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# INTEGRIN EXPRESSION IN COLON CANCER CELLS IS REGULATED BY THE CYTOPLASMIC DOMAIN OF THE β6 INTEGRIN SUBUNIT

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**We have previously reported that the αvβ6 integrin upregulates its own expression in a protein kinase C-dependent manner with increasing cell density. The wild-type β6 integrin subunit has also been shown to promote tumour growth *in vivo* and its growth-enhancing effect is regulated by both a MAP kinase binding motif on β6 and the 11 amino acid C-terminal cytoplasmic extension unique to the β6 subunit. Herein, we show that the 11 amino acid cytoplasmic extension is essential for the cell density-dependent increase in β6 expression and that the 11 amino acid tail exerts a dominant negative effect on cell density- and PKC-mediated β5 expression in αvβ6-expressing colon cancer cells. Cells that express β6 lacking the 11 amino acid tail respond to PKC simulation with increased expression of only the β5 subunit as seen for cells that lack constitutive αvβ6 expression. In contrast, loss of the ERK binding site on β6 markedly impairs cell density- and PKC-dependent expression of either β6 or β5 in the presence or absence of the 11 amino acid tail, respectively. Our findings suggest that in αvβ6-expressing cells, a hierarchy of kinase signalling cascades exists and that the β6-ERK2 interaction dominates over PKC-mediated signalling pathways responsible for integrin upregulation with cell confluence. Given the dominance of the β6-ERK2 interaction over PKC-mediated expression of both β5 and β6 integrin subunits, targeting the β6-ERK2 interaction may prove useful as an anticancer strategy in colon cancer.**
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**Key words:** *integrins; cell density; kinase signalling; colon cancer*

Amongst the various families of cell adhesion molecules, integrin expression patterns appear to be directly implicated in the progression of malignant disease.[1] Integrins are transmembrane glycoprotein receptors each comprising an alpha (α) and a beta (β) subunit in noncovalent association that mediate dynamic linkages between the actin cytoskeleton and the extracellular matrix as well as transducing signals to and from the cell interior.[2–4] Within the αv subfamily, the αvβ6 integrin is either not expressed or expressed at low levels in normal adult epithelia; however, it becomes highly expressed during tumourigenesis.[5,6] For example, induction of αvβ6 expression in oral leukoplakia appears to be a necessary prerequisite for progression to squamous cell cancer[7] and *de novo* expression of αvβ6 has been observed in oral squamous and colon cancers.[8,9] In lung cancer, approximately 50% of tumours exhibit upregulation of the αvβ6 subunit,[10] and in breast cancer αvβ6 expression has recently been linked to more advanced tumours.[11]

We have reported that high cell density in a 2-dimensional monolayer culture model selectively upregulates β6 integrin subunit expression in colon cancer cells in a protein kinase C (PKC)-dependent manner in preference to other β subunits.[12] Moreover, PKC activity has been shown to increase with cell confluence in colon cancer cells, and the rise in PKC activity is much greater for αvβ6-expressing cells than for colon cancer cells that lack αvβ6. Hence, we have proposed a system of integrin autoregulation whereby the integrin αvβ6 upregulates its own expression via PKC-mediated signalling as tumour cells become crowded.[12]

Integrin cytoplasmic domains mediate inside-out and outside-in signalling through interactions with cytoskeletal molecules and intracellular kinases. Functional links between both the major integrin subfamilies, β1 and αv, and the kinase families, PKC and mitogen-activated protein (MAP) kinases, are now well recognised. For example, phosphorylation of αv and β1 integrin cytoplasmic domains on serine have been shown to be PKC-dependent[13] and collagen I-stimulated MAP kinase activity is mediated specifically through the cytoplasmic tail of the α2 integrin subunit.[14] The cytoplasmic domain of the β6 integrin subunit contains an 11 amino acid C-terminal extension not shared by other β integrin subunits.[5] We have previously reported that heterologous expression of αvβ6 in colon cancer cells promotes tumour cell growth *in vitro* and *in vivo,* and this growth-enhancing effect requires the presence of the 11 amino acid cytoplasmic extension.[15] Moreover, αvβ6 expression in colon cancer cells leads to increased gelatinase B secretion in a PKC-dependent manner, which is also dependent upon the presence of this cytoplasmic extension.[16,17] The β6 cytoplasmic domain has recently been shown by us to bind directly to extracellular signal-regulated kinase 2 (ERK2), a member of the MAP kinase family, and this physical interaction defines a novel paradigm of integrin-mediated signalling in cancer.[18] This binding event occurs through a motif on the β6 cytoplasmic domain that is upstream of the 11 amino acid tail. In our study, we sought to examine the respective roles of the ERK2-binding site and the 11 amino acid tail in regulating expression of β integrin subunits that associate with αv in SW480 colon cancer cells.

## MATERIAL AND METHODS

### Antibodies and reagents

Monoclonal antibodies R6G9 and E7P6 against β6 and PIF6 against the β5 subunit have been described previously.[19] Monoclonal antibodies L230 and AP3 against the αv and β3 subunits, respectively, were prepared from hybridoma cells obtained from the American Type Culture Collection (ATCC, Rockville, MD). The monoclonal antibody against the β1 subunit (MAb 13) was purchased from Becton Dickinson (San Jose, CA). Phycoerythrin-

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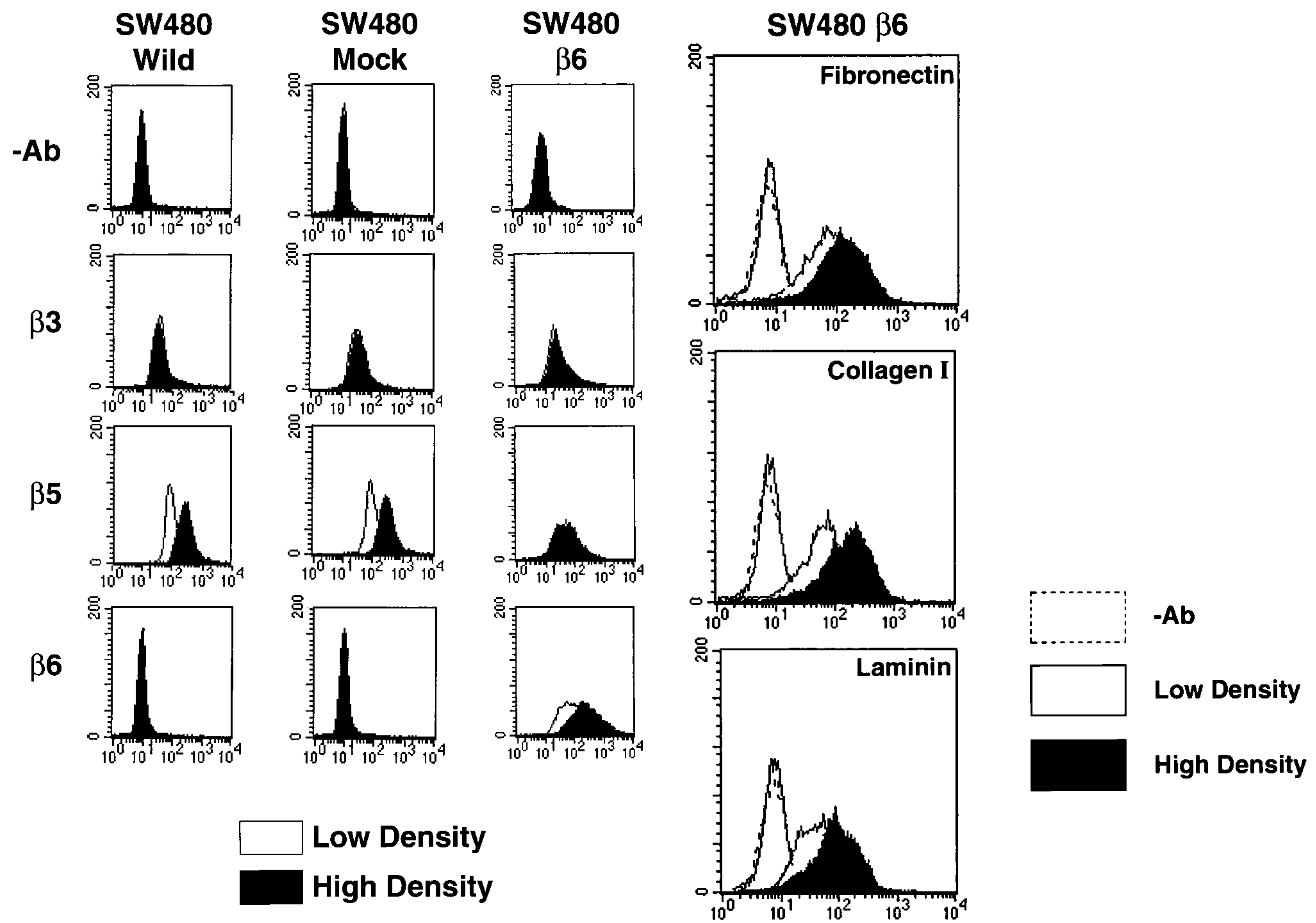


**Figure 1** – Effect of cell density on integrin expression in colon cancer cells. Nontransfected SW480 cells (SW480 wild-type) and cells transfected with either vector alone (SW480 mock) or the vector containing the β6 gene construct (SW480β6) were harvested from low- and high-density cultures and analysed for expression of β integrin subunits by FACScan as described in Material and Methods. Cells were incubated with either no primary antibody (−Ab) or monoclonal antibodies (MAbs) recognising the β3, β5 and β6 subunits (MAbs AP3, P1F6 and E7P6, respectively). White and black histograms represent cells harvested from low- and high-density cultures, respectively. Absence of white histograms indicate overlapping fluorescence intensity profiles from low- and high-density cultures. The data are representative of 3 similar experiments.

**Figure 2** – Ligand independency of cell density-dependent β6 expression. SW480β6 transfectants were seeded onto either fibronectin, collagen or laminin substrates (10 μg/ml) under serum-free conditions. Cells were harvested from low- and high-density cultures and analysed for β6 expression by FACScan. Dotted white histograms represent overlapping profiles for cells harvested from low- and high-density cultures and not incubated with primary antibody (−Ab). Solid-line white and black histograms represent cells harvested from low- and high-density cultures, respectively, and probed with MAb E7P6 (anti-β6). The upper 3 panels show β6 expression for SW480 cells transfected with wild-type β6 and the lowermost panel shows SW480 cells transfected with a β6 mutant (Subst. Mutant) unable to bind fibronectin.[21] The data are representative of 2 similar experiments.

conjugated goat anti-mouse IgG was obtained from Chemicon (Temecula, CA). Collagen type I, vitronectin, fibronectin and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). Mouse laminin was obtained from Becton Dickinson (Bedford, MA).

*Cell lines*

The colon cancer cell lines HT29 (which constitutively expresses αvβ6) and SW480 (which lacks αvβ6) were obtained from the ATCC. Stable transfectants of SW480 colon cancer cells were prepared containing β6 gene constructs in the expression plasmid pcDNA1neo under the control of the CMV promoter. The transfectants expressed either wild-type β6 (SW480β6), a truncated β6 (Trunc. Mutant; lacking the C-terminal 11 amino acids: $^{778}$EKQKVDLSTDC$^{788}$), β6 lacking the ERK2 binding site (Del. Mutant; lacking $^{746}$EAERSKAKWQTGTNPLYRG,$^{764}$ the ERK2 binding sequence is underlined), β6 lacking both the ERK2 binding site and the 11 amino acid tail (Double Del. Mutant), β6 with a single point mutation in the extracellular domain (Subst. Mutant; Asp$^{140}$ to Ala), or the expression plasmid only. These transfectants have been previously described.[5,15,18,20,21] The stably transfected cell lines were maintained in standard medium comprising Dulbecco's Modified Eagle's Medium (DMEM; 4.5 gm/litre of glucose) with 10% heat-inactivated foetal bovine serum (FBS) supplemented with HEPES, penicillin and streptomycin.

In cell cultures prepared for FACScan analyses, low-density cultures were established by seeding $5 \times 10^5$ cells in 2.5 ml of

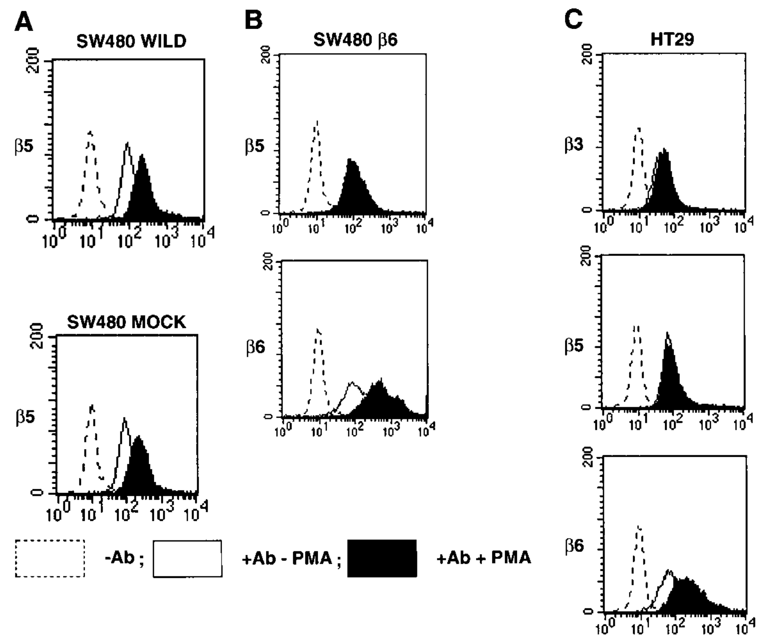


**FIGURE 3** – Effect of protein kinase C-stimulation on integrin expression. All cell lines were cultured at low density for 24 hr and then stimulated with phorbol myristate acetate (PMA, 50 nM for 30 min). The PMA was washed off and the cells maintained in culture for a further 24 hr followed by cell harvesting and analysis for integrin expression by FACScan. Cells were harvested from non-PMA-treated (full-line white histograms) and PMA-treated cultures (black histograms) and incubated with MAbs that recognise either β3, β5 or β6 integrin subunits (MAb AP3, P1F6 or E7P6, respectively). Overlapping histograms indicate no effect of PMA. Dotted-line white histograms represent cells not incubated with primary antibody. *(a)* β5 expression profiles for non-transfected SW480 cells (SW480 wild) and cells transfected with vector alone lacking the β6 gene construct (SW480 mock). *(b)* β5 and β6 expression profiles for SW480 cells expressing wild-type β6. *(c)* β3, β5 and β6 expression profiles for HT29 colon cancer cells that constitutively express the αvβ6 integrin. The data shown in *(a–c)* are representative of 3 similar experiments.

standard medium into either 6 cm diameter tissue culture dishes or 25 $cm^2$ tissue culture flasks (Corning, Corining, NY). In parallel, high-density cultures were established using identical cell numbers and culture medium volumes seeded into 24-well tissue culture plates (Falcon, Becton Dickinson). Culture medium for all cultures was changed daily until the termination of experiments at 48–72 hr, at which time low-density and high-density cultures were approximately 40% and 100% confluent, respectively. The cell number per surface area was in the range of $1.5–2 \times 10^5$ cells per $cm^2$ and $3.5–4 \times 10^5$ cells per $cm^2$ for low- and high-density cultures, respectively. Cells were harvested with trypsin/EDTA (Commonwealth Serum Laboratories, Victoria, Australia) for analysis of integrin expression. In some experiments, tissue culture surfaces were coated with matrix substrates (fibronectin, collagen or laminin; 10 μg/ml) followed by blocking nonspecific binding sites with 0.5% BSA in PBS before initiating low-/high-density cultures in serum-free medium. Serum-free medium was composed of DMEM supplemented with ITS (insulin, selenous acid and transferrin), HEPES and penicillin/streptomycin.

*FACScan analyses*

Monolayer cultures of SW480 transfectants or HT29 wild-type cells were harvested with trypsin/EDTA and then blocked with goat serum at 4°C for 10 min. Cells were washed once with PBS and incubated with primary antibodies against the β3, β5 and β6 subunits for 20 min at 4°C and then washed twice with PBS. Cells were then stained with secondary antibody (goat Anti-mouse IgG) conjugated to phycoerythrin for 20 min at 4°C, washed twice with PBS and resuspended in 0.5 ml PBS prior to FACScan analysis (Becton Dickinson, Rutherford, NJ).

*Cell adhesion assays*

Cell adhesion assays were performed essentially as previously described.[15] Briefly, wells of nontissue culture-treated polystyrene 96-well flat-bottom microtitre plates (NUNC, Roskilde, Denmark) were coated with 1 μg/ml fibronectin or 1 μg/ml vitronectin for 2 hr at 37°C, washed with PBS and then blocked with 0.5% BSA in PBS for 1 hr at 37°C. Harvested SW480 transfectants from low- and high-density cultures were seeded at a density of $10^5$ cells/well in 200 μl of a serum-free DMEM containing 0.5% BSA. To block adhesion, cells were incubated with either anti-β1 (MAb13) plus anti-αv (L230) for fibronectin adhesion assays and in the presence of only anti-αv for vitronectin assays for 15 min at 4°C before plating. The plates were centrifuged (top side up) at 10*g* for 5 min, then incubated for 1 hr at 37°C in humidified 5% carbon dioxide. Nonadherent cells were removed by centrifugation top side down at 48*g* for 5 min. The attached cells were fixed and stained with 0.5% crystal violet (in 20% methanol and 1% formaldehyde) and the wells washed with PBS. The relative number of cells in each well was evaluated by measuring the absorbance at 595 nm in a microplate reader (Bio-Rad, Hercules, CA), and background readings for wells coated with BSA were subtracted from readings for all matrix-coated wells. The data were expressed as the mean absorbance (± SEM) for triplicate wells.

*Western blotting and immunoprecipitation*

Cells were cultured as adherent monolayers in plastic tissue culture flasks in complete DMEM. Cells were then recovered using trypsin/EDTA, the trypsin neutralised with complete DMEM and cell pellets washed with ice-cold PBS before lysis in Buffer A (100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $CaCl_2$, 1% Triton X-100, 0.1% SDS, 0.1% NP-40, 1 mM vanadate, 1 μg/ml pepstatin, 1 mM PMSF, 5 μg/ml aprotinin and 1 μg/ml of leupeptin). Lysates stood at 4°C for 30 min and were clarified at 10,000*g* for 10 min at 4°C to remove detergent-insoluble material. Soluble lysate was recovered and used in all subsequent analyses. Integrin immunoprecipitations were performed following biotinylation of cell surface proteins with biotin-CNHS-ester in Buffer B (10 mM sodium borate, 150 mM NaCl, pH 8.8) to determine expression levels of integrin subunits. A portion of immunoprecipitated biotinylated integrin was separately electrophoresed for immunoblotting with anti-ERK antibody. All proteins were resolved by SDS-PAGE under nonreducing conditions. Separated proteins were electrophoretically transferred to nitrocellulose membrane and immunoblotted with either a monoclonal antibody that recognises phosphorylated ERK1/2 (MAb E10; New England BioLabs, Beverly, MA) or antibiotin MAb (Sigma). Where comparative immunoprecipitations were carried out, protein concentrations of detergent-soluble cell lysates were determined (BCA Protein Assay Kit; Pierce, Rockford, IL) and protein concentrations made equivalent (1 mg/ml of protein) with complete lysis buffer before immunoprecipitation. All lysates were then precleared with rabbit anti-mouse Ig coupled to Sepharose CL-4B beads.

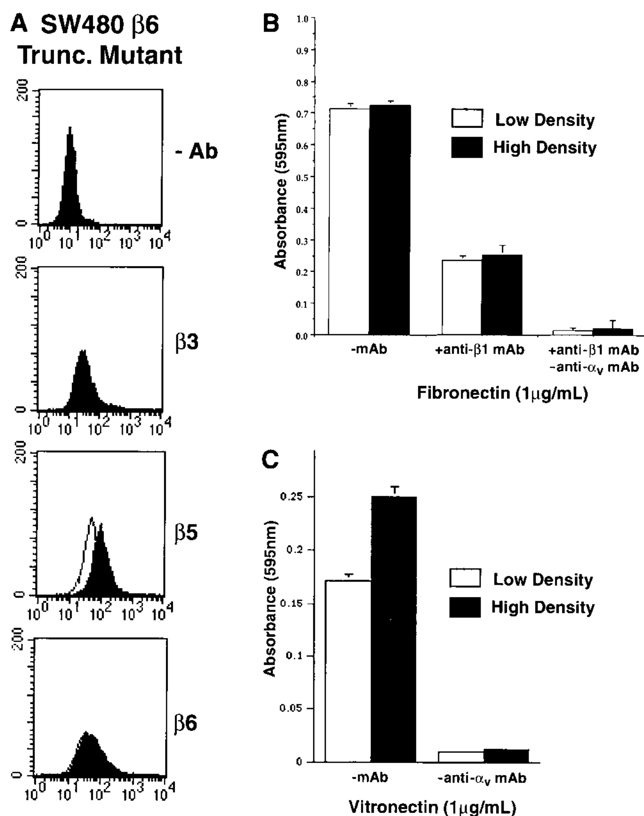


FIGURE 4 – Effect of the 11 amino acid C-terminal extension of the β6 cytoplasmic domain on cell density-dependent integrin expression and binding to ligand. *(a)* SW480 cells transfected with β6 lacking the 11 amino acid cytoplasmic tail (SW480 Trunc. Mutant) were harvested from low- and high-density cultures and analysed for expression of β integrin subunits by FACScan as described in Material and Methods. Cells were incubated with either no primary antibody (−Ab) or monoclonal antibodies that recognise the β3, β5 and β6 subunits (MAbs AP3, P1F6 and E7P6, respectively). White and black histograms represent cells harvested from low- and high-density cultures, respectively. Absence of white histograms indicate overlapping fluorescence intensity profiles from low- and high-density cultures, and the data are representative of 3 similar experiments. *(b)* SW480 truncation mutant (Trunc. Mutant) cells were harvested from low- and high-density cultures and cell adhesion assays were performed as described in Material and Methods. The ability of cells harvested from the 2 culture conditions to bind to fibronectin (coated at a concentration of 1 μg/ml) in a β1 integrin-independent manner (*i.e.,* in the presence of anti-β1 MAb 13) is shown in the pair of middle bars and the effect of addition of anti-αv MAb, L230, in the right-hand bars. *(c)* The ability of SW480 Trunc. Mutant cells harvested from low- and high-density cultures to bind to vitronectin (coated at a concentration of 1 μg/ml) in the absence/presence of anti-αv MAb L230 is shown in the left- and right-hand pair of bars, respectively. Data shown in *(b)* and *(c)* represent mean (+ SEM) values obtained from triplicate wells and are representative of 3 similar experiments.

## RESULTS

### *The β6 subunit exerts a dominant negative effect on β5 expression in cells at high density*

SW480 colon cancer cells express αvβ5, only minimal levels of αvβ3 and lack αvβ1 and αvβ6.[9] SW480 cells were stably transfected with either the β6 construct (SW480β6) or vector alone (SW480 mock) and the effect of high *vs.* low cell density on expression of β integrin subunits examined for SW480 wild-type cells and both transfected cell lines (Fig. 1). As shown in Figure 1, in the absence of β6 expression (SW480 wild-type and mock transfectants), high cell density led to increased surface expression of only the β5 subunit. In mock transfectants expressing vector alone (no β6), identical cell density-dependent changes in β5 expression were observed as for SW480 wild-type cells (Fig. 1). In contrast, in SW480 β6 transfectants, β6 expression increased at high cell density, and the density-dependent increase in β5 observed for non-β6-expressing cells was abolished as shown in Figure 1. Cells that express β6 under the control of the CMV promoter behave identically to cells that endogenously express β6 with respect to the effect of cell density and PKC stimulation on β6 expression.[12]

### *Cell density-dependent expression of β6 is ligand-independent*

Fibronectin is the major extracellular matrix ligand for the integrin αvβ6,[19] and we therefore examined the effect of relevant (fibronectin) and irrelevant (laminin and collagen) matrix substrates on cell density-dependent enhancement of β6 expression in SW480β6 transfectants. Fibronectin, collagen type I or laminin (10 μg/ml) was coated onto the surface of tissue culture flasks or wells and potential nonspecific binding sites blocked with BSA before establishing low-/high-density cultures of SW480β6 transfectants in serum-free medium. FACScan analyses of cells harvested after 48–72 hr in culture revealed similar density-dependent increases in β6 expression irrespective of the matrix substrate on which the cells had been cultured as shown in Figure 2.

However, SW480 cells themselves secrete fibronectin,[15] raising the possibility that these cells could adhere to their own matrix regardless of the substrate coated onto tissue culture surfaces. To further confirm the ligand-independent nature of density-dependent expression of β6, we tested a cell line that expresses β6 with a single point mutation in the extracellular domain ($Asp^{140}$ to Ala).[21] This mutation prevents binding between integrin αvβ6 and its ligand, fibronectin, and prevents localisation of the receptor to focal contacts.[21] The mutant subunit is completely capable of forming an integrin heterodimer with its normal partner, αv, and this heterodimer is well expressed on the surface of stably transfected cells. As shown in Figure 2 (bottom panel), expression of the β6 mutant (SW480β6 Subst. Mutant) is nevertheless increased on the cell surface at high cell density.

### *The β6 subunit exerts a dominant negative effect on protein kinase C (PKC)-mediated β5 expression*

We have recently reported that the cell density-dependent increase in β6 expression is PKC-mediated and inhibitable with PKC inhibitors.[12] To determine whether the effect of cell density on β5 expression is also PKC-mediated, nonconfluent low-density cultures of SW480 cells were exposed to PMA for 30 min and β5 expression assessed by FACScan 24 hr later. As shown in Figure 3*a*, exposure of the non-β6-expressing cell lines, SW480 wild-type and mock transfectants, to PMA enhanced β5 expression. However, exposure of SW480β6 transfectants to PMA enhanced β6 surface expression but had no effect on β5 expression (Fig. 3*b*). Similarly, exposure of HT29 cells, which constitutively express αvβ6, to PMA under low-density culture conditions resulted in increased cell surface expression of only the β6 subunit (Fig. 3*c*).

### *Cell density-dependent expression of the β6 subunit requires the 11 amino acid C-terminal extension of the β6 cytoplasmic domain*

The β6 integrin subunit contains an 11 amino acid C-terminal cytoplasmic extension not shared by other β integrin subunits.[5] Moreover, this cytoplasmic extension has been shown to be necessary for β6-mediated tumour growth *in vitro* and *in vivo* as well as PKC-dependent matrix metalloproteinase-9 secretion.[15,17] We therefore examined the effect of either high cell density or PKC stimulation of nonconfluent cultures on integrin expression in SW480 cells transfected with a β6 truncation mutant lacking the C-terminal 11 amino acids (SW480 Trunc. Mutant). As shown in Figure 4*a*, β5 surface expression increased at high cell density but β6 and β3 expression remained unchanged. We have previously reported that the increase in β6 seen in high-density cultures of cells expressing the β6 wild-type receptor is functional and is

associated with enhanced binding of cells to the β6 ligand, fibronectin, in a β1-integrin-independent manner.[12] Cells prepared from high-density cultures of the β6 truncation mutant bound no better to fibronectin than cells from low-density cultures, in contrast to the increased binding of cells from high-density cultures to the β5 substrate, vitronectin, as shown in Figure 4*b,c*. Inhibition of PKC activity with calphostin C abolished the density-dependent increase in β5 expression seen in cells expressing the β6 truncation mutant lacking the 11 amino acid cytoplasmic extension (Fig. 5*a*). Further, stimulation of the cells at low density with PMA enhanced β5 but not β6 expression as shown in Figure 5*b*.

*The ERK2 domain on β6 is necessary for the stimulatory effect of high cell density and PKC activation on expression of both β5 and β6 subunits in αvβ6-expressing cells*

We have recently reported a novel paradigm of integrin-mediated signalling, which operates through a direct interaction between the β6 cytoplasmic domain and the extracellular signal-

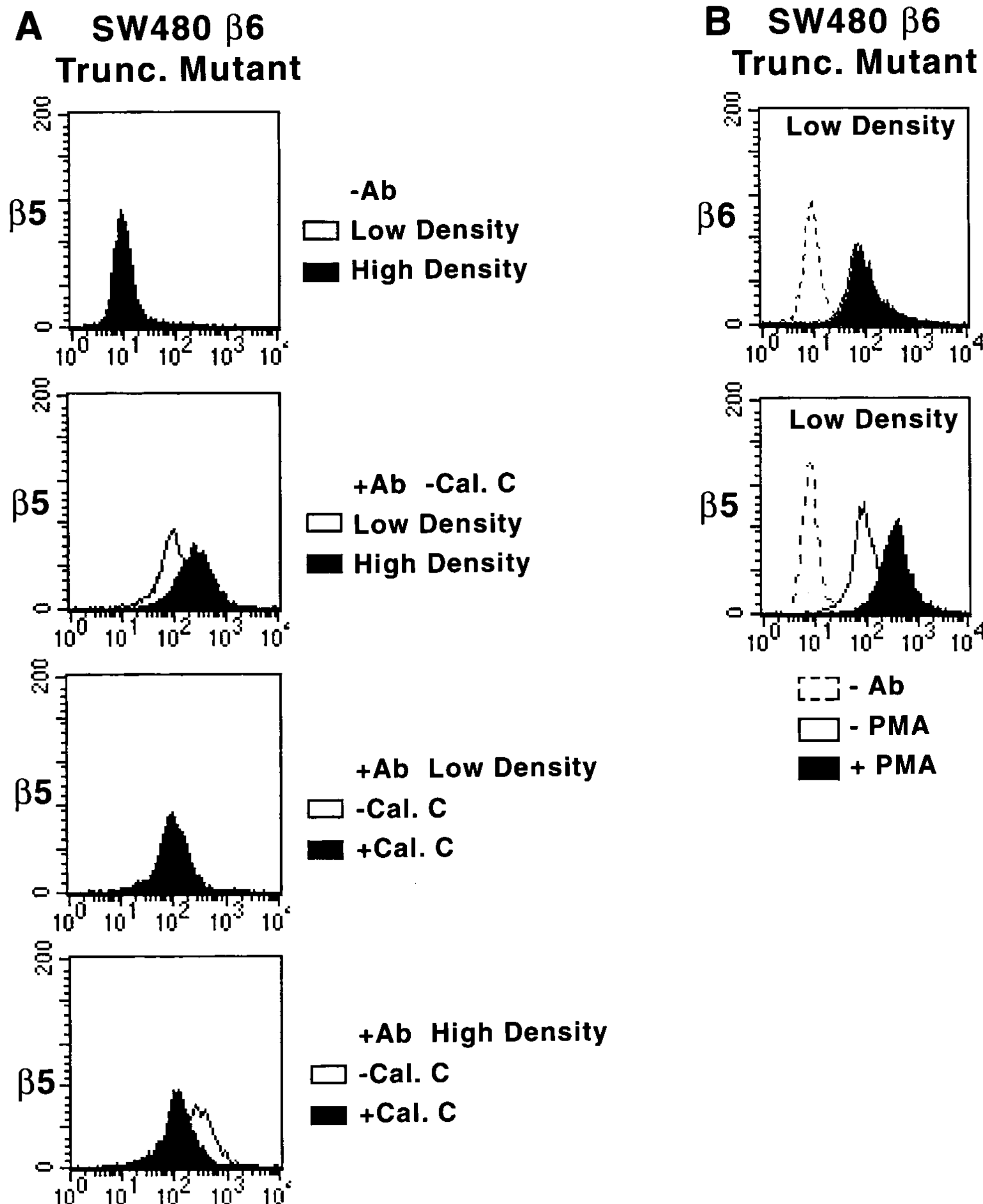


FIGURE 5 – Effect of inhibition/stimulation of protein kinase C on β5/β6 integrin subunit expression in SW480 cells expressing β6 lacking the 11 amino acid tail (SW480 Trunc. Mutant). *(a)* Cells were cultured under low- and high-density conditions in the absence/presence of calphostin-C (100 nM) and then harvested and analysed for β5 expression (MAb P1F6) by FACScan. Upper 2 panels: full-line white and black histograms represent cells from low- and high-density cultures, respectively, in the absence of calphostin-C. In the uppermost panel, in the absence of anti-β5 antibody low- and high-density background fluorescence intensity profiles are overlapped. Lower 2 panels: full line white and black histograms represent cells either not exposed or exposed to calphostin-C, respectively. At low density, the fluorescence intensity profiles in the absence/presence of calphostin-C are overlapped. *(b)* Cells were cultured at low density for 24 hr and then stimulated with PMA (50 nM for 30 min). The PMA was washed off and the cells maintained in culture for a further 24 hr followed by cell harvesting and analysis of β6/β5 expression by FACScan. Cells were harvested from non-PMA treated (full-line white histograms) and PMA-treated cultures (black histograms) and incubated with MAbs that recognise either β6 or β5 subunits (MAbs E7P6 or P1F6, respectively). Overlapping histograms indicate no effect of PMA. Dotted-line white histograms represent cells not incubated with primary antibody. The data shown in *(a)* and *(b)* are representative of 3 similar experiments.

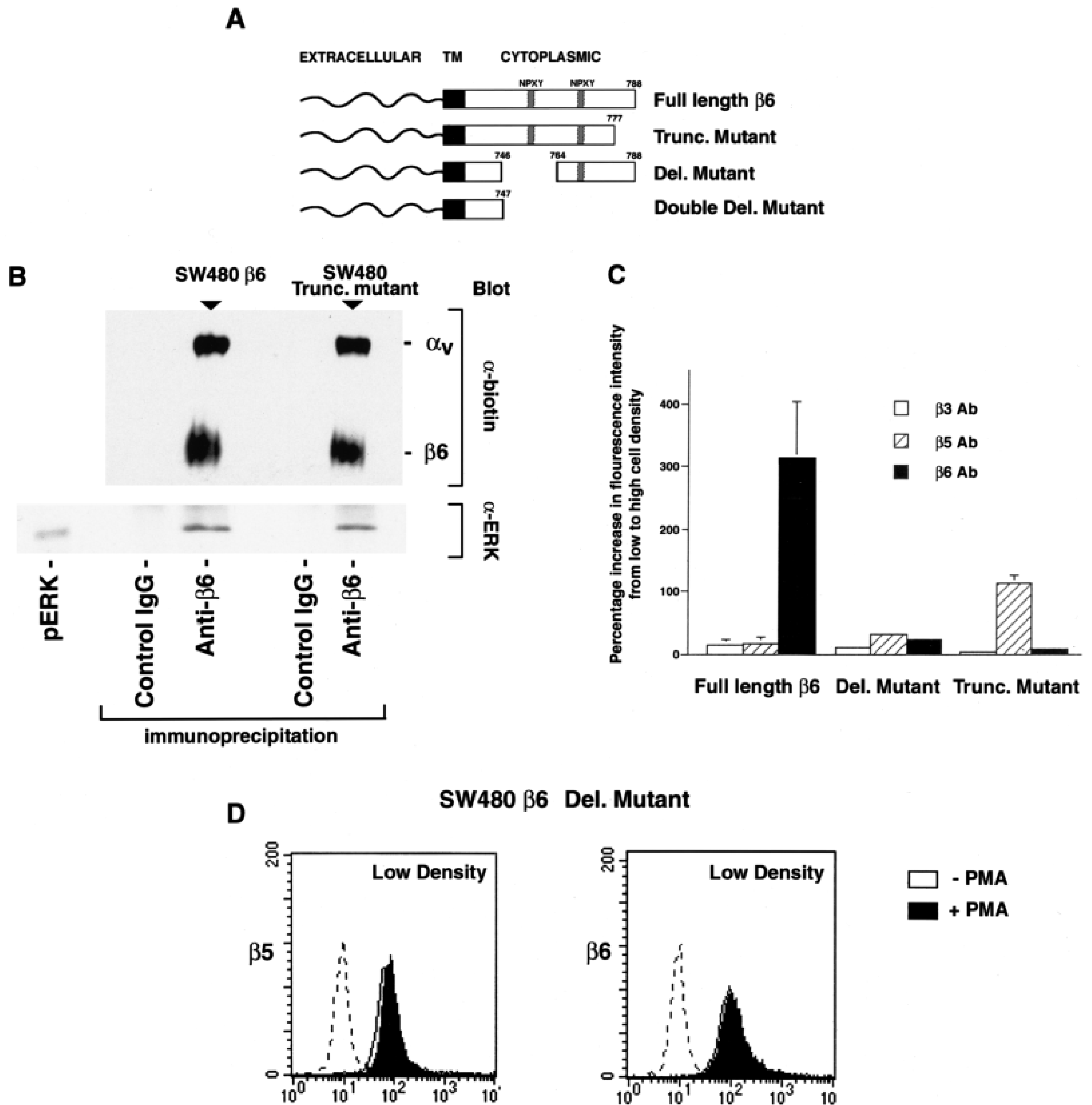


FIGURE 6 – Dependency of the β6-ERK2 association on the 11 amino acid cytoplasmic extension and effect of high cell density/PKC stimulation on integrin expression in the presence of β6 lacking the ERK2 binding site. *(a)* Diagrammatic representation of the extent of truncation of the β6 cytoplasmic domain. *(b)* β6-associated ERK2 identified by immunoprecipitations of the β6 integrin subunit. Cell lysates were prepared from surface biotinylated SW480 transfectants expressing either the wild-type β6 receptor (SW480β6) or β6 lacking the 11 amino acid C-terminal extension (SW480 Trunc. Mutant). Immunoprecipitations were performed with either anti-β6 antibody (R6G9) or matched isotype control antibody MAb ($IgG_{2a}$). The immunoprecipitated proteins were resolved by SDS-PAGE under nonreducing conditions. Separated proteins were electrophoretically transferred to nitrocellulose membrane and immunoblotted with an anti-biotin MAb or anti-ERK MAb (E10) against phosphorylated forms of ERK1/2. Purified, phosphorylated ERK2 is shown in the left-hand lane. ERK is shown coimmunoprecipitated with both wild-type β6 and β6 lacking the 11 amino acid tail. *(c)* Histogram showing the mean percentage increase (+ SEM) in fluorescence intensity from low to high cell density (assessed by FACScan as described in Material and Methods) for each of the αv-associated β3, β5 and β6 subunits from 3 independent experiments. The 2 SW480 mutant β6 cell lines shown are β6 lacking either the ERK2 binding site (Del. Mutant) and β6 lacking the 11 amino acid tail (Trunc. Mutant). No difference in background fluorescence intensity (in the absence of primary antibody) was observed between low and high cell density samples. The difference between the 2 mutants in terms of the mean percentage increase in β5 expression from low to high cell density was highly significant ($p < 0.005$). *(d)* The SW480 transfectant expressing β6 (lacking the ERK2 binding site, Del. Mutant) was cultured at low density in the absence/presence of PMA. The cell line was cultured at low density for 24 hr and then stimulated with PMA (50 nM for 30 min). The PMA was washed off and the cells maintained in culture for a further 24 hr followed by cell harvesting and analysis of integrin expression by FACScan. Cells were harvested from non-PMA-treated (full-line white histograms) and PMA-treated cultures (black histograms) and incubated with MAbs that recognise either β5 or β6 integrin subunits (MAbs P1F6 or E7P6, respectively). Dotted white-line histograms represent cells not incubated with primary antibody. The data shown in *(b–d)* are representative of 3 similar experiments.

regulated kinase 2 (ERK2).[18] The ERK2 binding motif on the β6 cytoplasmic domain consists of the 15-mer amino acid sequence $^{749}$RSKAKWQTGTNPLYR.$^{763}$ Expression of a deletion mutant lacking this domain ($^{746}$EAERSKAKWQTGTNPLYRG$^{764}$)[20] abrogates β6-mediated tumour cell proliferation *in vivo* as has also been reported for cells either expressing β6 lacking the 11 amino acid extension only or cells lacking both the ERK2 binding site and the 11 amino acid tail.[15] Given the possibility that loss of the 11 amino acid C-terminal extension of β6 could affect conformation of and, therefore, ERK2 binding to the nearby ERK2 binding site of the β6 cytoplasmic domain, we compared the ability of the truncated β6 mutant (Trunc. Mutant) and wild-type receptor to coimmunoprecipitate ERK. As shown in Figure 6*b*, β6 immunoprecipitants obtained from cell lysates prepared from SW480β6 transfectants (wild-type β6 receptor and truncated β6) coimmunoprecipitated similar amounts of phosphorylated ERK.

We then compared the effect of high cell density on β-integrin subunit expression for the 2 β6 cytoplasmic mutants, *i.e.,* the truncation mutant lacking the 11 amino acid C-terminal extension (Trunc. Mutant) and the deletion mutant lacking the ERK2 binding domain (Del. Mutant). The mean percentage increase in fluorescence intensity as assessed by FACScan for each of the αv-associated β subunits expressed by wild-type β6-expressing cells and the 2 mutant cell lines cultured at low and high density for 3 independent experiments is shown in Figure 6*c*. Loss of the ERK2 binding site on β6 markedly suppressed cell density-mediated surface expression of both β5 and β6 subunits when compared to the truncation mutant (Trunc. Mutant) as shown in Figure 6*c* (for 3 separate experiments). The mean percentage increase in fluorescent intensity from low to high cell density across 3 experiments for β6 expression was 148–478% in SW480 cells expressing wild-type β6 and 15–28% and 0–17% for SW480 cells expressing β6 lacking the ERK2 binding site and the 11 amino acid tail, respectively (Fig. 6*c*). Exposure of low-density cultures of SW480 cells transfected with the β6 deletion mutant (Del. Mutant lacking the ERK2 binding site) to PMA did not result in an increase in β6 surface expression at low cell density (Fig. 6*d*) as seen for cells either transfected with wild-type β6 or expressing endogenous β6 (Fig. 3*b,c*). Further, in cells transfected with β6 lacking the ERK2 binding site, the failure to increase β6 expression at high cell density was not associated with a switch to enhanced β5 expression (Fig. 6*c*) as seen for either SW480 wild-type cells and mock transfectants (that lack β6) or cells expressing β6 lacking the 11 amino acid tail (Figs. 1, 4*a*). In addition, PKC stimulation had no effect on expression of either β6 or β5 in cells expressing β6 that lacks the ERK2 binding site (Fig. 6*d*). In cells expressing β6 that lacked both the ERK2 binding site and the 11 amino acid tail (Double Del. Mutant, shown diagrammatically in Fig. 6*a*), neither high cell density nor PMA stimulation led to an increase in surface expression of either the β5 or the β6 subunit (Fig. 7*a,b*). These data demonstrate differential regulation of cell density-dependent β5/β6 expression between wild-type β6 and the 2 mutant variants that would not be expected if the CMV promoter acted independently of β6-derived autoregulation.

## DISCUSSION

We have reported that the αvβ6 integrin upregulates its own expression via PKC-mediated signalling in tumour cells upon cell crowding.[12] Both high cell density and stimulation of low-density cultures with PMA induced identical changes in β6 integrin subunit expression in cells that constitutively express αvβ6 and SW480 cells lacking αvβ6 but transfected with the β6 gene construct. While it has to be recognised that cell density-dependent integrin expression *in vitro* may not unambiguously apply to the *in vivo* situation, cell density-dependent changes in protein expression for the wild-type SW480 colon cancer cell line cultured as a monolayer have been shown to mimic events observed in primary tumours and their metastasis.[22]

Fibronectin is the major matrix ligand for αvβ6;[19] however, increased expression of β6 was observed at high cell density irrespective of whether cells were cultured on fibronectin or on irrelevant β6 ligands, such as collagen or laminin. Loss of the 11 amino acid C-terminal extension of the β6 subunit does not impair the ability of the receptor to bind fibronectin,[15] yet abolishes the density-dependent increase in β6 expression seen in cells expressing wild-type β6. In reverse experiments, cell density-dependent changes in β6 expression were observed for cells that had been stably transfected with β6 containing a single point mutation on the extracellular domain that is known to abolish β6-dependent binding to fibronectin.[21] Taken together, these data indicate that cell density-dependent β6 expression is ligand independent.

Little is known of the signalling mechanism(s) whereby integrins regulate their own expression as a function of cell density in human malignancies. In our study, we used 2 β6 cytoplasmic domain mutants to examine integrin cross-talk and integrin recruitment to the cell surface. One of these β6 mutants lacks the 11 amino acid C-terminal extension unique to the β6 integrin subunit, and we have previously shown that this is necessary for β6-mediated tumour cell growth.[5,15,17] Herein, we show that loss of this terminal cytoplasmic extension suppresses the ability of αvβ6 to upregulate its own expression via PKC-mediated signalling with cell crowding in contrast to findings previously reported for cells expressing wild-type β6.[12] Hence, the 11 amino acid extension is essential for the increase in β6 expression; however, loss of this cytoplasmic extension resulted instead in a cell density-dependent PKC-mediated increase in surface expression of the β5 but not the β3 subunit. Vitronectin binds to both αvβ3 and αvβ5 integrins.[4] This increase in β5 expression in cells harvested from high-density cultures was associated with an increase in the ability of these cells to bind to a vitronectin substrate. Similarly, stimulation of low-density cultures with PMA resulted in increased surface expression of only the β5 subunit but not the truncated β6 receptor just as seen for cells that lack wild-type β6, further implicating PKC-signalling pathways. Taken together, these data indicate that the 11 amino acid cytoplasmic extension of the β6 subunit exerts a dominant negative effect on cell density- and PKC-mediated β5 expression in αvβ6-expressing colon cancer cells.

The MAP kinase pathway acts a convergence point for diverse signalling events responsible for cell proliferation, differentiation and migration. We have recently shown, using another β6 cytoplasmic domain mutant, that tumour cell growth is dependent upon direct integrin-ERK2 binding. This led us to postulate that the β6 integrin subunit serves to direct growth factor-activated ERK to downstream cytoplasmic targets involved in regulating cell growth and/or cytoskeletal reorganisation.[18] The ERK2 binding site on the β6 cytoplasmic domain lies near the 11 amino acid C-terminal extension, and loss of this extension does not impair ERK2 binding to the β6 subunit. High cell density or PKC stimulation leads to a switch from increased β6 to increased β5 expression in cells expressing β6 lacking the 11 amino acid tail as shown in Figures 4 and 5. However, loss of the ERK2 binding site markedly impairs cell density- and PKC-dependent expression of either β6 or β5 in the presence or absence of the 11 amino acid tail, respectively. Our findings suggest that in αvβ6-expressing cancer cells, a hierarchy of kinase signalling cascades exists and that the β6-ERK2 interaction dominates over PKC-mediated signalling pathways responsible for integrin upregulation with cell confluence.

We have observed that *in vivo* tumour growth is less for colon cancer cells that express β6 lacking the ERK2 binding site compared to cells that constitutively lack the wild-type β6 receptor.[18] In cells that lack constitutive αvβ6, β5 subunit expression increases in response to cell density and PKC stimulation in contrast to cells expressing β6 that lacks the ERK2 binding site. The significance of cell density-dependent upregulation of β5 in non-β6-expressing colon cancer cells remains to be determined. Beta 5 is thought to play a role in mediating epithelial cell locomotion.[23] In endothelial cells, αvβ5-mediated cell migration has been shown

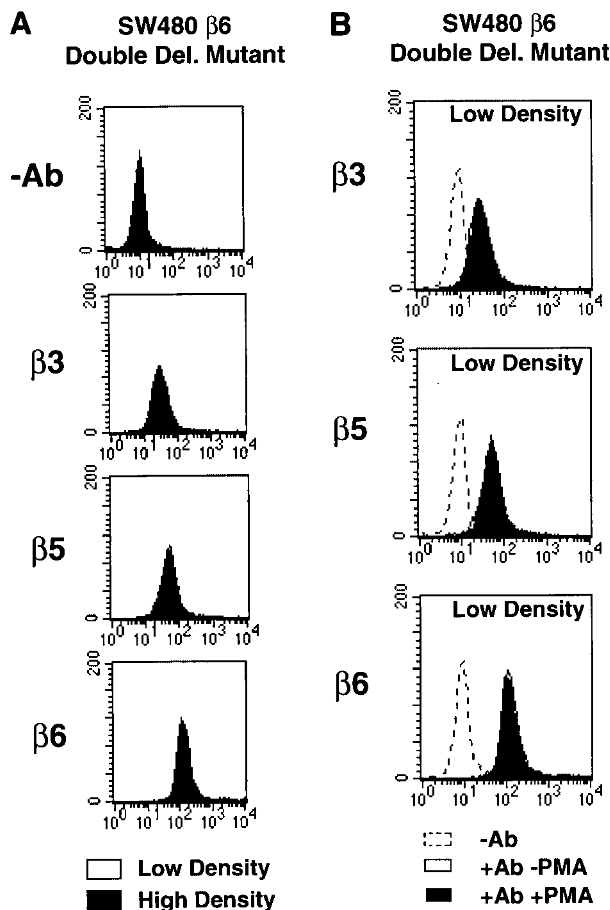


FIGURE 7 – Effect of deletion of both the ERK2 binding motif and the 11 amino acid tail on cell density- and PKC-dependent integrin expression. *(a)* SW480 cells transfected with the β6 gene construct lacking both the ERK2 binding site and the 11 amino acid tail (Double Del. Mutant) were harvested from low-/high-cell density cultures and analysed for β integrin subunits by FACscan. Cells were incubated with either no primary antibody (−Ab) or monoclonal antibodies (MAbs) recognising the β3, β5 and β6 subunits (MAbs AP3, P1F6 and E7P6, respectively). White and black histograms represent cells harvested from low- and high-density cultures, respectively. Absence of white histograms indicates overlapping fluorescence intensity profiles from low- and high-density cultures. *(b)* SW480β6 Double Del. Mutant cells were cultured at low cell density for 24 hr and then stimulated with phorbol myristate acetate (PMA, 50 nM for 30 min). The PMA was washed off and the cells maintained in culture for a further 24 hr followed by cell harvesting and analysis for integrin expression by FACScan. Cells were harvested from non-PMA-treated (full-line white histograms) and PMA-treated cultures (black histograms) and incubated with MAbs that recognise either β3, β5 or β6 integrin subunits (MAb AP3, P1F6 or E7P6, respectively). Overlapping histograms indicate no effect of PMA. Dotted-line white histograms represent cells not incubated with primary antibody. Data shown in *(a)* and *(b)* are representative of 3 similar experiments.

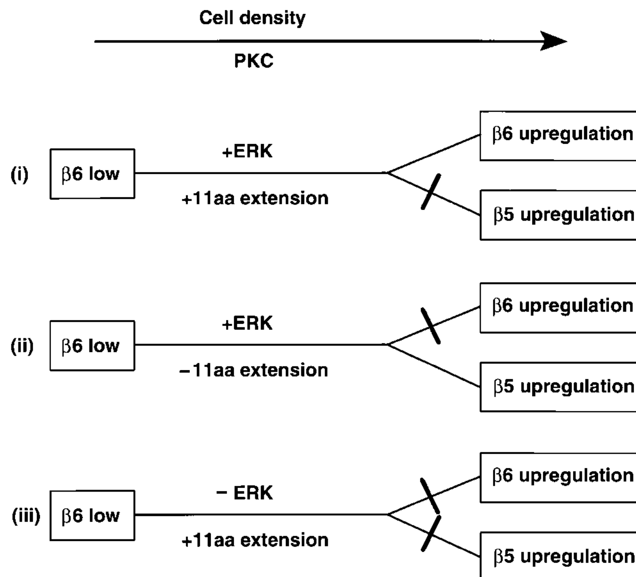


FIGURE 8 – Schema of β6 cytoplasmic domain regulation of β5/β6 integrin expression in response to high cell density. *(i)* With increasing cell density, only β6 is upregulated in β6-expressing cells, and the upregulation of β6 requires both the ERK-binding domain and the 11 amino acid extension. *(ii)* Disruption of the 11 amino acid extension abolishes upregulation of β6 while allowing the upregulation of β5. *(iii)* Disruption of the ERK-binding domain abolishes upregulation of both β subunits.

to be protein kinase C-dependent[24,25] and in breast cancers, tumour cell invasion mediated by the protease-activated receptor family requires cooperativity with the αvβ5 integrin.[26] Hence, the increased β5 expression may serve to promote cell migration and invasion at times when β6 is switched off or not expressed.

Our data provide the first evidence of integrin cross-talk at high cell density in cancer cells. In colon cancer, increased surface expression of beta integrin subunits at high cell density is specified by preferential expression of β6 over the β5 subunit. The model shown in Figure 8 summarises diagrammatically how 2 distinct motifs on the β6 cytoplasmic domain (ERK2-binding site and the 11 amino acid tail) regulate expression of individual integrin subunits in response to high cell density or PKC stimulation. The identification of cytoplasmic molecules that interact with the 11 amino acid C-terminal extension of β6 will lead to a better understanding of the signalling pathways responsible for suppression of β5 in β6-expressing cells at high cell density. However, given the dominance of the β6-ERK2 interaction over PKC-mediated expression of both integrin subunits, targeting the MAP kinase-β6 interaction may be more useful as a novel anticancer strategy in colon cancer than efforts to disrupt molecular interactions occurring at the C-terminal extension of the β6 integrin.

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