lines were cultured for 48 h either in the absence (A and B) or in the presence (C and D) of 1 μg/ml tetracycline and analyzed by FACS with TS2/16 mAb to human β_1 integrin, 7E2 mAb to hamster β_1 integrin, or 12CA5 as a negative control, followed by FITC goat anti-mouse immunoglobulin G. Fluorescence intensity is expressed in arbitrary units. FACS analysis of a representative clone for each β_1 variant is shown. Thick gray line, TS2/16; dotted line, 7E2; thin black line, 12CA5. (E) CHO stable cell lines were cultured as in A and B and surface-labeled with Na ^{125}I; exogenous β_1 integrins were immunoprecipitated with P4C10 mAb to human β_1 integrin (lanes 2 and 4). The immunoprecipitated material was then eluted from protein A–Sepharose with 50 mM Tris-HCl, pH 7.5, 2% SDS and boiled for 5 min. The immunocomplexes were then reprecipitated with rabbit antiserum to the β_{1C} cytoplasmic domain (lane 6) and separated on 7.5% SDS-PAGE. mAb 1C10 (lanes 1 and 3) or normal rabbit serum (lane 5) were used as negative controls. Lanes 1, 2, 5, and 6, β_{1C} CHO; lanes 3 and 4, β_{1A} CHO. Proteins were visualized by autoradiography. Prestained marker proteins (in kilodaltons) are shown.

β_{1C} Integrin Expression Does Not Affect FAK Phosphorylation or AKT Activation

To analyze the effect of β_{1C} on integrin-mediated intracellular signaling pathways, we used the CHO stable cell lines described above (Figures 1 and 2). It has been shown that integrin ligation leads to tyrosine phosphorylation of intracellular proteins, including FAK (Schwartz *et al.*, 1995).

To examine whether FAK phosphorylation was differentially affected by β_{1C} and β_{1A} integrin variants, FAK was immunoprecipitated from detergent cell extracts prepared from either β_{1C} or β_{1A} stable cell lines. FAK phosphorylation was analyzed by immunoblotting with an antibody that recognizes FAK only when phosphorylated on Tyr^{397} (Sieg *et al.*, 1999). As shown in Figure 4, cell adhesion to fibronectin induced FAK phosphorylation on Tyr^{397} in both β_{1C} and β_{1A} stable cell lines compared with cells in suspension (top panel). The results indicate that β_{1C} integrin expression does not affect FAK phosphorylation mediated by adhesion to fibronectin.

We then examined the ability of β_{1C} and β_{1A} integrins to activate AKT, a downstream effector of PI 3-kinase that promotes cell survival. AKT activity was first assayed on detergent cell extracts obtained from cells that attached to fibronectin for 10 or 30 min. As shown in Figure 5A, adhesion to fibronectin for 10 min induced comparable activation of AKT in both β_{1C} and β_{1A} stable cell lines as determined by in vitro kinase assay (top panel, lanes 2 and 5). However, upon adhesion to fibronectin for 30 min, a modest but consistent increase of AKT activation was observed in β_{1C} versus β_{1A} stable cell lines (top panel, lanes 3 and 6). Similar results were obtained by immunoblotting with a phospho-specific AKT antibody (Figure 5B); total lysates from cells that were either held in suspension or allowed to adhere for 30 min to fibronectin, TS2/16, or 7E2 were immunoblotted with phospho-specific antibody to Ser^{473}. As shown in panel B, a marked increase in AKT serine phosphorylation was observed in β_{1C} and β_{1A} stable cell lines upon adhesion to fibronectin (top panel, lanes 2 and 6), 7E2 (top panel, lanes 3 and 7), or TS2/16 (top panel, lanes 4 and 8) compared with cells in suspension and with cells on poly-L-lysine (our unpublished results). These results indicate that both β_1 variants activate AKT in CHO cells. Densitometric analysis performed on three separate experiments showed that cell adhesion to fibronectin, TS2/16, or 7E2 induced an increase in AKT Ser^{473} phosphorylation in β_{1C} (6.3 $\pm$ 1.4-fold, 4.4 $\pm$ 0.8-fold, and 5.5 $\pm$ 1.9-fold increase, respectively) as well as in β_{1A} (2.2 $\pm$ 0.5-fold, 2.1 $\pm$ 0.2-fold, and 2.7 $\pm$ 0.7-fold increase, respectively) stable cell lines (our unpublished results). No differences in AKT activation were detected upon adhesion to fibronectin between β_{1C} and β_{1A} stable cell lines cultured in the presence of tetracycline (Figure 5C, lanes 2, 3, 5, and 6) to prevent expression of exogenous β_1 variants. AKT phosphorylation in response to engagement of either endogenous integrins or exogenous β_{1C} and β_{1A} variants by

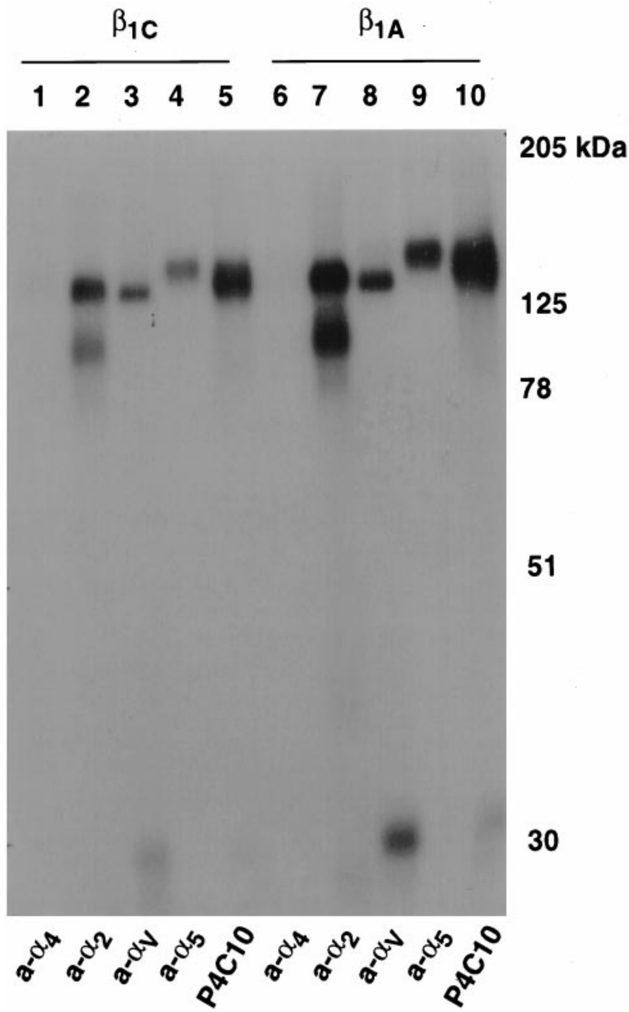


Figure 2. β_{1C} associates with α_5, α_V, and α_2 subunits. β_{1C} or β_{1A} CHO stable cell lines were cultured for 72 h in the absence of tetracycline and surface-labeled with iodine, and exogenous β_1 integrins were immunoprecipitated with P4C10 (lanes 5 and 10). The immunoprecipitated material was then eluted from protein A–Sepharose with 10 mM Tris-HCl, pH 7.5, 0.5% SDS for 10 min at 70°C, reprecipitated with rabbit antiserum to α_4 (lanes 1 and 6), α_2 (lanes 2 and 7), α_V (lanes 3 and 8), or α_5 (lanes 4 and 9), and separated by 10% SDS-PAGE. Lanes 1–5, β_{1C} CHO; lanes 6–10, β_{1A} CHO. Proteins were detected by autoradiography. Prestained marker proteins (in kilodaltons) are shown.

either fibronectin or TS2/16 was completely inhibited by wortmannin, a PI 3-kinase inhibitor (Figure 5B, top panel, lanes 10, 12, 14, and 16). These data show that β_{1C} and β_{1A} do not differentially affect PI 3-kinase/AKT pathway activation induced by fibronectin and that antibody-mediated engagement of β_{1C} and β_{1A} stimulates AKT phosphorylation.

β_{1C} *Integrin Expression Inhibits MAP Kinase Activation Stimulated by Fibronectin*

MAP kinase pathway activation by integrins is transient and is detectable soon after integrin engagement (maximum at

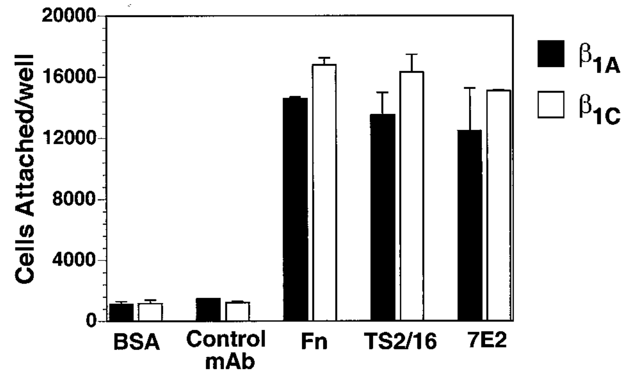


Figure 3. CHO cell adhesion is not affected by β_{1C} expression. β_{1C} or β_{1A} CHO stable cell lines were cultured as described for Figure 1. Cells were detached and labeled with ^{51}Cr in DMEM containing 10% FCS for 1 h at 37°C. Cells were then washed in serum-free medium, and 2.5 × 10^4 cells were allowed to adhere to fibronectin-coated (10 μg/ml), 7E2-coated (1 μg/ml), TS2/16-coated (1:10 dilution of culture supernatant), or negative control mAb-coated (1 μg/ml) or BSA-coated (10 mg/ml) wells at 37°C for 30 min. Attached cells were then washed and lysed, and radioactivity was measured by liquid scintillation counting. Duplicate observations with two separate clones for each β_1 variant were performed in each experiment, and the experiments were repeated at least twice with similar results.

10 min in CHO cells; Figure 6A). We examined the ability of β_{1C} and β_{1A} to modulate ERK2 activation in CHO stable cell lines. Endogenous and exogenous integrins were engaged with fibronectin (Figure 6, A–C), whereas exogenous human

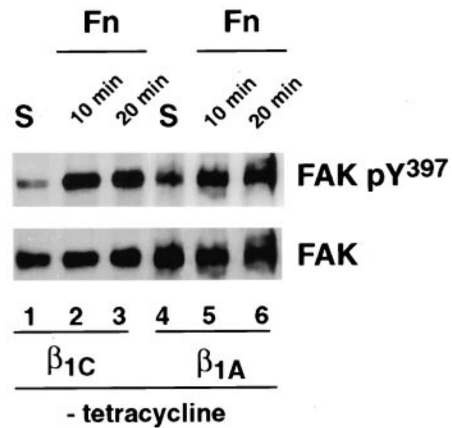


Figure 4. Expression of β_{1C} integrin does not affect FAK activation. β_{1C} or β_{1A} CHO stable cell lines were cultured for 48 h in the absence of tetracycline and serum-starved during the last 24 h of the 48-h culture. The cells were detached and either held in suspension (S; lanes 1 and 4) or plated on tissue culture plates coated with fibronectin (Fn; lanes 2, 3, 5, and 6) for either 10 or 20 min at 37°C. FAK was immunoprecipitated from 500 μg of total cell lysate with 0.5 μg of affinity-purified antibody to FAK, and its phosphorylation was analyzed by immunoblotting with 0.2 μg/ml phospho-specific antibody to Tyr^{397}. FAK protein levels were analyzed with 0.1 μg/ml affinity-purified antibody to FAK, and proteins were visualized by enhanced chemiluminescence. The experiments were repeated twice with consistent results.

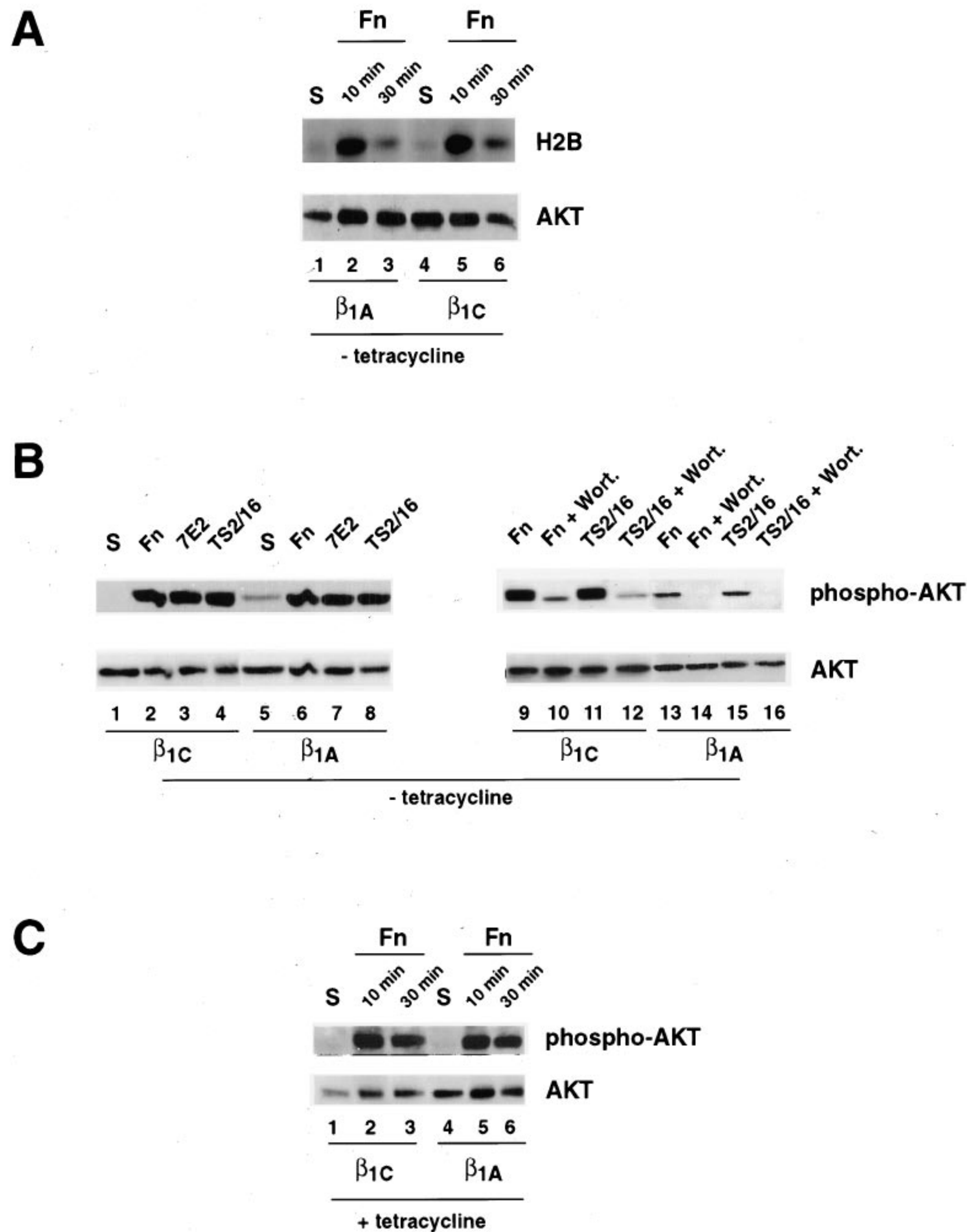


Figure 5. AKT activation in β_{1C} and β_{1A} transfectants. AKT activation was analyzed by in vitro kinase assay (A) and by immunoblotting (B and C). β_{1C} or β_{1A} CHO stable cell lines were cultured for 48 h either in the absence (A and B) or in the presence (C) of 1 μg/ml tetracycline and serum-starved during the last 24 h of the 48-h culture. The cells were detached and either held in suspension (S; A and C, lanes 1 and 4; B, lanes 1 and 5) or seeded on tissue culture plates coated with fibronectin (Fn; A and C, lanes 2, 3, 5, and 6; B, lanes 2, 6, 9, 10, 13, and 14), TS2/16 (B, lanes 4, 8, 11, 12, 15, and 16), or 7E2 (B, lanes 3 and 7) for either 10 min (A and C) or 30 min (A–C) at 37°C. Cells were also incubated with 100 nM wortmannin (Wort.) for 15 min at 4°C before plating on either fibronectin (B, lanes 10 and 14) or on TS2/16 (B, lanes 12 and 16). (A) AKT was immunoprecipitated from total cell lysate with 0.1 μg of affinity-purified antibody to AKT, and its kinase activity was analyzed by in vitro kinase assay with histone H2B (H2B) as a substrate. Phosphorylated H2B was visualized by autoradiography (top panel). (B and C) Detergent cell extracts were analyzed with 0.05 μg/ml phospho-specific antibody that recognizes AKT only when phosphorylated at Ser^{473} (top panels). The levels of AKT expression were examined with 0.1 μg/ml control AKT antibody (phosphorylation state independent; A–C, bottom panels). Proteins were visualized by enhanced chemiluminescence. The experiments were repeated twice with consistent results.

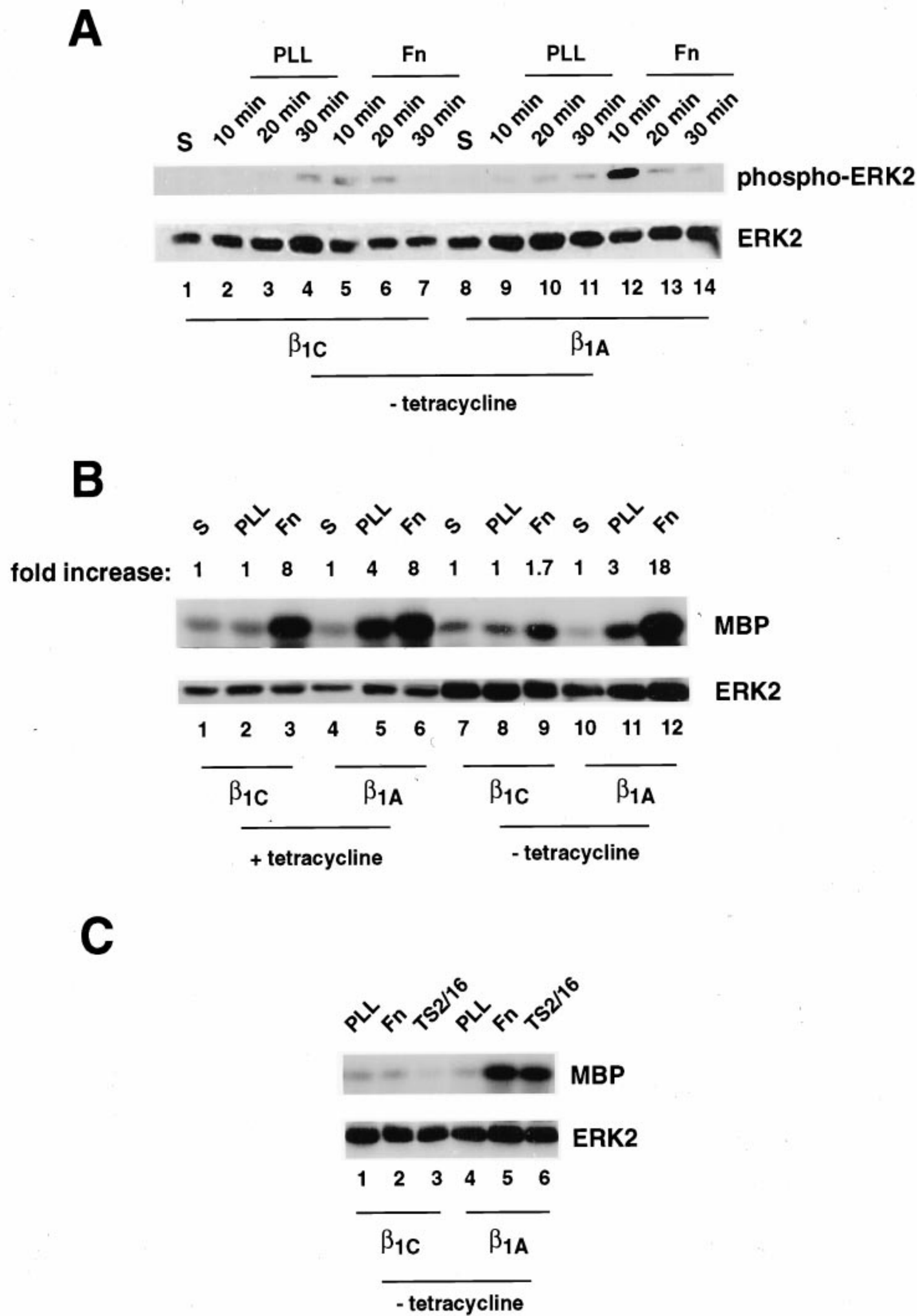


Figure 6. β_{1C} prevents ERK2 activation mediated by fibronectin. β_{1C} or β_{1A} CHO stable cell lines were cultured as described for Figure 5. The cells were detached and either held in suspension (S; A, lanes 1 and 8; B, lanes 1, 4, 7, and 10) or seeded on tissue culture plates coated with poly-L-lysine (PLL; A, lanes 2–4 and 9–11; B, lanes 2, 5, 8, and 11; C, lanes 1 and 4), fibronectin (Fn; A, lanes 5–7 and 12–14; B, lanes 3, 6, 9, and 12; C, lanes 2 and 5), or TS2/16 (C, lanes 3 and 6) for either 10 min (A–C) or 20 or 30 min (A) at 37°C. Cells were lysed, and ERK2 activation was analyzed by immunoblotting (A) or by in vitro kinase assay (B and C). (A) Detergent cell extracts were analyzed with 0.5 μg/ml mAb E10, which recognizes ERK2 only when phosphorylated at Thr^{202}/Tyr^{204} (top panel). (B and C) ERK2 was immunoprecipitated from 50 μg of total cell lysate with 0.5 μg of affinity-purified antibody to ERK2, and its kinase activity was analyzed by in vitro kinase assay with myelin basic protein (MBP) as a substrate. Phosphorylated MBP was visualized by autoradiography (top panels). The levels of expression of ERK2 were analyzed with 0.1 μg/ml rabbit affinity-purified antibody to ERK 2 (A–C, bottom panels). Proteins were visualized by enhanced chemiluminescence. In B, ERK2 activation is expressed as fold increase over the activity detected in cells held in suspension. The experiments were repeated at least twice with two separate clones for each variant with consistent results.

β_{1C} or exogenous human β_{1A} integrins were engaged with TS2/16 (Figure 6C). The activation of ERK2 was analyzed by immunoblotting with E10 mAb, which recognizes the Thr^{202}/Tyr^{204} phosphorylated form of ERK2 (Figure 6A, top panel), and by in vitro kinase assay (Figure 6, B and C, top panels); comparable amounts of ERK2 were used in the kinase assays (Figure 6, B and C, bottom panels). ERK2 activation was reduced significantly in β_{1C} compared with β_{1A} stable cell lines in response to integrin engagement by fibronectin as determined by immunoblotting (Figure 6A, top panel, lanes 5 and 12) and by in vitro kinase assay (Figure 6, B, top panel, lanes 9 and 12, and C, top panel, lanes 2 and 5). In the presence of tetracycline, adhesion to fibronectin mediated by endogenous integrins induced comparable ERK2 activation in both β_{1C} and β_{1A} stable cell lines (Figure 6B, top panel, lanes 3 and 6). Exogenous expression of β_1 variants in CHO cells did not alter the expression levels of endogenous hamster β_1 subunit or $\alpha_5\beta_1$ integrin as assessed by FACS analysis (Figure 1, A and B; our unpublished results), indicating that the differences in ERK2 activation on fibronectin between β_{1C}- and β_{1A}-expressing cells were not due to changes in endogenous $\alpha_5\beta_1$ integrin expression, the major fibronectin receptor in CHO cells.

Ligation of β_{1C} integrin by TS2/16 compared with poly-L-lysine did not induce activation of ERK2 as assessed by in vitro kinase assay (Figure 6C, top panel, lanes 1 and 3) or by immunoblotting with mAb E10 (our unpublished results). However, attachment of β_{1A} stable cell lines to TS2/16 resulted in activation of ERK2 compared with poly-L-lysine (Figure 6C, top panel, lanes 4 and 6). These results show that β_{1C} has an inhibitory effect on ERK2 activation mediated by fibronectin and, at variance with β_{1A}, is not able to stimulate ERK2 activity. These results also show that ERK2 activity is inhibited in cells attached to fibronectin for 10 min when both FAK and AKT are activated.

β_{1C} Integrin Expression Inhibits Fibronectin-mediated Ras Activation

Several reports have shown the role of Ras as an important effector of integrin-mediated activation of the MAP kinase pathway (Schlaepfer *et al.*, 1994, 1998; Clark and Hynes, 1996; Wary *et al.*, 1996; King *et al.*, 1997; Mainiero *et al.*, 1997; Schlaepfer and Hunter, 1997; Wei *et al.*, 1998). The data presented above indicate that β_{1C} has an inhibitory effect on ERK2 activity. Therefore, to determine whether β_{1C} mediated this effect at the level of Ras, Ras activation was assessed through its ability to bind the Ras-binding domain of Raf-1. This interaction has been shown to require GTP binding to Ras (Taylor and Shalloway, 1996). Adhesion of β_{1A} cell transfectants to fibronectin as well as engagement of endogenous integrins by fibronectin in β_{1C} stable cell lines cultured in the presence of tetracycline stimulated Ras activation (Figure 7A, top panel, lanes 1, 2, 9, and 10). Maximal activation of Ras in CHO cells in the presence of tetracycline was observed at 10 min (Figure 7A, top panel, lanes 10–12). In contrast, in the absence of tetracycline, β_{1C} expression nearly abolished Ras activation mediated by fibronectin (Figure 7A, top panel, lanes 5–8). We investigated whether Ras could overcome the β_{1C} inhibitory effect on fibronectin-mediated ERK2 activation by expressing a constitutively active form of Ras, Ras 61 (L). Transfection of β_{1C} CHO stable cell lines with constitutively active Ras 61 (L) restored fibronectin-induced ERK2 activation to the levels observed in cells transfected with vector alone and cultured in the presence of tetracycline (Figure 7B, top panel, lanes 4 and 6). These data indicate that β_{1C} inhibits the MAP kinase pathway by preventing Ras activation.

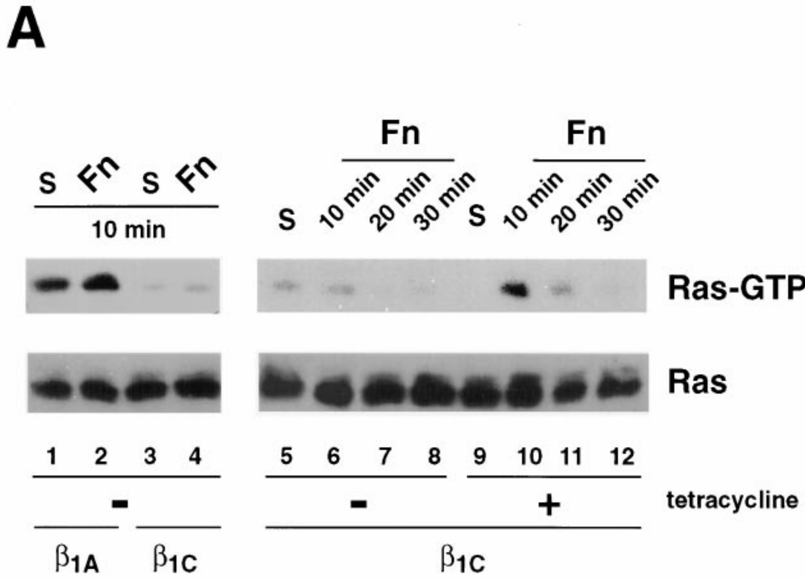
Inhibition of Cell Proliferation in β_{1C} Transfectants Is Rescued by MEK

To evaluate whether down-regulation of ERK2 activity causes inhibition of cell proliferation in β_{1C} transfectants, we transfected β_{1C} CHO stable cell lines with either MEK WT or MEK EE. The levels of expression of both MEK WT and MEK EE were comparable as determined by immunoblotting with 12CA5 mAb to hemagglutinin (our unpublished results). As expected, β_{1C} expression in CHO cells had an inhibitory effect on cell proliferation, whereas β_{1A} did not affect cell proliferation in response to serum (Figure 8A). Transfection of β_{1C} CHO stable cell lines with MEK EE restored cell proliferation to an extent similar to the level observed in cells cultured in the presence of tetracycline (Figure 8B). Thus, expression of constitutively active MEK rescues the inhibitory effect on cell proliferation exerted by β_{1C}.

DISCUSSION

In this study, as indicated in the model shown in Figure 9, we demonstrate that β_{1C} integrins inhibit ERK2 activation in response to cell adhesion to fibronectin by preventing Ras activation. It is also shown that β_{1C} inhibits Ras and ERK2 activation without affecting either FAK phosphorylation or AKT activity. Engagement of β_{1C} activates AKT but is not able to stimulate the MAP kinase pathway; this indicates that its unique cytodomain allows selective activation of the AKT kinase pathway in response to engagement of the common β_1 extracellular domain. Furthermore, constitutively active MEK restored cell proliferation in β_{1C} transfectants, suggesting that the negative effect of β_{1C} on the Ras/ERK pathway causes inhibition of cell proliferation.

The aim of this investigation was to determine the roles of two integrin variants, β_{1C} and β_{1A}, in modulating specific signaling pathways that control cell proliferation and survival. Specifically, we studied MAP kinase, FAK, and AKT pathways. MAP kinase pathway involvement in mediating cell cycle progression and gene expression, as well as the ability of FAK and AKT to support cell survival and prevent anoikis, have been well documented (Frisch *et al.*, 1996; Hungerford *et al.*, 1996; Xu *et al.*, 1996; Khwaja *et al.*, 1997; Robinson and Cobb, 1997; Downward, 1998; Guadagno and Ferrell, 1998; Brunet *et al.*, 1999; Cary and Guan, 1999). The mechanisms of integrin-mediated activation of the MAP kinase cascade comprise Ras-dependent and Ras-independent activation of ERK2 by integrins (Howe *et al.*, 1998). Our results show that, in contrast to β_{1A}, β_{1C} has an inhibitory effect on Ras and ERK2 activation mediated by fibronectin. Selective inhibition of the Ras/MAP kinase pathway by β_{1C} indicates that this integrin has the ability to either interfere with Ras membrane localization or inhibit positive regulators of Ras, or increase the activity of negative regulators of this molecule (Rebollo and Martinez-A, 1999). FAK has been shown to mediate Ras activation through Grb2/Sos binding (Schlaepfer and Hunter, 1998). However, in our system, we



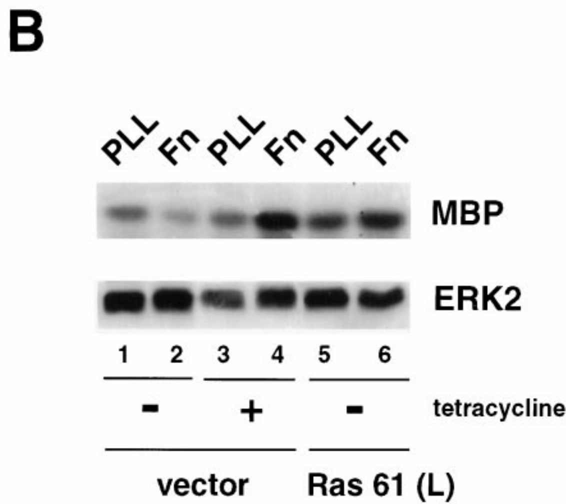


Figure 7. β_{1C} prevents Ras activation stimulated by fibronectin. (A) β_{1C} or β_{1A} CHO stable cell lines were cultured for 48 h either in the absence (lanes 1–8) or in the presence (lanes 9–12) of 1 μg/ml tetracycline and serum-starved during the last 24 h of the 48-h culture. The cells were detached and either held in suspension (S; lanes 1, 3, 5, and 9) or seeded on tissue culture plates coated with fibronectin (Fn; lanes 2, 4, 6–8, and 10–12) for 10 min (lanes 1–6, 9, and 10), 20 min (lanes 7 and 11), or 30 min (lanes 8 and 12) at 37°C. Cells were lysed, and Ras activation was analyzed by affinity precipitation with GST-RBD (top panels). Ras proteins were detected by immunoblotting with 2 μg/ml mAb to Ras (bottom panels). (B) β_{1C} CHO stable cell lines were transiently transfected with constitutively activated Ras [Ras 61 (L); lanes 5 and 6] or vector alone (vector; lanes 1–4). Cells were cultured for 48 h either in the absence (lanes 1, 2, 5, and 6) or in the presence (lanes 3 and 4) of 1 μg/ml tetracycline and starved during the last 24 h of the 48-h culture. Transfected cells were then detached and plated on dishes coated with either poly-L-lysine (PLL; lanes 1, 3, and 5) or fibronectin (Fn; lanes 2, 4, and 6) for 10 min at 37°C. ERK2 in vitro kinase activity (top panel) and expression (bottom panel) were analyzed as described for Figure 6. In A and B (bottom panels), proteins were visualized by enhanced chemiluminescence. The experiments were repeated twice with consistent results.

do not expect β_{1C} to act through FAK because β_{1C} inhibits ERK2 activity without affecting integrin signaling to FAK. This is the first description of a selective inhibitory role of the integrin cytoplasmic domain on a member of the MAP kinase family. In one instance, integrin down-regulation of FAK tyrosine phosphorylation and MAP kinase activity has been described (Sastry *et al.*, 1999). Here we show that FAK phosphorylation and AKT activation can occur in the absence of ERK2 activation, indicating that β_{1C} inhibits either a pathway downstream of FAK or AKT or a FAK- and AKT-independent pathway (Figure 9A). It has been described that PI 3-kinase is required for maximal fibronectin-mediated ERK2 activation and that it functions downstream of Ras (King *et al.*, 1997); in our β_{1C}-expressing cells, the PI 3-kinase/AKT pathway is active even though ERK2 is inhibited, suggesting that PI 3-kinase alone is not sufficient to activate ERK2 in the absence of Ras activation. It was reported recently that PKC inhibition selectively prevents ERK2 activation in response to integrin without affecting FAK tyrosine phosphorylation (Miranti *et al.*, 1999). Thus, expression of β_{1C} might down-regulate ERK2 activity in response to fibronectin adhesion via inhibition of PKC, which has been shown to act upstream of Ras (Miranti *et al.*, 1999).

The β_{1C} and β_{1A} variants have a different subcellular distribution (Meredith *et al.*, 1995); β_{1A} localizes to focal contacts, whereas β_{1C} remains diffuse on the cell surface. Thus, our results indicate that MAP kinase inhibition ob-

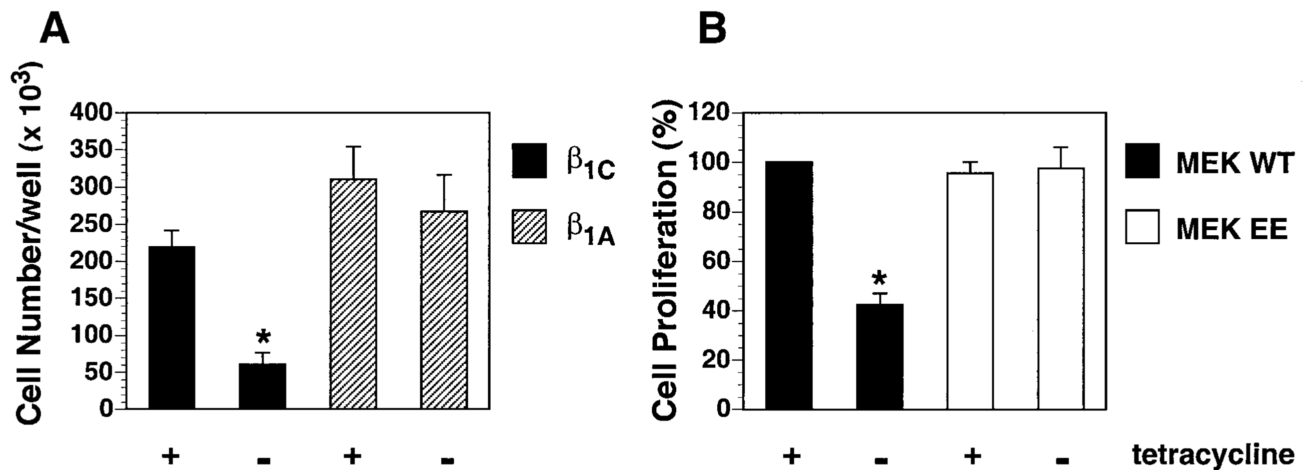


Figure 8. Constitutively activated MEK rescues β_{1C} inhibition of CHO cell proliferation. (A) β_{1C} or β_{1A} CHO stable cell lines were cultured as described for Figure 7A. Cells were detached, resuspended in serum-free medium, and plated (10,000 cells/well) on tissue culture plates coated with 1 μg/ml fibronectin for 1 h at 37°C. Attached cells were cultured for 96 h at 37°C in growth medium containing 5% FCS either in the absence or in the presence of 1 μg/ml tetracycline. Cells were washed, fixed with 3% paraformaldehyde, and stained overnight with 0.5% toluidine blue. Cell number was evaluated as described in MATERIALS AND METHODS. (B) β_{1C} CHO stable cell lines were transiently transfected with either MEK WT or MEK EE. Cells were cultured for 48 h either in the absence or in the presence of 1 μg/ml tetracycline and starved during the last 24 h of the 48-h culture. Cells were detached, plated, and cultured for 72 h, and cell number was analyzed as described for A. Cell proliferation is expressed as percent relative to the value for MEK WT cultured in the presence of tetracycline. Shown is the average ± SEM from two separate experiments. Group differences were compared with one-way analysis of variance. In A, the differences in proliferation either between β_{1C} CHO stable cell lines in the absence (*) and in the presence of tetracycline or between β_{1C} CHO stable cell lines cultured in the absence of tetracycline (*) and β_{1A} CHO stable cell lines cultured either in the presence or in the absence of tetracycline are statistically significant ($p < 0.05$). In B, the differences in cell proliferation either between MEK WT in the absence of tetracycline (*) and in the presence of tetracycline or between MEK WT in the absence of tetracycline (*) and MEK EE cultured either in the presence or in the absence of tetracycline are statistically significant ($p < 0.05$).

served in β_{1C} transfectants does not require β_{1C} recruitment to focal adhesion complexes. In a previous report, we had attempted to study ERK2 activation in response to β_{1C} or β_{1A} engagement by TS2/16. However, we had not detected either β_{1C} or β_{1A} integrin-mediated ERK2 activation because of the low integrin levels and the low number of cells transfected in the transient expression system (Fornaro *et al.*, 1999). Here, using stable cell lines that have higher levels of expression, we show the failure of β_{1C} to activate ERK2, although we detect MAP kinase activation in response to β_{1A} engagement (Figure 9B). In this study, it is also shown that AKT phosphorylation is observed in response to β_{1C} engagement (Figure 9B). Therefore, specific domains in the extreme carboxy-terminal region of β_1 are not required to activate the PI 3-kinase/AKT pathway. In our cell system as well as in the cell systems of others (King *et al.*, 1997), AKT activation is PI 3-kinase dependent, because wortmannin completely prevents AKT serine phosphorylation in response to either endogenous or exogenous integrin engagement. Ras is a potent activator of PI 3-kinase, in addition to Raf and non-Raf pathways (Rebollo and Martinez-A, 1999); thus, in our experimental system, in which Ras is inhibited, stimulators of PI 3-kinase different from Ras are expected to be active. FAK is a potential candidate; PI 3-kinase is activated by FAK (Chen *et al.*, 1996a). In our system, a causal effect of FAK activation on PI 3-kinase/AKT pathway stimulation, in response to either β_{1C} or β_{1A} engagement, remains to be investigated. Recent evidence points also to integrin-linked kinase (ILK) as a candidate effector for activation of AKT in response to integrin engagement, because ILK mediates PI 3-kinase–dependent AKT activation and binds the integrin β_1 cytodomain (Hanningan *et al.*, 1996; Delcommenne *et al.*, 1998). However, ILK binds the integrin β_1 cytodomain in a region that is not found in β_{1C} (S. Dedhar, personal communication). Thus, although it is crucial for signaling pathways activated in response to β_{1A} ligation, ILK is unlikely to play a role in the activation of AKT in β_{1C} transfectants.

Cell adhesion to fibronectin or to β_1 ligands is unaffected in response to β_{1C} expression. Furthermore, the β_{1C} variant associates with the same α subunits as β_{1A}, indicating that up-regulation of β_{1C} allows the cell to preserve the interaction with the extracellular matrix but, at the same time, to inhibit cell cycle progression. Therefore, we suggest that by expressing variant β_1 intracellular domains, cells may accomplish the delicate task of inhibiting proliferation without affecting either selective downstream survival signals (FAK and AKT) mediated by integrins or interactions with the extracellular environment. This observation is very important because in vivo, β_{1C} is expressed in nonproliferative and differentiated epithelium (Fornaro *et al.*, 1998) and is selectively down-regulated in prostatic adenocarcinoma (Fornaro *et al.*, 1996). Thus, the ability of β_{1C} to sustain activation of signals that stimulate survival and differentiation (FAK and AKT) (Downward, 1998; Jiang *et al.*, 1999) might be crucial to preventing apoptosis while blocking cell cycle progression and maintaining a differentiated phenotype. Failure to maintain a differentiated phenotype is be-

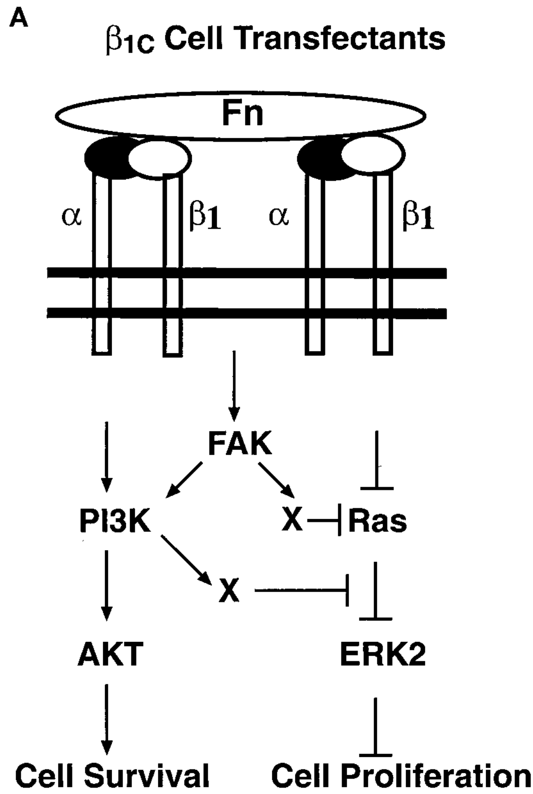
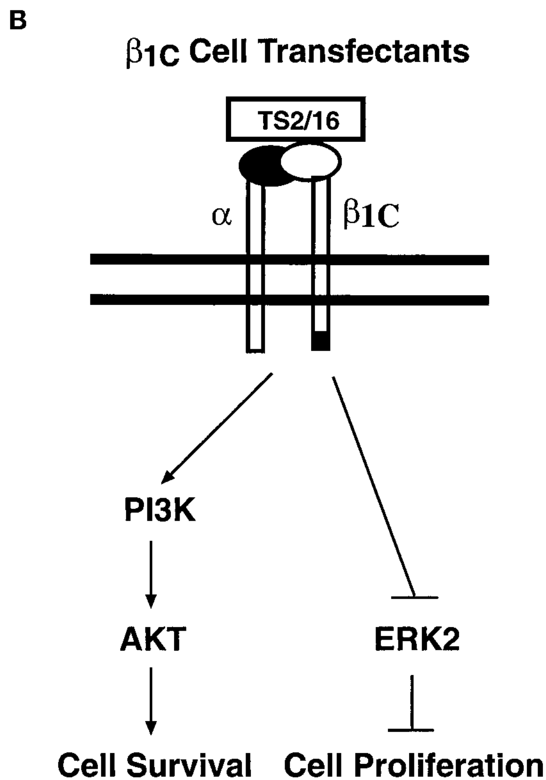
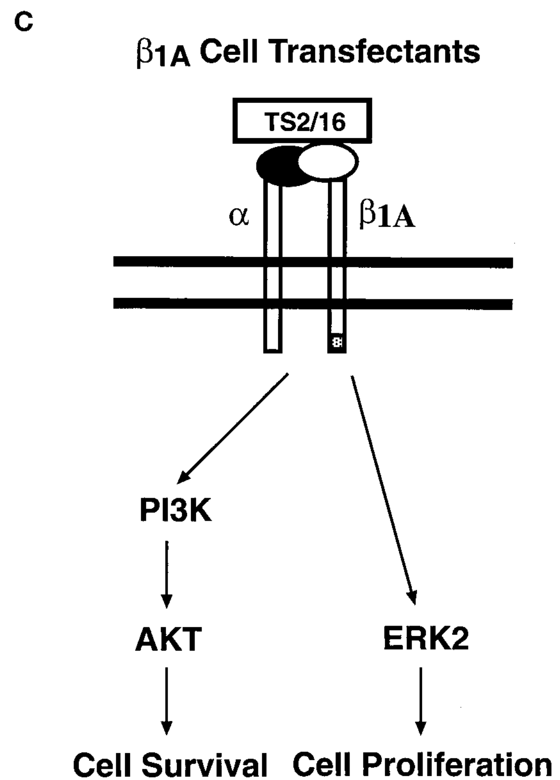


Figure 9. Differential effect of β_{1C} and β_{1A} integrin cytoplasmic variants on FAK, AKT, and MAP kinase pathways. The schematic drawings illustrate the β_{1C} effect on intracellular signaling pathways in response to exogenous and endogenous integrin engagement by fibronectin (Fn; A) or in response to either exogenous β_{1C} (B) or exogenous β_{1A} (C) ligation by TS2/16. The inhibitory effect of β_{1C} on Ras/ERK2, but not on FAK and AKT pathways, is shown in A. The failure of β_{1C} to induce ERK2 activation is shown in B. A previously described activation of the MAP kinase pathway by PI 3-kinase, downstream of Ras (King *et al.*, 1997), is blocked in our model (A). It is also shown that AKT is activated in response to fibronectin (A), β_{1C} (B), or β_{1A} (C) engagement.





lieved to be an early event in cancer progression (Hunter, 1997), suggesting that loss of β_{1C} might activate a cascade that contributes to a transformed phenotype.

We have shown previously that β_{1C} expression increases p27^{kip1} protein levels (Fornaro *et al.*, 1998). This cyclin kinase inhibitor is highly expressed in nonproliferative, quiescent cells, and its forced overexpression is sufficient to inhibit cell proliferation (Sherr and Roberts, 1995) and apoptosis (Hiromura *et al.*, 1999). In prostate cancer, loss of p27^{kip1} is an adverse prognostic factor that correlates with poor patient survival (Catzavelos *et al.*, 1997; Loda *et al.*, 1997; Porter *et al.*, 1997; Tsihlias *et al.*, 1998; Yang *et al.*, 1998). A report has indicated that oncogenic Ras-induced degradation of p27^{kip1} occurs through activation of the MAP kinase cascade (Kawada *et al.*, 1997). Thus, it is conceivable that by blocking Ras activation, β_{1C} expression, at variance with β_{1A} expression, achieves the goal of inhibiting ERK2 activation and, consequently, p27^{kip1} degradation and cell proliferation.

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