

## Direct integrin $\alpha v\beta 6$ -ERK binding: implications for tumour growth

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**Blockade of the mitogen-activated protein (MAP) kinase pathway suppresses growth of colon cancer *in vivo*. Here we demonstrate a direct link between the extracellular signal-regulated kinase ERK2 and the growth-promoting cell adhesion molecule, integrin  $\alpha v\beta 6$ , in colon cancer cells. Down-regulation of  $\beta 6$  integrin subunit expression inhibits tumour growth *in vivo* and MAP kinase activity in response to serum stimulation. In  $\alpha v\beta 6$ -expressing cells ERK2 is bound only to the  $\beta 6$  subunit. The increase in cytosolic MAP kinase activity upon epidermal growth factor stimulation is all accounted for by  $\beta 6$ -bound ERK. Deletion of the ERK2 binding site on the  $\beta 6$  cytoplasmic domain inhibits tumour growth and leads to an association between ERK and the  $\beta 5$  subunit. The physical interaction between integrin  $\alpha v\beta 6$  and ERK2 defines a novel paradigm of integrin-mediated signalling and provides a therapeutic target for cancer treatment.**  
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### Introduction

Amongst the different intracellular kinase families, mitogen activated protein (MAP) kinases behave as a convergence point for diverse receptor-initiated growth-signalling events initiated at the plasma membrane. In turn, MAP kinases drive cell cycle progression through modulation of cyclin D1 expression and associated cyclin-dependent kinase activity (Lavoie *et al.*, 1996). Extracellular signal-regulated kinases (ERKs) comprise a sub-family of MAPs that are activated by phosphorylation of tyrosine and threonine residues by MAP kinase kinases (MEKs) (Payne *et al.*, 1991). This signalling cascade begins at

the plasma membrane via non-receptor tyrosine kinases and growth factors acting through membrane-associated receptor tyrosine kinases (Boulton *et al.*, 1991; Schlaepfer and Hunter, 1997; Giancotti and Ruoslahti, 1999). The importance of the MAP kinase pathway in promoting cancer growth *in vivo* is now no longer in question. For example, MAP kinases have been shown to be important in experimental tumour metastases (Mansour *et al.*, 1994) and MAP kinases have been shown to be highly activated during the late progression of colorectal cancer (Licato *et al.*, 1997). In a large study of primary tumours of diverse origins, high frequencies of MAP kinase activation have been observed (Hoshino *et al.*, 1999), although the precise cause of the constitutive activation of the MAP kinase signalling pathway remains unclear. In a recent breakthrough in this field, a highly potent inhibitor of MAP kinase activation has been identified which is capable of inhibiting human cancer growth in immune-deficient mice (Sebolt-Leopold *et al.*, 1999).

Integrins comprise a family of cell surface adhesion receptors that also activate the MAP kinase cascade leading to transcription of growth-promoting genes (Hynes, 1992; Clark and Brugge, 1995; Giancotti and Ruoslahti, 1999). Each integrin consists of an alpha ( $\alpha$ ) and beta ( $\beta$ ) subunit molecule in close association which form a structural and functional bridge between the extracellular matrix scaffolding outside cells and cytoskeletal proteins within a cell (Hynes, 1992). The cytoplasmic tails of integrins are generally short and transduce signals by associating with adaptor proteins that connect the integrin, cytoskeleton, cytoplasmic kinases and transmembrane growth factor receptors (Giancotti and Ruoslahti, 1999). Activation of ERKs in response to integrin ligation is thought to be especially important when the concentration of growth factors available to a cell is limited (Giancotti and Ruoslahti, 1999). In addition, integrin ligation with extracellular matrix substrates has recently been shown to be a pre-requisite for translocation of activated ERK not only to the nucleus but also to plasma membrane focal adhesion sites (Aplin *et al.*, 2001; Fincham *et al.*, 2000).

Within the  $\alpha v$  integrin subfamily, the  $\alpha v\beta 6$  integrin is not expressed in normal epithelial cells; however, it

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becomes highly expressed during tumorigenesis and the  $\beta 6$  integrin subunit is thought to be widespread in cancers of various origins including lung, breast, pancreas, ovary, skin as well as in the tracheal airway epithelium of heavy smokers (Sheppard *et al.*, 1990; Breuss *et al.*, 1995). For example, induction of  $\alpha v\beta 6$  expression in oral leukoplakia appears to be a necessary pre-requisite for progression to squamous cell cancers (Hamidi *et al.*, 2000) and *de novo* expression of  $\alpha v\beta 6$  in oral squamous and colon cancers is seen particularly at the advancing tumour edge (Thomas *et al.*, 1997; Agrez *et al.*, 1996). In breast cancer,  $\alpha v\beta 6$  expression has recently been linked to more advanced tumours (Arihiro *et al.*, 2000) and we have previously reported that heterologous expression of  $\alpha v\beta 6$  in colon cancer promotes tumour cell proliferation (Agrez *et al.*, 1994). In the present study, we sought to identify the signalling pathway by which the  $\beta 6$  integrin subunit enhances colon cancer growth.

## Results

### *Antisense $\beta 6$ suppresses tumour growth in vivo and MAP kinase activity*

WiDr and HT29 cells, which constitutively express  $\alpha v\beta 6$  (Niu *et al.*, 2001), were stably transfected with  $\beta 6$  in the antisense orientation resulting in markedly reduced  $\beta 6$  subunit expression as shown for HT29 parent transfectants and three WiDr clones at the transcript and protein levels (Figure 1a–d). Control transfectants contained vector alone (mock). The  $\alpha v$  subunit binds multiple  $\beta$  integrin subunits besides  $\beta 6$ , none of which was down-regulated as a consequence of antisense transfection in these stable transfectants (Figure 1b). To examine the effect of  $\alpha v\beta 6$  suppression on *in vivo* tumour growth, cells expressing antisense  $\beta 6$  were inoculated into the flanks of immune-deficient athymic mice. Xenografts removed after 1 week from antisense  $\beta 6$  and mock transfectants (vector alone) appeared histologically similar (data not shown) but the antisense  $\beta 6$  expressing tumours were already smaller at that time (Figure 1e,f). Of a total of 40 mice injected with cells expressing antisense  $\beta 6$  (HT29, 10 mice, and three distinct WiDr clones, 30 mice) tumours disappeared in 93% of animals and in the remaining three animals diminished to 1 mm<sup>2</sup> in size during the 6-week period. In contrast, of a total of 40 mice inoculated with cells expressing vector alone (mock transfectants; HT29, 10 mice, and three distinct WiDr clones, 30 mice) progressive tumour growth was observed in all animals. The tumour growth curves shown in Figure 1(e,f) represent tumour sizes for three WiDr mock and three WiDr antisense  $\beta 6$  clones (10 mice each) and HT29 mock and antisense  $\beta 6$  transfectants (10 mice each).

We then measured the effect of down-regulating constitutive  $\beta 6$  expression on MAP kinase activity in both cell lines, WiDr and HT29. The percentage

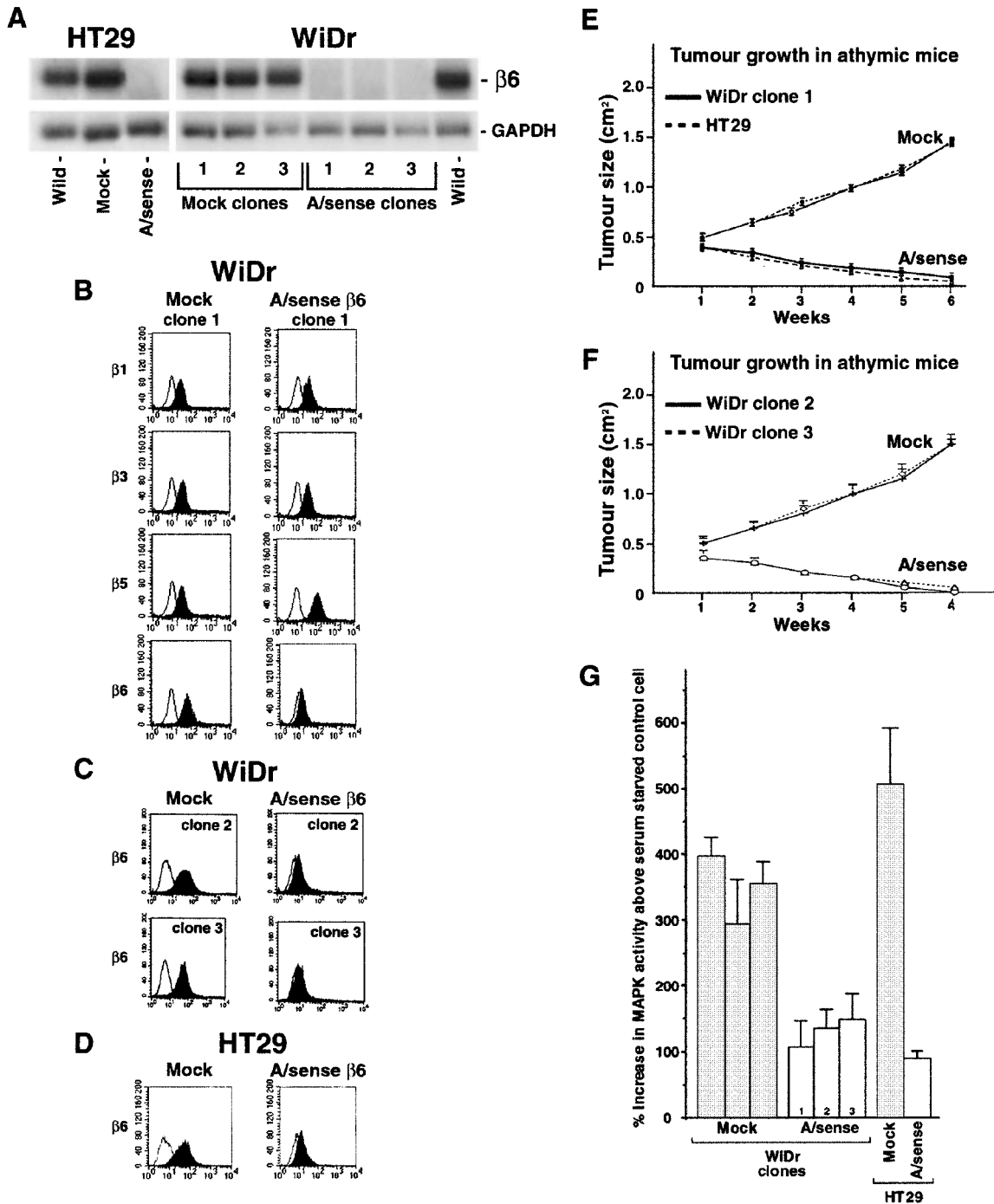
increase in MAP kinase activity for serum-starved cells stimulated for 30 min with serum for the cell lines WiDr (three mock and three antisense  $\beta 6$  clones) and HT29 (mock and antisense  $\beta 6$ ) is shown in Figure 1g. In  $\beta 6$ -expressing cells transfected with vector alone (mock) serum stimulation increased MAP kinase (ERK1/2) activity threefold above the activity levels observed for serum-starved cells. In contrast, only a onefold increase in MAP kinase activity was observed for the antisense  $\beta 6$  transfectants (Figure 1g).

### *The $\beta 6$ integrin subunit co-immunoprecipitates with ERK*

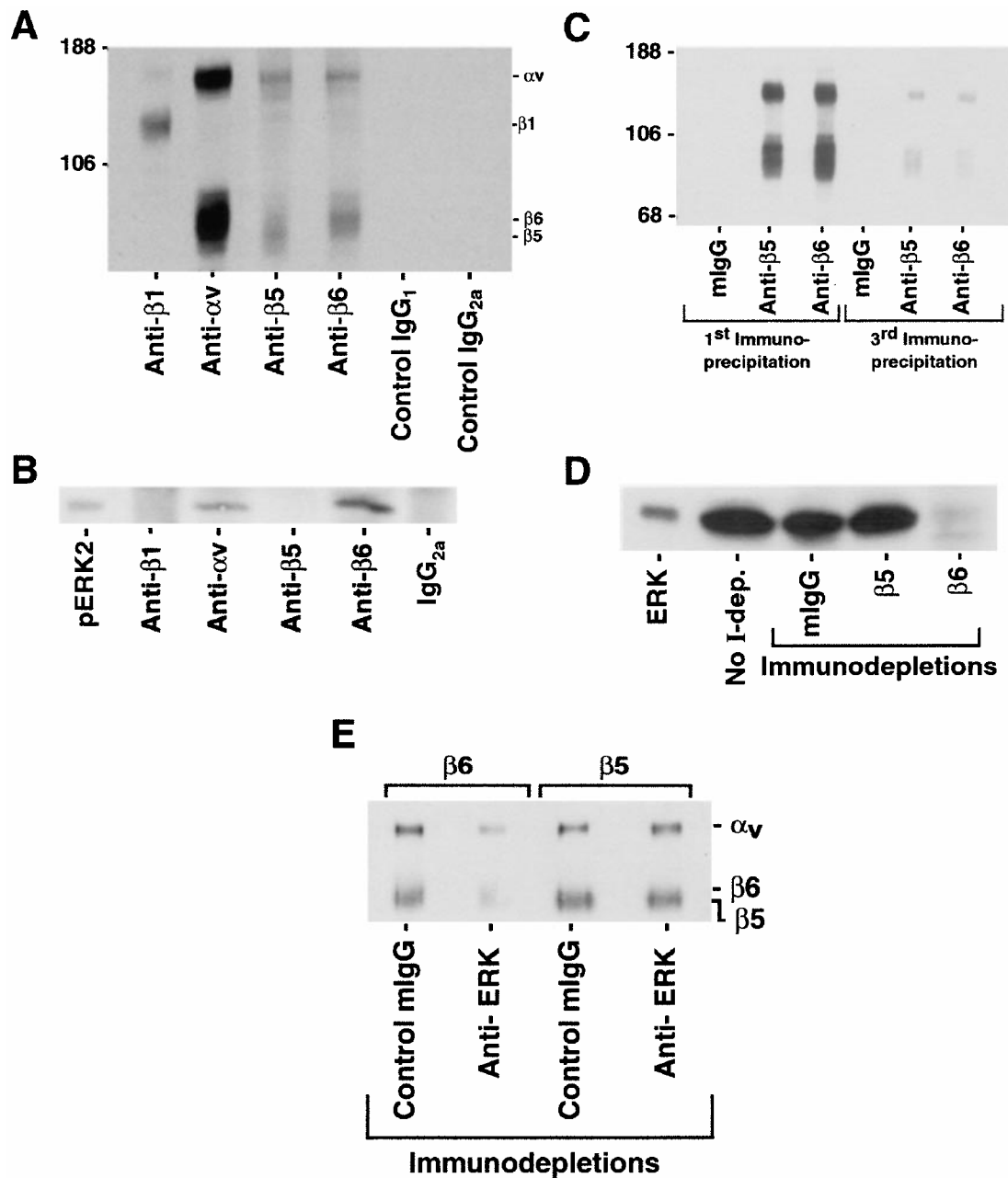
To search for  $\beta 6$ -interacting proteins that might play a role in MAP-kinase signalling we used phage display to screen a  $\lambda$ GT11 cDNA colon cancer cell library (CLONTECH Laboratories GmbH Heidelberg, Germany). A bait comprising the  $\beta 6$  cytoplasmic domain was presented as a biotinylated peptide and an interaction observed with a clone which precisely encoded the last 130 amino acids of ERK2 (Boulton *et al.*, 1991). We therefore probed integrin immunoprecipitates from soluble lysates derived from WiDr wild-type cells (Figure 2a) with an anti-ERK antibody and identified a protein band with a relative mobility identical to that of purified phosphorylated ERK2 co-immunoprecipitating only with  $\beta 6$  and not with the other major integrin  $\beta$  subunits,  $\beta 1$  and  $\beta 5$ , expressed on colon cancer cells (Agrez *et al.*, 1996) (Figure 2b). Three rounds of  $\beta 6/\beta 5$  integrin subunit immunoprecipitation markedly depleted cells lysates of  $\beta 6$  and  $\beta 5$  integrin subunits as shown in Figure 2c. Immunodepletion of  $\beta 6$  but not the  $\beta 5$  integrin subunit substantially reduced the levels of total ERK using an anti-ERK antibody (Santa Cruz, SC1647) that recognizes both non-phosphorylated and phosphorylated ERK (Figure 2d). This was confirmed in reverse experiments by immunodepleting WiDr tumour cell lysates of ERK using anti-ERK antibody, SC1647, which reduced levels of  $\beta 6$  and its partner subunit  $\alpha v$  but not the  $\beta 5$  integrin subunit (Figure 2e). In similar experiments we were unable to co-immunoprecipitate either JNK or p38 kinases with  $\beta 6$  (data not shown).

### *The increase in cytosolic MAP kinase activity upon growth-factor stimulation is all accounted for by $\beta 6$ -bound ERK*

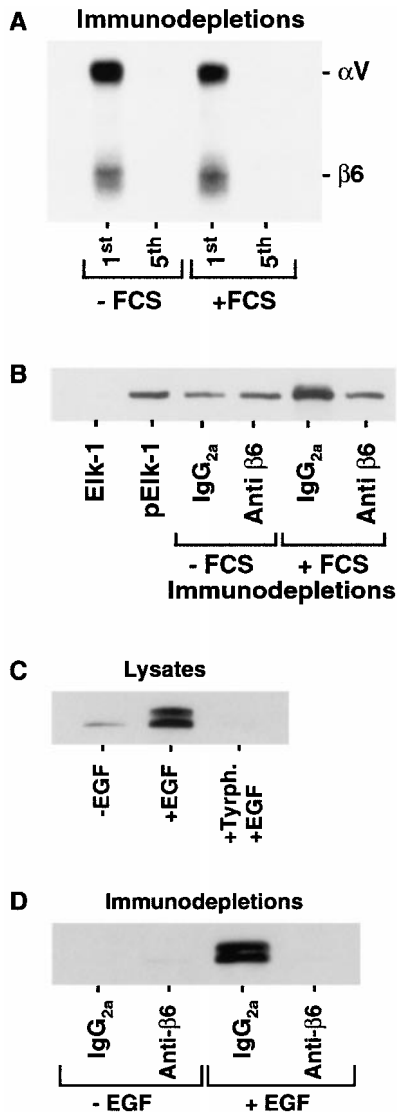
To determine the amount of ERK activity associated with  $\beta 6$  following activation of the MAP kinase pathway we compared ERK activity in non- $\beta 6$  immunodepleted lysates with that in  $\beta 6$ -immunodepleted lysates derived from cells before and after serum stimulation. In HT29 cells  $\beta 6$  could be completely immunodepleted from the soluble cell lysate after five rounds of sequential  $\beta 6$  immunoprecipitation (Figure 3a) resulting in the loss of all serum-induced MAP kinase activity (Figure 3b). Growth factors act through receptor tyrosine kinases, which in turn activate MAP kinases to modulate cell growth (Davis, 1993). The



**Figure 1** Down-regulation of  $\beta 6$  expression leads to inhibition of both tumour growth and MAP kinase activity. (a) Northern analysis of  $\beta 6$  mRNA expression in HT29 and WiDr cells lines containing either vector alone (mock), antisense  $\beta 6$  (A/sense) or wild type cells (wild). (b) FACS analysis of  $\beta$  integrin subunit expression in WiDr cells containing either vector alone (mock) or antisense  $\beta 6$  (A/sense). The data shown are derived from two clones shown in (a) (one transfected with vector alone and the other with antisense  $\beta 6$ ). Analyses with anti-integrin antibodies against  $\beta 1$ ,  $\beta 3$ ,  $\beta 5$  and  $\beta 6$  subunits were performed as previously described (Niu *et al.*, 2001). White and black histograms represent analyses in the absence and presence of primary antibodies. (c) FACS analysis of  $\beta 6$  expression in WiDr cells for the remaining mock clones (clones 2 and 3) and antisense  $\beta 6$  clones (clones 2 and 3) as shown in (a). (d) FACS analysis of  $\beta 6$  expression in HT29 cells transfected with either vector alone (mock) or antisense  $\beta 6$  (A/sense). (e) Average tumour size determined at weekly intervals over 6 weeks for subgroups of 10 animals each (WiDr and HT29 mock and antisense  $\beta 6$  transfectants) is shown following subcutaneous inoculation of cells into the flanks of immune-deficient mice. The WiDr data are derived from mock clone 1 and antisense  $\beta 6$  clone 1 as shown in (a) and (b). (f) Average tumour size determined at weekly intervals over six weeks for subgroups of 10 animals for each cell line. The data are derived from WiDr mock clones 2 and 3 and antisense  $\beta 6$  clones 2 and 3 shown in (a) and (c). (g) Percentage increase in MAP kinase activity for serum-starved cells upon stimulation with 10% FCS for 30 min for the cell lines WiDr (three mock and three antisense  $\beta 6$  clones) and HT29 (mock and antisense  $\beta 6$ ). The data represent mean ( $\pm$ s.e.m.) percentage increase in MAP kinase activity for four separate experiments



**Figure 2**  $\beta 6$  integrin-associated ERK. **(a)** Immunoprecipitation of integrin subunits from wild-type WiDr colon cancer cells. The cell lysates were immunoprecipitated with mAbs QE2E5 (anti- $\beta 1$ ), L230 (anti- $\alpha v$ ), P1F6 (anti- $\beta 5$ ), R6G9 (anti- $\beta 6$ ), isotype matched control Ab for R6G9 and QE2E5 (IgG<sub>2a</sub>) or isotype matched control Ab for L230 and P1F6 (IgG<sub>1</sub>). Anti-beta integrin antibody concentrations against individual  $\beta$  subunits were optimised to permit capture of approximately equivalent amounts of specific target protein.  $\alpha v$  which binds the  $\beta 1$ ,  $\beta 5$  and  $\beta 6$  subunits in these cells (Agrez *et al.*, 1996) was not equilibrated in this manner to ensure sufficient recovery of beta subunit-associated ERK. Molecular weight (kDa) markers are shown at left. **(b)**  $\beta 6$ -associated ERK in the  $\beta 6$  and  $\alpha v$  immunoprecipitants shown in **(a)**. The immunoprecipitated integrin subunits in **(a)** were immunoblotted with anti-ERK mAb (E10) against phosphorylated ERK1/2 (New England BioLabs, Beverly, MA, USA). L230 (anti- $\alpha v$ ) acts as a control for P1F6 (anti- $\beta 5$ ) as an isotype-matched antibody. Purified, phosphorylated ERK2 is shown in the left hand lane. **(c)** Three rounds of sequential immunoprecipitation of cell lysates derived from WiDr wild-type cells with either anti- $\beta 5$  or anti- $\beta 6$  mAb resulting in immunodepletion of target integrin. Immunoprecipitation with mouse immunoglobulins was used as a control. Molecular weight (kDa) markers are shown at left. **(d)** Total ERK (phosphorylated and non-phosphorylated) in integrin-immunodepleted cell lysates from WiDr wild-type cells shown in **(c)**. The immunodepleted cell lysates and lysates not immunodepleted (No I-dep.) were immunoblotted with anti-ERK mAb SC1647 that recognizes both phosphorylated and non-phosphorylated forms of ERK1/2. Purified ERK is shown in the left hand lane. **(e)** Six rounds of sequential immunodepletion of ERK1/2 from the lysates of surface biotinylated WiDr wild-type cells using anti-ERK mAb SC1647 (that recognizes both phosphorylated and non-phosphorylated ERK1/2) or control IgG followed by immunoprecipitation of  $\beta 5$  and  $\beta 6$  integrin subunits with mAbs P1F6 and R6G9, respectively, and immunoblotting with anti-biotin antibody. Results shown in **(a–e)** are representative of at least three separate experiments



**Figure 3**  $\beta 6$  integrin-associated ERK activity. (a)  $\beta 6$  recovery in the first and fifth rounds of sequential immunodepletion of  $\beta 6$  from HT29 wild-type cell lysates using mAb R6G9. Cells were serum-starved for 24 h and then stimulated with 10% FCS for 10 min before surface biotinylation, lysis and  $\beta 6$  immunoprecipitation from soluble lysate. Precipitated proteins were visualized by immunoblotting with anti-biotin antibody. (b) MAP kinase activity of immunoprecipitated ERK from cell lysates and determined by the *in vitro* phosphorylation of GST-Elk-1. Bands show MAP kinase activity from immunodepleted cell lysates (anti- $\beta 6$  or control IgG immunodepletions, five rounds each) prepared from HT29 wild-type cells before and after serum stimulation. Purified non-phosphorylated and phosphorylated GST-Elk-1 are shown in the left hand two lanes. (c) *In vitro* phosphorylation of GST-Elk-1 by immunoprecipitated ERK from cell lysates of HT29 wild-type cells  $\pm$  50 ng/ml EGF. Addition of 500 nM tyrphostin AG1478 for 30 min prior to addition of EGF completely inhibited the growth factor-induced MAP kinase activity. (d) MAP kinase activity of immunoprecipitated ERK from cell lysates and determined by the *in vitro* phosphorylation of GST-Elk-1. Bands show MAP kinase activity from immunodepleted cell lysates (anti- $\beta 6$  or control IgG immunodepletions, five rounds each) prepared from HT29 wild-type cells before and after EGF stimulation. Basal levels of MAP kinase activity in serum-starved cells prior to addition of EGF or FCS varied from undetectable as in (d) to a maximum as seen in (b). Results shown in (a–d) are representative of at least three separate experiments

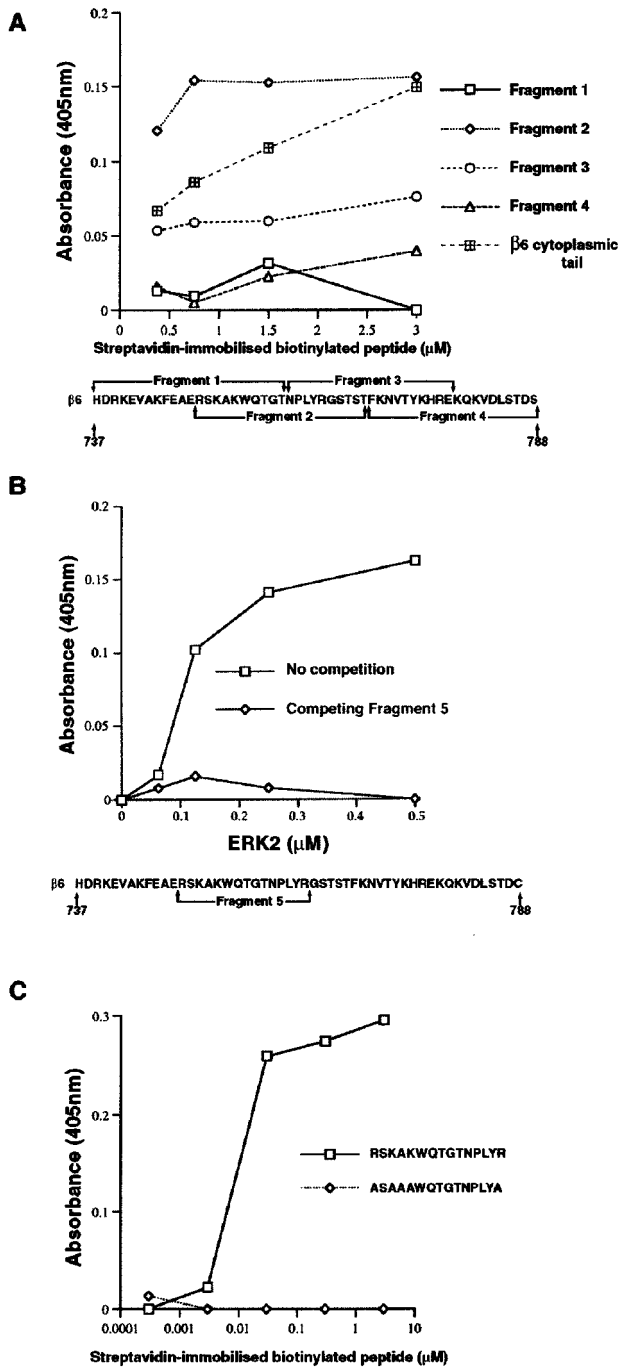
biological activity of EGF is mediated by the intrinsic tyrosine kinase activity of the EGF receptor (EGFR). Specific tyrphostins, which inhibit EGFR-dependent tyrosine kinase activity, have been shown to block growth of colorectal cancer cells *in vitro* (Partik *et al.*, 1999). Stimulation of serum-starved HT29 cells with EGF markedly enhanced the *in vitro* MAP kinase phosphorylation of a GST-Elk-1 fusion protein and this could be abrogated by either prior exposure of the cells to tyrphostin AG1478 or by totally immunodepleting  $\beta 6$  from the cell lysates (Figure 3c,d, respectively). These results demonstrate that in  $\beta 6$ -expressing cells, the rise in cytosolic MAP kinase activity above basal levels upon either serum or EGF stimulation is completely accounted for by  $\beta 6$ -bound ERK.

*The  $\beta 6$  cytoplasmic domain binds directly to ERK*

To identify the precise site of interaction on  $\beta 6$ , a series of 20-mer peptide fragments corresponding to overlapping regions of the  $\beta 6$  cytoplasmic domain (amino acids 737–788) were tested for their ability to bind ERK2 in a direct enzyme-linked immunosorbent assay (ELISA). Binding of the full-length  $\beta 6$  cytoplasmic domain to ERK2 increased in a dose-dependent manner and the binding motif on  $\beta 6$  primarily responsible for this event resided in a 20-mer peptide designated Fragment 2 (Figure 4a). A  $\beta 6$  peptide designated Fragment 5 which lacked the C-terminal five amino acids from the active Fragment 2 (Figure 4a) was equally effective in binding to ERK2 as shown in a competition assay (Figure 4b). However, substitution of the charged arginine/lysine residues of Fragment 5 with alanine resulted in complete loss of binding to ERK2 (Figure 4c). These results demonstrate a direct interaction between  $\beta 6$  and ERK2.

*Deletion of the ERK2 binding site on the  $\beta 6$  cytoplasmic domain inhibits tumour growth*

Transfection of wild-type  $\beta 6$  into colon cancer cells lacking  $\alpha v\beta 6$  has been shown to enhance tumour growth *in vitro* and *in vivo* (Agregz *et al.*, 1994). We therefore investigated the ability of a  $\beta 6$  cytoplasmic domain mutant (Cone *et al.*, 1994) which lacks the sequence <sup>746</sup>EAERSKAKWQTGTNPLYRG<sup>764</sup> (the ERK2 binding sequence is underlined) to bind to ERK and induce tumour formation. Surface expression of mutant and wild-type  $\beta 6$  subunit were similar as shown by FACScan analysis (Figure 5a) and  $\beta 1$ -independent adhesion of the deletion mutant to fibronectin was minimally affected (<20% reduction compared with wild-type  $\beta 6$ ; data not shown). Cell lysates containing equal protein loads were prepared from transfected SW480 cells expressing either wild-type  $\beta 6$ , the deletion mutant or no  $\beta 6$  (mock transfectants) and immunoprecipitated with either mAb R6G9 (anti- $\beta 6$ ) or isotype matched control antibody as shown in Figure 5b. Western blotting of these  $\beta 6$  immunoprecipitants with mAb E10 (anti-ERK



**Figure 4** Direct binding between  $\beta 6$  and ERK2 determined by ELISA. (a) Binding of full-length  $\beta 6$  cytoplasmic domain peptide to ERK2 ( $1 \mu\text{M}$ ) compared with binding of four 20-mer peptide fragments corresponding to overlapping regions of the  $\beta 6$  cytoplasmic domain. (b) Competition assay showing inhibition of binding between biotinylated peptide Fragment 5 ( $^{749}\text{RSKAKWQTGTNPLYR}^{763}$ ;  $1 \mu\text{M}$ ) and ERK2 following pre-incubation of immobilized ERK2 at increasing concentrations with non-biotinylated Fragment 5 ( $10 \mu\text{M}$ ). (c) Comparative binding of ERK2 ( $1 \mu\text{M}$ ) to  $^{749}\text{RSKAKWQTGTNPLYR}^{763}$  with and without alanine substitutions at the charged residues. The data shown in (a–c) are representative of  $\beta 6$  peptide-ERK2 binding profiles from either duplicate or triplicate wells of at least three independent experiments

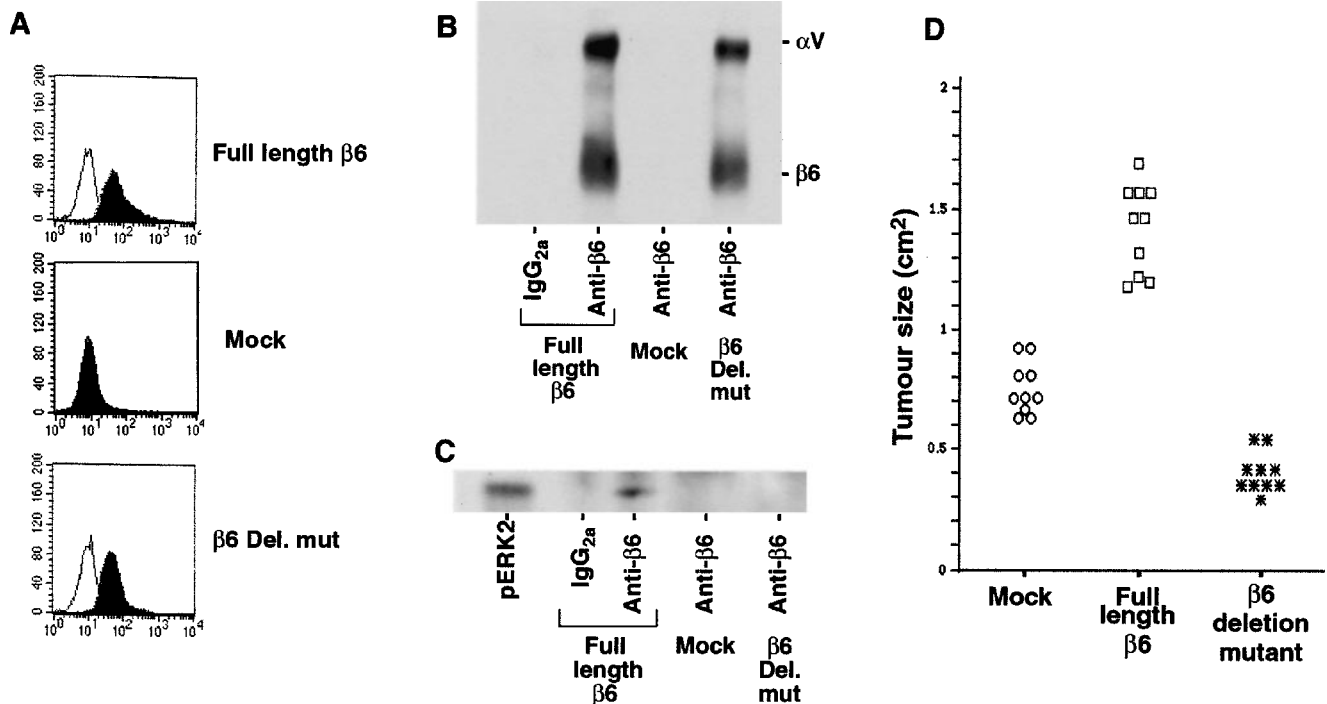
recognizing phosphorylated ERK1/2) revealed no  $\beta 6$ -bound ERK for either mock transfectants or the deletion variant compared with the wild-type receptor (Figure 5c). Inoculation for  $10^6$  tumour cells into the flanks of athymic mice resulted in tumours which were 2–3-fold larger for cells expressing wild-type  $\beta 6$  than for cells expressing either no  $\beta 6$  or the deletion mutant lacking the binding motif for ERK2 (Figure 5d). Basal ERK activity in the absence of serum is less for the  $\beta 6$  deletion mutant than for mock transfectants (data not shown) possibly accounting for the observation that the  $\beta 6$  deletion mutant forms smaller tumours than non- $\beta 6$  expressing cells.

*The  $\beta 5$  integrin subunit binds ERK in cancer cells that either lack  $\beta 6$  or express  $\beta 6$  lacking the binding site for ERK2*

Recently published data have shown that activated ERK translocates to the plasma membrane upon integrin ligation in non- $\beta 6$ -expressing cells (Fincham *et al.*, 2000). The SW480 cell line expresses abundant  $\alpha\beta 5$  but only minimal levels of  $\alpha\beta 3$  and lacks  $\alpha\beta 1$  and  $\alpha\beta 6$  (Agrez *et al.*, 1996). SW480 cells provide a model, therefore, with which to test whether the  $\beta 5$  integrin subunit can co-immunoprecipitate with ERK either in the absence of constitutive  $\beta 6$  expression or in the presence of  $\beta 6$  that lacks the binding site for ERK2. Cell lysates containing equal protein loads were prepared from SW480 wild-type cells and SW480 cells transfected with either vector alone (mock transfectants), wild-type  $\beta 6$  or the  $\beta 6$  deletion mutant (lacking the binding site for ERK2). The lysates were immunoprecipitated with mAbs R6G9 (anti- $\beta 6$ ), P1F6 (anti- $\beta 5$ ) or murine IgG (mIgG). Western blotting of the immunoprecipitates with mAb E10 recognizing phosphorylated ERK1/2 revealed no  $\beta 5$ -bound ERK in the SW480  $\beta 6$  transfectants expressing wild-type  $\beta 6$  in contrast to  $\beta 5$ -associated ERK observed for the cell lines which either lack  $\beta 6$  or express the deletion mutant lacking the ERK2 binding sequence (Figure 6a,b). To determine whether  $\beta 5$  can bind ERK2 *in vitro* we tested the ability of a 15-mer peptide fragment derived from the  $\beta 5$  cytoplasmic domain corresponding to the ERK binding domain on  $\beta 6$ , for its ability to bind ERK2 in an ELISA assay. Dose-dependent binding of the  $\beta 5$  fragment ( $^{761}\text{RSRARYEMASNPLYR}^{775}$ ) to ERK2 was observed as shown in Figure 6c although less avidly than the 15-mer  $\beta 6$  peptide. In contrast, a synthetic peptide derived from the cytoplasmic domain of the  $\beta 1$  subunit ( $^{765}\text{KFEKEKMNAKWDGTGENPIYK}^{784}$ ) which corresponds to the ERK binding domain on  $\beta 6$  failed to bind ERK2 (Figure 6c).

**Discussion**

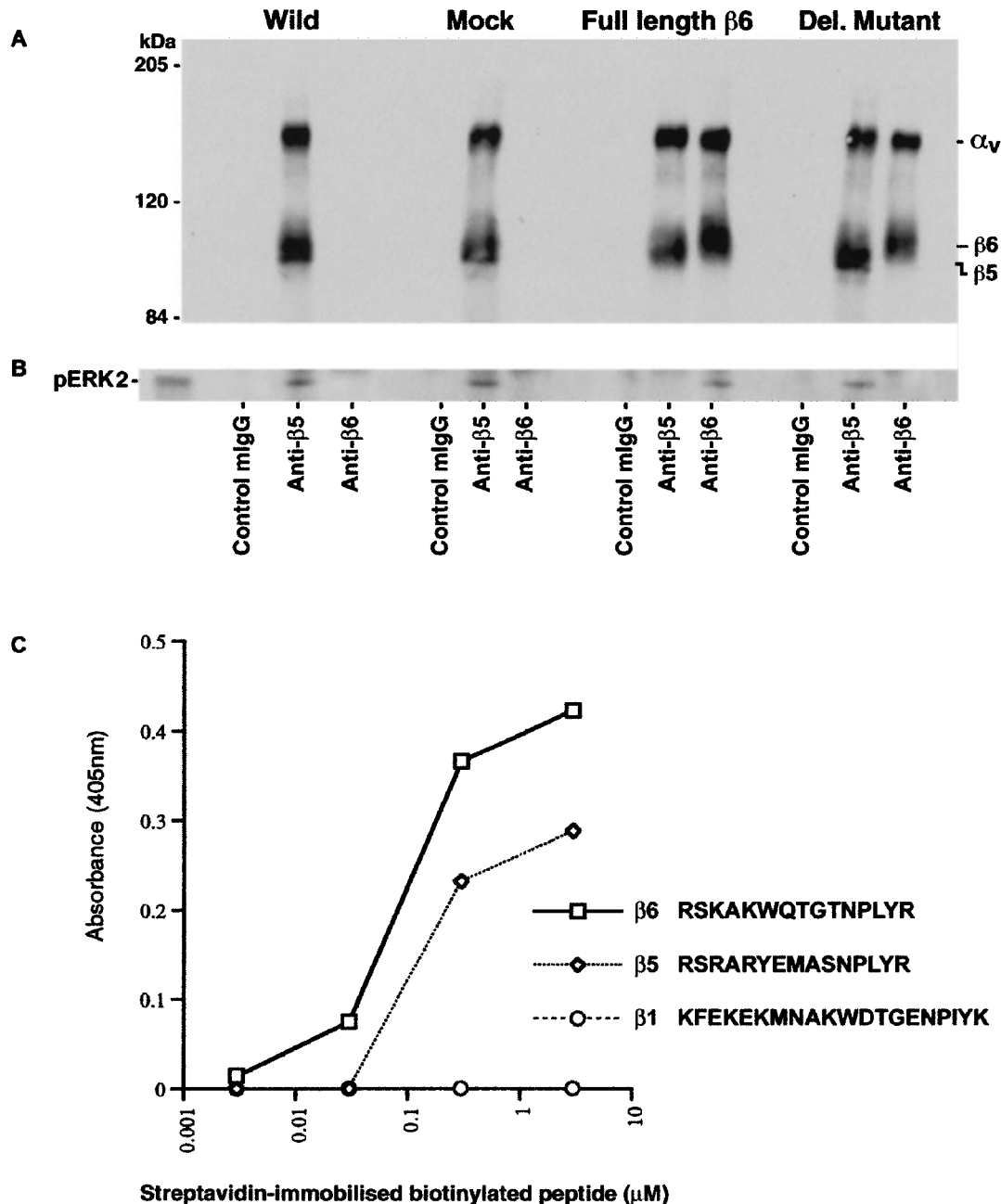
It is now becoming clear that MAP kinases are translocated to various cellular compartments to facilitate processes such as cell spreading, migration and proliferation and the proportion of activated ERK



**Figure 5** Deletion of the  $\beta 6$  binding site for ERK2 suppresses tumour growth. (a) FACS analysis of  $\beta 6$  subunit expression in SW480 cells which either lack  $\beta 6$  (mock transfectants) or express either wild-type  $\beta 6$  or  $\beta 6$  lacking the binding site for ERK2. White and black histograms represent analyses in the absence and presence, respectively, of primary antibody recognizing  $\beta 6$  (mAb, E7P6). The  $\beta 6$ -cytoplasmic domain mutant lacks the sequence <sup>746</sup>EAERSKAKWQTGTNPLYRG<sup>764</sup> (the ERK2 binding sequence is underlined) (Cone *et al.*, 1994). (b)  $\beta 6$  immunoprecipitants (mAb R6G9) from cell lysates derived from surface-biotinylated SW480 cells which either lack  $\beta 6$  (mock transfectants) or express either wild-type  $\beta 6$  or  $\beta 6$  lacking the binding site for ERK2 ( $\beta 6$  Del. Mutant). (c)  $\beta 6$  immunoprecipitants (mAb R6G9) from SW480 cell lysates shown in (b) immunoblotted with anti-ERK mAb (E10) against phosphorylated forms of ERK1/2. Purified, phosphorylated ERK2 is shown in the left-hand lane. (d) Tumour growth after 6 weeks following subcutaneous inoculation of  $10^6$  SW480 cells either expressing wild-type  $\beta 6$ , mutant  $\beta 6$  lacking the binding site for ERK2, or lacking  $\beta 6$  (mock transfectants) into immunodeficient mice. Tumour size is shown for 10 mice per group

which is found to be nuclear-associated varies with the cell type as well as the anti-ERK antibody used (Reszka *et al.*, 1995; Wolf *et al.*, 2001). For example, in non- $\beta 6$ -expressing rat embryo fibroblasts active ERK has been shown to be targeted to newly formed focal adhesions upon integrin engagement (Fincham *et al.*, 2000). MAP kinases also play a role in microtubule dynamics in various cell types and Reszka *et al.* (1995) have shown that half of all cellular MAP kinase activity generated by mitogenic stimulation is microtubule-associated indicating that a significant proportion of MAP kinase activity is directed at substrates outside the nucleus. Our study did not permit us to estimate the proportion of total ERK (nuclear plus cytosolic) that is represented by  $\beta 6$ -bound ERK. Importantly, epitopes targeted by specific anti-MAP kinase antibodies have been shown to be masked by MAP kinase-microtubule interactions possibly accounting for reported differences in active ERK translocation to the nucleus upon growth factor stimulation (Reszka *et al.*, 1995). Despite the possibility of masked ERK epitopes, removal of  $\beta 6$  from soluble cell lysates by  $\beta 6$  immunodepletion resulted in the near-complete disappearance of cytosolic ERK demonstrating the specificity of the  $\beta 6$ -ERK interaction and suggesting that  $\beta 6$  binds both active and inactive ERK.

The physical interaction between an integrin involved in tumorigenesis and ERK2 defines a novel paradigm of integrin-mediated signalling in cancer. We have shown that suppression of either wild-type  $\beta 6$  expression or expression of  $\beta 6$  deficient in the binding domain for ERK2 dramatically inhibits colon cancer cell growth. Upon either serum or EGF stimulation, all activated ERK within the cytoplasm is bound to the  $\beta 6$  integrin subunit as shown by a MAP kinase activity assay that measures phosphorylation of the ERK substrate, Elk-1. Taken together these results demonstrate that tumour growth is dependent upon direct integrin-ERK2 binding. In this model, we propose that  $\beta 6$  serves to direct growth factor-activated ERK to downstream cytoplasmic targets involved in regulating cell growth and/or cytoskeletal reorganization. Activation of ERK by serum-derived growth factors is greatly amplified in cancer cells expressing  $\alpha v\beta 6$ . Whether non-phosphorylated ERK, when bound to  $\beta 6$ , is more efficiently phosphorylated at that location because of conformational changes at the phosphorylation lip of ERK remains unknown. It is also possible that activated ERK, when bound to  $\beta 6$ , is protected from deactivation by cellular phosphatases.



**Figure 6**  $\beta 5$  integrin-associated ERK in cancer cells either lacking  $\beta 6$  or the  $\beta 6$  binding site for ERK2. (a) Immunoprecipitations of  $\beta 5$  and  $\beta 6$  integrin subunits with mAbs P1F6 and R6G9, respectively, or control murine IgG (mIgG) followed by immunoblotting with anti-biotin antibody. The  $\alpha v/\beta 5/\beta 6$  bands are shown for the SW480 cell line: wild-type cells, mock transfectants (vector alone) or cells expressing either full length  $\beta 6$  or the  $\beta 6$  cytoplasmic domain deletion mutant lacking the  $\beta 6$  binding site for ERK2. Molecular weight (kDa) markers are shown at left. (b) Identical amounts of precipitated proteins shown in (a) were immunoblotted with anti-ERK mAb (E10) against phosphorylated ERK1/2. Purified, phosphorylated ERK2 is shown in the left hand lane. (c) Comparative binding of ERK2 (1  $\mu M$ ) to synthetic peptides comprising either the 15-mer ERK binding motif on  $\beta 6$  or peptide sequences derived from the  $\beta 5$  and  $\beta 1$  cytoplasmic domains that correspond to the  $\beta 6$ -ERK binding site

The 15-mer ERK-binding motif identified on the  $\beta 6$  cytoplasmic domain has considerable homology with the distinguishing characteristics of the MAP kinase docking domains of several MAP kinase binding molecules (Sharrocks *et al.*, 2000) including a number of positively charged amino acid residues clustered at the amino terminus. Alanine substitution of all charged

residues in the  $\beta 6$  15-mer ERK binding motif resulted in complete loss of binding of the  $\beta 6$  peptide to ERK2. Charge alone, however, is not the absolute determinant of the integrin-MAP kinase interaction as indicated by the inability of  $\beta 5$  in  $\beta 6$  expressing cells to bind ERK2. The  $\beta 5$  peptide fragment also shown to bind ERK2 in the ELISA assay, albeit less effectively than  $\beta 6$ , shares

50% homology with the  $\beta 6$ -ERK2 binding sequence and contains a cluster of similarly distributed basic amino acid residues at the amino terminus of the domain. Importantly, in cells that either lack constitutive  $\beta 6$  expression or express  $\beta 6$  lacking the ERK2 binding domain, ERK appears associated with the  $\beta 5$  integrin subunit. The significance of the  $\beta 5$ -ERK binding event in the absence of  $\beta 6$  expression remains to be determined, but does raise the possibility that a hierarchy of integrin-ERK2 interactions exists within cancer cells with preferential binding of the kinase to the growth-promoting  $\beta 6$  subunit when it is expressed.

The inhibition of *in vivo* tumour growth consequent upon either down-regulation of  $\beta 6$  expression or loss of the ERK binding domain on  $\beta 6$  may be due to a combination of factors. For example, we have previously reported a direct positive correlation between levels of  $\beta 6$  expression in colon cancer cells and secretion of matrix metalloproteinase-9 (MMP-9) (Agrez *et al.*, 1999). Furthermore, exposure of tumour cells to a specific MMP inhibitor abolishes  $\beta 6$ -mediated tumour cell proliferation and colony spreading within a collagen matrix (Agrez *et al.*, 1999). Maximal expression of both MMPs and  $\beta 6$  has been observed at the invading margin of tumour cell islands in colon cancer (Hewitt *et al.*, 1995; Agrez *et al.*, 1996) and MMP-9 production has been shown to be directly dependent on the activation of ERK signalling in epithelial cells (Zeigler *et al.*, 1999; McCawley *et al.*, 1999; Johansson *et al.*, 2000). Downstream targets for ERKs include not only nuclear transcription factors such as *c-myc* but also cytosolic targets (e.g., 90 kDa ribosomal S6 protein kinase family; RSKs) involved in regulating cell growth (Kerkhoff *et al.*, 1998; Bonni *et al.*, 1999). In non-epithelial cells RSKs have been shown to catalyse phosphorylation of the pro-apoptotic protein BAD (Bonni *et al.*, 1999). Another critical target of RSKs is the transcription factor CREB (cyclic AMP response element-binding protein) (Xing *et al.*, 1996). Hence, the MAP kinase signalling pathway can promote cell survival by modulating the cell death machinery through inhibition of the pro-apoptotic protein BAD and by inducing expression of pro-survival genes in a CREB-dependent manner (Bonni *et al.*, 1999). Whether or not the  $\beta 6$ -ERK interaction in colon cancer cells mediates similar downstream events is currently under investigation.

A highly potent inhibitor of MAP kinase activation has been shown to inhibit human colon cancer growth in immuno-deficient mice (Sebolt-Leopold *et al.*, 1999). However, tumour cell specificity remains a potential problem with this approach because MAP kinases are involved in a wide range of normal cellular processes (Duesbery *et al.*, 1999). Given that *de novo*  $\beta 6$  expression occurs in tumorigenesis, targeting either  $\beta 6$  or its MAP kinase interaction may offer greater therapeutic specificity in cancer treatment. The interaction and reagents described herein may also provide a suitable method for high-throughput screening of inhibitors of this binding event.

## Materials and methods

### Antibodies and reagents

The monoclonal antibody (mAb) L230 (IgG<sub>1</sub>) against the integrin  $\alpha v$  subunit was prepared from hybridoma cells obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). Monoclonal antibodies E7P6 (IgG<sub>1</sub>) and R6G9 (IgG<sub>2a</sub>) against the integrin  $\beta 6$  subunit and PIF6 (IgG<sub>1</sub>) against the integrin  $\beta 5$  subunit, have been described previously (Weinacker *et al.*, 1994). Monoclonal antibodies QE2E5 (IgG<sub>2a</sub>) and mAb 13 (IgG<sub>2a</sub>) against the integrin  $\beta 1$  subunit were obtained from G Russ (Queen Elizabeth Hospital, Adelaide, Australia) and Becton Dickinson (San Jose, CA, USA), respectively. Phycoerythrin-conjugated goat anti-mouse IgG was obtained from Chemicon (Temecula, CA, USA) and rabbit anti-mouse immunoglobulin from DAKO (Denmark). Protein A-Sepharose CL-4B was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Reagents for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and molecular weight markers were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Mouse immunoglobulins, epidermal growth factor (EGF) and tyrphostin AG1478 were purchased from Sigma-Aldrich (St Louis, MO, USA).

### Cell lines and culture conditions

The human colon cancer cell lines, WiDr, HT29 and SW480 were obtained from the ATCC and maintained as monolayers in standard medium comprising Dulbecco's Modified Eagle's Medium (DMEM; 4.5 gm/litre of glucose) containing 10% heat-inactivated foetal calf serum (FCS) and supplemented with 20 mM HEPES, 100 IU/ml penicillin and 100  $\mu$ g/ml streptomycin. SW480 colon cancer cells, which lack constitutive  $\alpha v\beta 6$  expression, were stably transfected with pcDNA1neo constructs containing either the  $\beta 6$  gene construct or the expression plasmid only as previously described (Agrez *et al.*, 1994). WiDr and HT29 cells, which express endogenous  $\beta 6$ , were stably transfected with  $\beta 6$  in antisense orientation.  $\beta 6$  cDNA was excised from the vector pcDNA1neo $\beta 6$  and re-ligated into the vector pEF.PGK.puro (a gift from D Huang, The Walter and Eliza Hall Institute, Melbourne, Australia), which uses the promoter from the human polypeptide chain elongation factor-1 $\alpha$ . Orientation of  $\beta 6$  in pEFpuro was confirmed by sequence analysis and stable transfectants were selected continuously in puromycin (WiDr, 1  $\mu$ g/ml; HT29, 2.5  $\mu$ g/ml). Mock transfectants were transfected with vector alone. Three mock and three antisense  $\beta 6$  transfected clones were established from the WiDr cell line. The  $\beta 6$  cytoplasmic domain deletion mutant transfected into the SW480 cell line has been previously reported (Cone *et al.*, 1994) and was a gift from D Sheppard (Lung Biology Centre, University of California, San Francisco, CA, USA).

### FACScan and $\beta 6$ transcript analysis

FACScan analyses with anti-integrin antibodies were performed as previously described (Niu *et al.*, 2001). For  $\beta 6$  Northern analyses, a  $\beta 6$  probe was prepared by restriction endonuclease digest of full-length  $\beta 6$  from pcDNA1neo $\beta 6$  followed by restriction digest of the  $\beta 6$  insert with SSP1. The larger of the two fragments obtained (1700 bp) was labelled with  $\alpha^{32}$ P-dCTP using a Prime It II Random Primer Labelling kit (Stratagene, La Jolla, CA, USA). A total cellular RNA of 2–3  $\mu$ g was separated on a 1% agarose gel containing formaldehyde and transferred to Hybond N<sup>+</sup>

membrane. Following hybridization with the  $\beta 6$  probe and autoradiographic development, membranes were stripped and rehybridized with a radiolabelled DNA probe for GAPDH.

#### Tumorigenicity assay

The ability of human colon cancer cells to form tumours in immuno-deficient athymic nude mice (Animal Resource Centre, Perth, Western Australia) was assessed by measuring length and breadth of tumours during a 6-week period following subcutaneous inoculation of  $10^6$  viable tumour cells suspended in  $100 \mu\text{l}$  of standard complete culture medium (DMEM). Formalin-fixed sections of tumours were excised after 1 week and stained with haematoxylin-eosin.

#### Western blotting and immunoprecipitation

Cells were cultured as adherent monolayers in plastic tissue culture flasks in complete DMEM before being serum starved for 24 h prior to addition of either FCS (10% for 30 min) or EGF (50 ng/ml for 10 min). Cells were then recovered using Trypsin/EDTA, the Trypsin neutralized 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  $\text{CaCl}_2$ , 1% Triton X-100, 0.1% SDS, 0.1% NP-40, 1 mM vanadate, 1  $\mu\text{g/ml}$  pepstatin, 1 mM PMSF, 5  $\mu\text{g/ml}$  aprotinin and 1  $\mu\text{g/ml}$  leupeptin). Lysates were stood at  $4^\circ\text{C}$  for 30 min and clarified at  $10000 g$  for 10 min at  $4^\circ\text{C}$  to remove detergent-insoluble material. Soluble lysate was recovered and used in all subsequent analyses. Integrin immunoprecipitations either before or after ERK immunodepletion 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 non-reducing conditions. Separated proteins were electrophoretically transferred to nitrocellulose membrane and immunoblotted with either a monoclonal antibody which recognizes phosphorylated ERK1/2 (mAb E10, New England BioLabs, Beverly, MA, USA), both phosphorylated and non-phosphorylated ERK1/2 (mAb SC1647; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-biotin mAb (Sigma). Where comparative immunoprecipitations were carried out protein concentrations of detergent soluble cell lysates were determined (BCA Protein Assay Kit, Pierce, Rockford, IL, USA) and protein concentrations made equivalent (1 mg/ml of protein) with complete lysis buffer before immunoprecipitation. All lysates were then pre-cleared with rabbit-anti-mouse Ig coupled to Sepharose CL-4B beads. Anti-beta integrin antibody concentrations against individual  $\beta$  subunits were optimized to permit capture of approximately equivalent amounts of specific target protein. In ERK immunodepletion experiments six rounds of ERK immunodepletions were performed using mAb SC1647 which recognizes both phosphorylated and non-phosphorylated ERK1/2. Preliminary experiments showed that six sequential immunoprecipitations completely removed all soluble ERK from the lysate (data not shown).

#### MAP kinase assay

Cultures of WiDr and HT29 mock and antisense  $\beta 6$  transfectants were established by seeding  $1 \times 10^6$  cells/5 ml of complete culture medium in  $25 \text{ cm}^2$  tissue culture flasks. Cells were incubated at  $37^\circ\text{C}$  in humidified  $\text{CO}_2$  for 24 h

before serum starvation in serum-free medium for the next 16 h. FCS was then added to a final concentration of 10% for 30 min and the cells then washed twice with ice cold PBS before resuspension in extraction buffer (10 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 2 mM DTT, 1 mM orthovanadate, 1 mM PMSF, 4  $\mu\text{g/ml}$  aprotinin, 2  $\mu\text{g/ml}$  leupeptin and 1  $\mu\text{g/ml}$  pepstatin, pH 7.4). The cell suspension was sonicated on ice at a setting of 7 using a Soniprep 150 watt ultrasonic disintegrator using three 30 s pulses with an interval of 30 s between each pulse. Cellular debris was removed by centrifugation at  $900 g$  for 10 min at  $4^\circ\text{C}$ . A MAP kinase assay was performed on sonicates from equal cell numbers using a MAP kinase assay system (Amersham Pharmacia Biotech). The ability of cell-derived sonicates to transfer phosphate from [ $\gamma$ - $^{32}\text{P}$ ]ATP to a synthetic peptide containing a p42/p44 MAP kinase specific phosphorylation site was quantitated as described in the manufacturer's instructions. Briefly,  $^{32}\text{P}$ -labelled peptides were spotted onto PE1-cellulose paper, non-peptide bound radioactivity removed by washing with 75 mM phosphoric acid and bound  $^{32}\text{P}$ -labelled peptides measured by liquid scintillation counting. Protein estimation was performed on each cell sonicate used and enzyme activity calculated as described in the manufacturer's instructions.

*In vitro* MAP kinase activity assays were also performed using a non-radioactive assay kit (Cell Signaling Technology, Beverly, MA, USA) which measures phosphorylation of the ERK1/2 substrate Elk-1 presented as a fusion protein comprising the Elk-1 codons 307-428 coupled to GST. In this assay ERK kinase was first immunoprecipitated from complete or integrin-immunodepleted soluble cell lysates and relative enzyme activity determined by the *in vitro* phosphorylation of GST-Elk-1.

#### Enzyme-linked immunosorbent assay (ELISA)

Biotin-coupled synthetic peptide fragments corresponding to regions of the  $\beta 6$  cytoplasmic domain (amino acids 737–788) were purchased from Auspep (Melbourne, Australia). A serine residue was substituted for the C-terminal cysteine in  $\beta 6$  to avoid dimerization during synthesis of the 20 mer  $\beta 6$  peptide fragments but not the 52 mer full-length  $\beta 6$  cytoplasmic domain. ERK2 was expressed as a GST-fusion protein in the pGEX 4T vector (from C Marshall, Institute of Cancer Research, London, UK). The fusion protein (GST-ERK2) was cleaved using thrombin to cleave GST and GST (both free and remnant uncleaved fusion protein) removed by clearance with glutathione-agarose. ERK2 purity was routinely assessed by silver stained SDS-PAGE. Biotinylated peptides were attached onto streptavidin-coated 96-well polystyrene plates obtained from Pierce (Rockford, IL, USA, Cat. No. 15125) and ELISAs performed according to the manufacturer's instructions. The efficiency of binding of ERK2 to immobilized peptides was determined using anti-ERK antibody (SC1647, Santa Cruz, CA, USA) followed by an anti-species alkaline phosphatase conjugated antibody and colourimetric development. In competition assays, ERK2 was coated directly onto plastic ELISA plates (Nunc) and the binding of a biotinylated peptide determined in the absence/presence of the competing non-biotinylated peptide using anti-biotin antibody (Sigma-Aldrich).

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