Inhibition of Cell Cycle Progression by the Alternatively Spliced Integrin β_{1C}

Jere Meredith Jr., Yoshikazu Takada, Mara Fornaro, Lucia R. Languino, Martin A. Schwartz

Integrins regulate cell growth, differentiation, and behavior in many systems. Integrin β_{1C} (β_{1S}) is an alternatively spliced variant of integrin β_1 with a specific cytoplasmic domain and is expressed in several human tissues. Human β_{1C} transiently expressed in mouse 10T1/2 fibroblasts showed a diffuse pattern of cell surface staining, whereas β_1 localized to focal adhesions. Moderate concentrations of β_{1C} had no effect on actin stress fibers or focal adhesions, but markedly inhibited DNA synthesis. Inhibition by β_{1C} mapped to the late G_1 phase of the cell cycle, near the G_1-S boundary. Thus, alternative splicing of β_1 results in transmission of distinct signals that may regulate growth in vivo.

Integrins are αβ heterodimers that mediate adhesion of cells to extracellular matrix proteins and to other cells (*1*). They regulate many intracellular signaling pathways, including tyrosine phosphorylation, cytoplasmic alkalinization, intracellular Ca^{2+} fluctuations, and inositol lipid metabolism (*1*, *2*).

The β_{1C} chain is an alternatively spliced variant of the integrin β_1 subunit (*3*), a widely expressed subunit that pairs with α_v and α_1 through α_7 and binds to extracellular matrix and cell surface ligands (*2*). β_{1C} contains a distinct 48–amino acid sequence that replaces the COOH-terminal 21 residues of the β_1 cytoplasmic domain. This alternative cytoplasmic domain shares 29% identity with the COOH-terminal half of the Src homology 2 (SH2) domain (*3*). The β_{1C} protein is expressed in epithelial cells of the liver (*4*) and in endothelial cells exposed to tumor necrosis factor (*5*). β_{1C} mRNA is present in placenta, platelets, and hematopoietic cell lines (*3*).

To investigate the function of β_{1C}, we transiently transfected mouse 10T1/2 fibroblasts by microinjecting complementary DNAs (cDNAs) that encode human β_1 and β_1 variants. Microinjected cells showed surface staining with monoclonal antibodies (mAbs) specific to the extracellular domain of human β_1 [TS2/16 (*6*) or P5D2 (*7*)] (Fig. 1). This observation indicates that β_{1C} can pair with endogenous mouse α subunits, consistent with data showing that the β_1 cytoplasmic domain has little effect on expression and pairing (*8*). Surface expression was detected 1 hour after cDNA injection in all expressing cells. β_{1C} showed a diffuse staining pattern (Fig. 1C), whereas human β_1 protein was localized to focal adhesions (Fig. 1A). A diffuse staining pattern was also detected for the other alternatively spliced variant of β_1, β_{1B} (*9*), suggesting that residues replaced by alternative splicing are required for localization to focal adhesions. Small or moderate amounts of β_{1C} did not disrupt endogenous focal adhesions, as revealed by the continued presence of actin stress fibers (Fig. 1, B and D) and vinculin localization in expressing cells (*10*), and had no detectable effect on cell morphology (Fig. 1, A and C).

Despite achieving expression in transient assays, we repeatedly failed to obtain stable cell lines that express β_{1C}, suggesting that β_{1C} might affect cell growth or survival. To test this hypothesis, we assayed cell growth in transiently transfected cells. Quiescent 10T1/2 cells in 0.25% serum were microinjected with either β_1 or β_{1C} cDNA, then placed in medium containing 10% serum and the thymidine analog bromodeoxyuridine (BrdU) to stimulate entry into the cell cycle. After 18 to 24 hours, cells were fixed and stained for both expression of human integrins and BrdU incorporation. Whereas expression of β_1 had no significant effect on DNA synthesis compared to uninjected cells, β_{1C} inhibited DNA synthesis (Fig. 2A). Similar amounts of β_1 and β_{1C} protein were expressed on the cell surface when immunofluorescence of single cells was quantitated by microfluorimetry (*11*). Even after 48 hours of continuous labeling, β_{1C} blocked DNA synthesis (5% of the β_{1C}-expressing cells entered the S phase of the cell cycle, compared with 96% of uninjected cells) (*10*).

To determine the amount of β_{1C} protein expressed relative to endogenous mouse β_1, we took advantage of the fact that human endothelial cells and 10T1/2 cells express similar amounts of β_1 as determined by immunoblot analysis with an antibody to the highly conserved cytoplasmic domain (*10*). Both 10T1/2 cells expressing human β_{1C} and human endothelial cells were stained with an antibody to human β_1, and specific fluorescence per cell was quantitated. The amount of β_{1C} protein in microinjected cells was 19 ± 5% of the endogenous β_1 according to this assay. However, when both expression and DNA synthesis were analyzed, even cells that expressed only one-third of this amount of β_{1C} protein (6% of endogenous β_1) showed marked inhibition of DNA synthesis (*10*). Because smaller amounts of β_{1C} expression cannot be accurately quantitated by this method, these values provide only a lower limit for the potency of β_{1C} as a growth inhibitor.

To determine at which point in the cell cycle β_{1C}-expressing cells were arrested, we injected quiescent cells with β_{1C} cDNA at various times after addition of 10% serum. Preliminary experiments showed that addition of serum triggered DNA synthesis after 12 to 18 hours (half-time, 15 hours). DNA synthesis became insensitive to inhibition by β_{1C} late in G_1 (half-time, 12 hours), ~3 hours before the beginning of the S phase (Fig. 2B). To analyze the cell cycle block further, we scored β_{1C}-expressing cells for the expression of cyclins E and A. Initial experiments showed that cyclin E nuclear staining was detectable 11 hours after the addition of serum and was maximal by 14 hours, in agreement with previous studies (*12*). Cyclin A nuclear staining was detectable 16 hours after addition of serum, consistent with its expression during the S phase

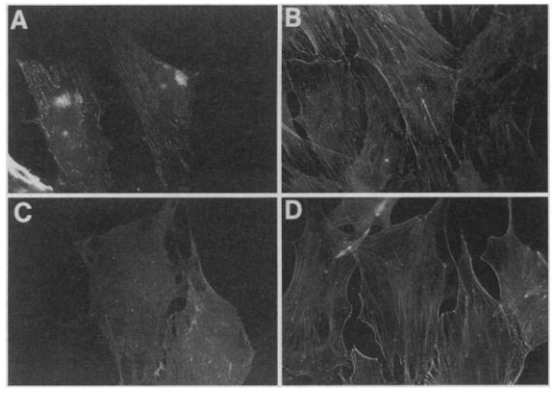

Fig. 1. Expression of human β_1 and β_{1C} in 10T1/2 cells. Mouse 10T1/2 fibroblasts were microinjected with expression vectors that contained cDNA encoding human integrin β_1 (**A** and **B**) or β_{1C} (**C** and **D**) (*17*, *19*). After 24 hours of incubation, cells were fixed and stained with a mAb specific for human β_1 (TS2/16 or P5D2) [(A) and (C)] or with rhodamine-phalloidin [(B) and (D)] (*16*). The same cells are shown in (A) and (B) and the same cells are shown in (C) and (D). Magnification, ×1300.

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(*13*). Expression of β_{1C} blocked the appearance of cyclin A, consistent with the failure to enter the S phase (Fig. 2C). In contrast, β_{1C} had no effect on the appearance of cyclin E in the nucleus. However, the fact that the loss of sensitivity to β_{1C} coincides with the expression of cyclin E suggests that β_{1C} may block cell cycle progression by affecting cyclin E–Cdk (cyclin-dependent kinase) activity or downstream functions.

Because integrin signaling events are usually dependent on receptor ligation (*1*, *2*), the role of ligand binding in growth inhibition by β_{1C} was investigated. The mutation D130A ($Asp^{130} \rightarrow Ala$) blocks binding of several ligands to β_1 integrins (*14*). Expression of a β_{1C} variant containing this mutation also inhibited DNA synthesis (Fig. 2A), and incubation of β_{1C}-expressing cells with antibody 13 (which blocks ligand binding to β_1 integrins) at concentrations that induced cell rounding of human endothelial cells did not affect β_{1C} inhibition (*10*). Thus, occupancy of the ligand binding site of β_{1C} appears not to be required for inhibition of cell cycle progression; however, we cannot rule out binding of unidentified ligands through sites on the α or β subunit that are not blocked by the D130A mutation or the antibody.

It is possible that β_{1C} inhibits growth because it possesses a disrupted cytoplasmic domain, rather than because it actively transmits a signal. However, expression of a β_1 variant with a truncated cytoplasmic domain in amounts equivalent to β_{1C} had no effect on DNA synthesis (Fig. 3A). This result suggests that specific β_{1C} sequences are required for growth inhibition. To map the growth-inhibitory domain of β_{1C}, we created three β_{1C} deletions by introducing stop codons within the β_{1C} coding sequence (Fig. 3B). Microinjection of these deletion mutants showed that removal of only 13 residues from the COOH-terminus of β_{1C} prevented inhibition of DNA synthesis (Fig. 3A), indicating that the growth-inhibitory function of β_{1C} depends on an intact β_{1C} domain. This observation also suggests that growth inhibition by β_{1C} is not a result of expression of β_{1C} mRNA sequences.

Only a few receptors are known to inhibit cell growth, primarily members of the transforming growth factor–β, tumor necrosis factor, or interferon receptor families (*15*); β_{1C} can now be added to this group. The fact that β_{1C} has been observed by immunocytochemistry in human tissues (*4*), together with our data showing that the smallest amount of β_{1C} detectable by immunofluorescence markedly inhibited DNA synthesis, suggests that β_{1C} plays a physiological role in preventing uncontrolled cell growth. Many important questions remain concerning the mechanisms of action and physiological or pathological functions of β_{1C}. Our results show that alternative splicing generates an integrin with specific functional properties, which presumably result from the transmission of specific signals via its cytoplasmic domain.

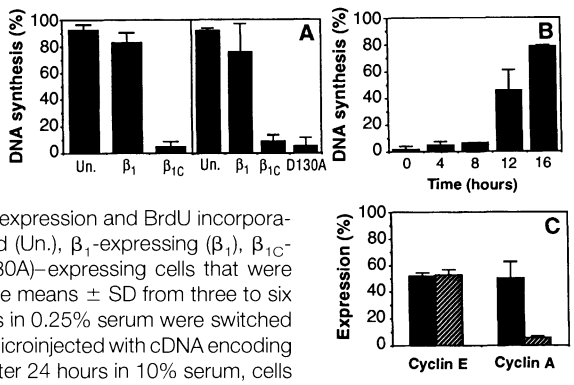
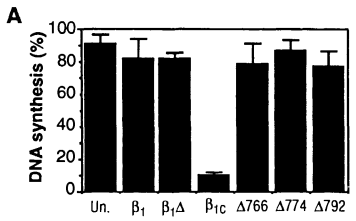


Fig. 2. The effect of β_{1C} on DNA synthesis and cyclin expression. (**A**) Quiescent 10T1/2 cells in 0.25% serum were microinjected with cDNA encoding β_1, β_{1C}, or the β_{1C} extracellular domain mutant D130A (*20*). Cells were incubated in 10% serum and BrdU for 24 hours, then fixed and stained for human β_1 integrin expression and BrdU incorporation (*18*). The percentages of uninjected (Un.), β_1-expressing (β_1), β_{1C}-expressing (β_{1C}), and β_{1C} D130A (D130A)–expressing cells that were labeled with BrdU are shown. Values are means ± SD from three to six experiments. (**B**) Quiescent 10T1/2 cells in 0.25% serum were switched to medium containing 10% serum and microinjected with cDNA encoding β_{1C} at the indicated times thereafter. After 24 hours in 10% serum, cells were fixed and stained for β_{1C} expression and BrdU incorporation (*21*). The percentage of β_{1C}-expressing cells that were labeled with BrdU is shown. Values are means ± SD from two experiments. Similar results were obtained in three other experiments. (**C**) Quiescent 10T1/2 cells in 0.25% serum were switched to 10% serum and either left uninjected (solid bars) or were microinjected with cDNA encoding β_{1C} (striped bars). Cells were fixed 18 hours later and stained for β_{1C} and either cyclin E or cyclin A (*22*). Values are means ± SD from three experiments.



B

β_1	KLLML...AKWDT GENPIYKSAVTTVVNPKYEGK
$\beta_1\Delta$	KL
β_{1C}	KLLML...AKWDT SLSVAQPGVQWCDISSLQPLTSRFQQFSCLSLPSTWDYRVKILFIRVP
Δ766	KLLML...AKWDT SLSVAQPGV
Δ774	KLLML...AKWDT SLSVAQPGVQWCDISSL
Δ792	KLLML...AKWDT SLSVAQPGVQWCDISSLQPLTSRFQQFSCLSLPST

Fig. 3. Effects of β_1 and β_{1C} variants on DNA synthesis. (**A**) Quiescent 10T1/2 cells in 0.25% serum were microinjected with cDNA encoding β_1, β_{1C}, a cytoplasmic deletion of β_1 (β_1^{Δ}), or one of three β_{1C} deletion mutants (Δ766, Δ774, or Δ792) (*23*). Cells were incubated in 10% serum and BrdU for 24 hours, then fixed and stained for human β_1 integrin expression and BrdU incorporation (*18*). The percentage of uninjected (Un.) or expressing cells that were labeled with BrdU is shown. Values are means ± SD from four experiments. (**B**) Amino acid sequences of the β_1 cytoplasmic domain variants. Sequences begin at residue 732. Residues 736 to 753 are not shown. Amino acid numbering is based on the mature protein. Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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16. Microinjected cells were washed twice with phosphate-buffered saline and then fixed with 3.7% formaldehyde in 100 mM Pipes (pH 6.8), 2 mM $MgCl_2$, and 1 mM EGTA for 20 min at room temperature. Fixed cells were permeabilized in 0.2% Triton X-100 and blocked in 10% normal goat serum (Gibco) in phosphate-buffered saline. Primary and secondary antibodies were diluted in blocking solution and incubated with cells for 30 to 60 min at 37°C. Cover slips were mounted in Fluoromount G (Fisher Scientific, Pittsburgh, PA), and cells were examined with a Nikon Diaphot inverted microscope.
17. Human cDNAs were subcloned into the pBJ-1 expression vector, a derivative of the pCD-Srα expression vector [Y. Takebe *et al.*, *Mol. Cell. Biol.* **8**, 466 (1988)] that was created by replacing the Xho I fragment of pCD-SRα with a polylinker containing restriction sites for Xho I, Xba I, Sfi I, Not I, Eco RI, Eco RV, Hind III, and Cla I. The β_1 and β_{1C} expression vectors were created by subcloning a combination of two cDNA subfragments into pBJ-1. An Xba I–Hind III fragment containing the 5′ end of β_1 cDNA (to nucleotide 2357 of the β_1 cDNA sequence) was isolated from the β_1 cDNA clone B3 (*14*). Fragments containing the 3′ end of either β_1 or β_{1C} cDNA (beginning at nucleotide 2358 of the β_1 cDNA sequence) were isolated as Hind III–Eag I fragments from a PCR-1000 vector (Invitrogen, San Diego, CA) containing the appropriate polymerase chain reaction–amplified regions (*3*). A combination of 5′ and 3′ fragments was subcloned into the Xba I–Not I sites of pBJ-1 (Eag I and Not I are compatible).
18. C3H 10T1/2 cells were plated on etched glass cover

slips in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and incubated for 24 hours. Cells were then incubated in DMEM containing 0.25% FBS for 48 hours before microinjection. One to 2 hours after microinjection, cells were switched to medium containing 10% FBS and a 1:1000 dilution of BrdU (Amersham, Arlington Heights, IL). After 18 to 24 hours of incubation, cells were fixed and processed (*16*). After permeabilization, cells were denatured with 1 M HCl for 30 min. For detection of human integrin expression, cells were probed with the human integrin β_1–specific mAbs TS2/16 (*6*) or P5D2 (Developmental Hybridoma Bank). After incubation with secondary antibodies, cells were blocked with mouse immunoglobulin IgG before staining with fluorescein isothiocyanate–labeled antibodies to BrdU (Becton Dickinson, San Jose, CA). The cDNAs were injected at a concentration of 0.1 mg/ml. In control experiments, injection of pBJ-1 vector alone at twice the molar concentration of β_{1C} cDNA had no effect on DNA synthesis. Injection of 10 times this concentration of vector alone inhibited DNA synthesis by 46%, substantially lower than the inhibition by β_{1C} (95%). Approximately 100 expressing cells, with equivalent expression levels as determined by microfluorimetry (*11*), were analyzed per variant.

19. C3H 10T1/2 cells were cultured in DMEM (Gibco, Grand Island, NY) supplemented with 10% FBS (Gemini Bioproducts, Calbasas, CA). Nuclear microinjections were performed as described [L. D. Chong *et al.*, *Cell* **79**, 507 (1994)]. Twenty-four hours before microinjection, cells were plated on etched glass cover slips. In a typical experiment, ~50 cells were injected with either β_1 (0.1 mg/ml) or β_{1C} (0.2 mg/ml) expression vector [cDNA concentrations were adjusted to generate cells that expressed equivalent amounts of protein as determined by microfluorimetry (*11*)]. Approximately 50% of the injected cells expressed protein.
20. The D130A mutation of β_1 was generated as previously described (*14*). An Xba I–Hind III fragment of the 5′ end of β_1 cDNA containing this mutation was then used to construct D130A β_{1C} (*17*).
21. Quiescent 10T1/2 cells that had been maintained in 0.25% serum (*18*) were injected with β_{1C} cDNA (0.2 mg/ml) 0, 4, 8, 12, or 16 hours after the addition of DMEM containing 10% FBS. BrdU (1:1000 dilution) was added 18 hours after the switch to 10% FBS, and after incubation for an additional 6 hours, cells were fixed and stained as previously described (*18*). In control experiments, maximal expression was achieved 1 hour after cDNA injection. Approximately 100 expressing cells were analyzed per time point.
22. Quiescent 10T1/2 cells that had been maintained in 0.25% serum (*18*) were injected with β_{1C} cDNA (0.1 mg/ml) 1 to 4 hours after the addition of DMEM supplemented with 10% FBS. After 18 hours, cells were fixed and stained (*16*). Cells were probed with mAb P5D2 to integrin β_1 and affinity-purified rabbit antibodies to cyclin A or cyclin E [M. Ohtsubo, A. M. Theodoras, J. Schumacher, J. M. Roberts, M. Pagano, *Mol. Cell. Biol.* **15**, 2612 (1995)]. Approximately 50 expressing cells were analyzed for each cyclin.
23. The cytoplasmic truncation of β_1 (β_1^{Δ}) was generated by inserting an Xba I linker into the Hind III site of the β_1 cDNA clone B3 (*14*). The COOH-terminal sequence of β_1^{Δ} is Gly-Leu-Ala-Leu-Leu-Leu-Ile-Trp-Lys-Leu^{733}. An Xba I fragment containing this mutation was then subcloned into the Xba I site of pBJ-1 to generate β_1^{Δ}. The β_{1C} cytoplasmic truncations Δ766, Δ774, and Δ792 were generated, with the use of site-directed mutagenesis [W. P. Deng and J. A. Nickoloff, *Anal. Biochem.* **200**, 81 (1992)], by introducing stop codons at positions 767, 775, and 793 of β_{1C}, respectively. Mutants were verified by DNA sequencing.
24. We thank W. Puzon for technical assistance, B. Cessna for help in preparing the manuscript, M. Davis for the pBJ vector, M. Hemler for mAb TS2/16, K. Yamada for antibody 13, and J. Roberts and M. Ohtsubo for providing purified antibodies to cyclins A and E. Supported by U.S. Public Health Service grants R01 GM47214 and P01 HL48728 (M.A.S.) and R01 GM47157 and R01 GM49899 (Y.T.).

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Association of the Yeast Pheromone Response G Protein βγ Subunits with the MAP Kinase Scaffold Ste5p

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The mating response pathway of the yeast *Saccharomyces cerevisiae* includes a heterotrimeric guanine nucleotide–binding protein (G protein) that activates a mitogen-activated protein (MAP) kinase cascade by an unknown mechanism. An amino-terminal fragment of the MAP kinase scaffold protein Ste5p that interfered with pheromone-induced cell cycle arrest was identified. A haploid-specific interaction between the amino terminus of Ste5p and the G protein β subunit Ste4p was also detected in a two-hybrid assay, and the product of a signaling-defective allele of *STE4* was defective in this interaction. In cells with a constitutively activated pheromone response pathway, epitope-tagged Ste4p was coimmunoprecipitated with Ste5p. Thus, association of the G protein and the MAP kinase cassette via the scaffolding protein Ste5p may transmit the G protein signal.

The pheromone response pathway of *S. cerevisiae* is a well-studied system of signal transduction (*1*, *2*). Many genes that are required for haploid yeast cells to mate have been identified in genetic screens. Characterization of the products of these genes has provided an outline of the molecular mechanism of this pathway, which includes a G protein encoded by the genes *GPA1* (Gα subunit) (*3*), *STE4* (Gβ subunit), and *STE18* (Gγ subunit) (*4*). This G protein is coupled to cell type–specific receptor proteins encoded by the *STE2* and *STE3* genes (*5*). The βγ subunits of the activated G protein stimulate the downstream elements of the pathway (*4*), which include the Ste20p protein kinase (*6*, *7*) and a set of kinases that are similar to MEK kinase, MEK (MAP kinase kinase), and MAP kinase of higher cells and are encoded by the *STE11* (*8*), *STE7* (*9*), and *FUS3/KSS1* (*10*) genes, respectively.

The *STE5* gene encodes a protein (Ste5p) of 917 amino acids, different regions of which physically interact with the members of the MAP kinase cascade (*11*–*13*). Thus, Ste5p has been proposed to act as a scaffold to organize the kinases (*2*, *11*). However, it is not clear how the elements of this cascade are activated in response to the βγ subunits of the G protein. Because MAP kinase activation is the hallmark of many signaling pathways in mammalian cells, characterization of this process is of general importance. We now show that, in the yeast pheromone response pathway, the G protein β subunit can associate with the NH_2-terminus of the MAP kinase scaffold Ste5p, suggesting a mechanism for direct activation.

Oligonucleotide-directed random mutagenesis (*14*, *15*) was performed on two regions of *STE5* (*16*, *17*), and the mutant libraries were screened to identify dominant-negative mutants of Ste5p (*18*). For this screen, the *STE5* libraries were transformed into strain YEL106 (W303-1a *sst1::LEU2*) and tested for galactose-dependent interference with the α factor–specific growth arrest signal (*15*). Plasmids from clones with a strong interference phenotype were rescued and retransformed into YEL106 to confirm that the phenotype was plasmid dependent. Four of the mutations resulted in major structural changes to Ste5p; CW1-2 was a deletion of amino acids 376 to 546, and CW4-8, CW3-1, and CW2-1 were COOH-terminal truncations leaving the NH_2-terminal 394, 241, and 214 amino acids, respectively. In addition, CW2-8 was a deletion of amino acids 215 and 216. All these mutants blocked the constitutive cell cycle arrest caused by deletion of *GPA1*. The two internal deletions, CW2-8 and CW1-2, allowed weak mating of a *STE5*-deleted strain, whereas the three COOH-terminal deletions did not.

Although expression of mutant CW2-1 blocked both the constitutive cell cycle arrest caused by deletion of *GPA1* and cell cycle arrest induced by mating pheromone (Fig. 1), it did not block cell cycle arrest caused by overexpression of the hyperactive *ste11-1* allele (Fig. 1A). This observation suggests that the Ste5p dominant-negative mutant may act at a step between the G protein and the MAP kinase cascade, a result consistent with the placement of Ste5p function in the pheromone response pathway by analysis of hyperactive and null

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