

# The $\alpha v \beta 6$ Integrin Promotes Proliferation of Colon Carcinoma Cells through a Unique Region of the $\beta 6$ Cytoplasmic Domain

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**Abstract.** Cell-matrix interactions are assumed to be important in regulating differentiation and tumor cell growth; however, the precise roles of individual matrix receptors in producing cellular responses are still unclear. We have previously described the  $\alpha v \beta 6$  integrin, an epithelial cell fibronectin receptor expressed in many carcinoma cell lines. Here we show that heterologous expression of  $\alpha v \beta 6$  in a human colon carcinoma cell line (SW480) enhances the proliferative capacity

of these cells, both in vitro and in vivo in nude mice. This property of  $\alpha v \beta 6$  correlates with the presence of an 11-amino acid region at the COOH terminus of the  $\beta 6$  cytoplasmic domain. This 11-amino acid sequence is required for the growth stimulatory effect, but not for other functions of the  $\beta 6$  cytoplasmic domain, such as promoting cell adhesion and focal contact localization.

**I**NTEGRINS are heterodimeric cell adhesion receptors composed of two subunits ( $\alpha$  and  $\beta$ ), both of which contain a large extracellular domain, a transmembrane domain and a short, COOH-terminal cytoplasmic domain (Hynes, 1992). At least 20 different integrins are known to interact with a variety of extracellular matrix components, including fibronectin, vitronectin, collagens, and laminins. Integrins have been shown to influence a wide array of cellular functions, presumably as a result of interactions between their cytoplasmic domains and intracellular signaling molecules.

The  $\beta$  subunit cytoplasmic domain is required for linking integrins to the cytoskeleton (Hynes, 1992). In many cases, this linkage is reflected in localization to focal contacts, which is believed to lead to the assembly of signaling complexes that include  $\alpha$ -actinin, talin, and the focal adhesion kinase (p125-FAK) (Guan and Shalloway, 1992; Kornberg et al., 1992; Otey et al., 1990). At least three different regions (Reszka et al., 1992) that are required for focal contact localization of  $\beta 1$  integrins have been delineated. These regions contain conserved sequences that are also found in the cytoplasmic domains of the  $\beta 2$ ,  $\beta 3$ ,  $\beta 5$ ,  $\beta 6$ , and  $\beta 7$  integrin subunits. The functional differences between these cytoplasmic domains with regard to their signaling capacity have not yet been established.

The  $\alpha v \beta 6$  integrin is a fibronectin receptor expressed predominantly by epithelial cells (Breuss et al., 1993). In

healthy adult primate tissues,  $\beta 6$  mRNA and protein are rarely detected (Breuss et al., 1993, and unpublished data). In contrast,  $\beta 6$  is expressed during fetal development, wound healing, and in a variety of epithelial tumors (unpublished data). These observations suggested a possible distinct role for this receptor in epithelial cell migration or proliferation.

In the present study, we heterologously expressed the  $\beta 6$  subunit in a colon carcinoma cell line, SW480, from which it is normally absent, and examined the effects of  $\alpha v \beta 6$  on a number of aspects of cell behavior. Expression of this receptor conferred on these cells an enhanced capacity to proliferate. By performing similar experiments with a series of cytoplasmic domain truncation mutants, we identified an 11-amino acid COOH-terminal region, unique to the  $\beta 6$  subunit, that is required for the proliferation-enhancing activity of the  $\alpha v \beta 6$  integrin.

## Materials and Methods

### Antibodies and Reagents

Monoclonal antibodies, E7P6 and R6G9, directed to the extracellular domain of human  $\beta 6$ , were prepared and characterized in our laboratory, as previously described (Weinacker et al., 1994). Monoclonal antibody L230 against  $\alpha v$  integrins was prepared in our laboratory from hybridoma cells obtained from the Amer. Type Culture Collection (Rockville, MD) (Weinacker et al., 1994). Monoclonal antibodies PIH5 against  $\alpha 2$ , and P3D10 against  $\alpha 5$  were gifts from Elizabeth Wayner (University of Minnesota, Minneapolis, MN). Antibodies PIB5, against  $\alpha 3$ , and JIB5, against  $\alpha 6$ , were obtained from Telios (San Diego, CA), and antibody 9G2 against  $\beta 3$  was a gift from Jin Kim (Genentech, South San Francisco, CA). Monoclonal antibody to vinculin was obtained from Sigma Chem. Co. (St. Louis, MO). Biotinylated sheep anti-mouse IgG and streptavidin-fluorescein were obtained from Amersham Corp. (Arlington Heights, IL). Enzymes BstEII, XbaI, NotI, and XhoI were purchased from Promega Corp. (Madison, WI).

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Geneticin (neomycin analog G418), type I collagen, and BSA were purchased from Sigma Chem. Co. Fibronectin and vitronectin was prepared from human plasma as described (Engvall and Ruoslahti, 1977; Yatohgo et al., 1988). Cells were grown in DMEM supplemented with 4.5 gm/liter glucose, L-glutamine, 10% FBS and penicillin/streptomycin, unless stated otherwise.

### Preparation of Expression Plasmids

A cDNA containing the entire coding region of human  $\beta 6$  in the mammalian expression vector pcDNAIneo (Invitrogen, San Diego, CA) was constructed as previously described (Weinacker et al., 1994). To construct truncated versions of pcDNAIneo $\beta 6$  that lacked selected portions of the cytoplasmic domain, cDNA fragments were amplified by PCR using a 5' upstream primer corresponding to nt 2050-2070 and 3' downstream primers at the following positions: (1) nt 2534-2557 to prepare pcDNAIneo $\beta 6$ (777t); (2) nt 2516-2536 to prepare pcDNAIneo $\beta 6$ (770t); (3) nt 2447-2467 to prepare pcDNAIneo $\beta 6$ (747t); and (4) nt 2417-2440 to prepare pcDNAIneo $\beta 6$ (738t). The full-length plasmid, pcDNAIneo $\beta 6$ , was used as a template. The 3' primers introduced stop codons to replace amino acids E778, N771, E748, and R739, respectively, and each primer included an XbaI recognition sequence to facilitate ligation into the unique XbaI site in the pcDNAIneo polylinker. Each PCR fragment included a unique BstEII site in  $\beta 6$  (nt 2087). The PCR products were digested with XbaI and BstEII and ligated into pcDNAIneo $\beta 6$  that had been digested with the same enzymes (Cone et al., 1994). The authenticity of each mutant clone was confirmed by dideoxy sequencing using Sequenase 2.0 (Un. States Biochemical Corp., Cleveland, OH).

### Transfection of Mammalian Cells

SW480 colon carcinoma cells obtained from the Amer. Type Culture Collection and the adenovirus-transformed human embryonic kidney cell line 293 (obtained from Sarah Bodary, Genentech) were transfected with pcDNAIneo constructs containing wild-type or mutant forms of human  $\beta 6$ , or the expression plasmid only (pcDNAIneo) by the calcium phosphate precipitation method in which the calcium phosphate/DNA precipitates were added to  $10^6$  suspended cells that had been removed from tissue culture dishes by trypsinization (Sambrook et al., 1989). After transfection, the cells were grown for two days in their usual medium, and then transfectants were selected by growth in the same medium supplemented with the neomycin analog, G418. Individual transfected clones were prepared by limiting dilution cloning in 96-well plates. Stably transfected cells were further cotransfected with expression plasmids containing cDNA encoding firefly luciferase (pRkluc) and a similar plasmid containing a hygromycin resistance gene (pRkhyg) (Crowley et al., 1993), and transfectants were selected by growth in both G418 and hygromycin. pRkluc and pRkhyg were gifts from Robert Cohen (University of California, San Francisco, CA). Transfected clones were analyzed with a Luciferase Assay System (Promega), and light intensity was measured with a scintillation counter (Nguyen et al., 1988) (LS 5000, Beckmann, Mountain View, CA). The luciferase activity per cell of mock, full-length  $\beta 6$ , and truncated  $\beta 6$  transfectants selected for use in *in vivo* experiments did not differ by more than 6%. Luciferase activity of each clone was measured on at least two occasions on at least three months apart, and remained essentially constant over that time period.

### Fluorescence-activated Cell Sorting

$\beta 6$ -transfected cells were sorted by flow cytometry in the following manner: Transfected cells were allowed to grow in selection medium to ~60–80% confluence in 100-mm tissue culture plates. The cells were then harvested by trypsinization and blocked with normal goat serum at 4°C for 10 min. Cells were washed once with PBS, incubated with primary antibody E7P6 for 20 min at 4°C, and then washed twice with PBS. Cells were then stained with secondary antibody conjugated with phycoerythrin (Boehringer Mannheim, Indianapolis, IN) for 20 min at 4°C, washed twice with PBS, and resuspended in 0.5 ml of PBS with 5% normal goat serum. The stained cells were sorted using FACS 440 (Becton Dickinson, Rutherford, NJ). The cells in the upper tenth percentile of fluorescence intensity were collected and grown in selection medium. Sorted transfectants were periodically analyzed for  $\alpha\beta 6$  expression using the labeling method described above and comparison and fluorescence intensity with wild-type SW480 cells as negative control using FACScan (Becton Dickinson). Expression of the integrin subunits  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 5$ ,  $\alpha 6$ ,  $\beta 1$ ,  $\beta 3$ , and  $\beta 5$  was also analyzed for selected transfectants using monoclonal antibodies PIH5, PIB5, P3D10, JIB5, P5D2, 9G2, and PIF6, respectively.

### Cell Proliferation Assays

Bilayer collagen gels comprising a 500- $\mu$ l cell-free underlayer and a 500- $\mu$ l upper cell-containing layer were prepared in 24-well tissue culture plates. Cell-free base layers consisted of equal volumes of collagen type I (Sigma, 3.0 mg/ml in 40 mM acetic acid) and a 2 $\times$  concentrate DMEM without FCS. The pH of the mixture was adjusted to 7.4 by addition of 1 M sodium hydroxide and the collagen-medium mix then supplemented with 5% FCS. For the upper collagen layer, harvested cells suspended in FCS were added to the collagen-medium mix. After gelation, 500  $\mu$ l of culture medium (containing 5% FCS) were added to each well. In all experiments, triplicate gels were prepared containing  $10^4$  cells and incubated at 37°C in 5% CO<sub>2</sub> for 7 d. At the termination of experiments, the collagen matrix was dissolved with collagenase (Sigma Chem. Co., Cat. No. C5138, 15 mg/ml in PBS) and total cell number per well estimated by counting cells in a hemocytometer. For antibody inhibition experiments, antibodies were added directly to the collagen medium mix before gelation, and were added to the overlying culture medium 4 d after gelation.

To analyze proliferation in standard culture dishes, 6-well plates were coated with fibronectin (10  $\mu$ g/ml) or collagen I (10  $\mu$ g/ml), and then blocked with 1% BSA. Cells plated in duplicate at a density of  $5 \times 10^3$  cells/well, were allowed to proliferate in DMEM supplemented with selenium (5 ng/ml), hydrocortisone (1  $\mu$ g/ml), insulin (5  $\mu$ g/ml), transferrin (5  $\mu$ g/ml), and nonessential amino acids (ICN Biomedicals, Irvine, CA), in the presence or absence of 5% FCS. On days 1 and 3 after plating, duplicate wells were labeled with [<sup>3</sup>H]thymidine for 4 h, cells were washed twice with cold PBS, and incorporated label was precipitated with 15% TCA. Precipitates were washed twice with cold 70% ethanol to remove unincorporated label, resuspended in 1 M NaOH, and neutralized with 1 M HCl. Thymidine incorporation was quantified by scintillation counting (Beckmann LS5000). In parallel triplicate wells, cells were removed and counted at each time point without labeling.

### Cell Adhesion Assays

Cell adhesion assays were performed as previously described (Busk et al., 1992). Briefly, wells of non-tissue culture-treated polystyrene 96-well flat-bottom microtiter plates (Linbro/Titertek, Flow Laboratories, McLean, VA) were coated with various concentrations of fibronectin, vitronectin, or type I collagen, washed with PBS, and then blocked with 1% BSA in serum-free DMEM. Cells were incubated in the presence or absence of the  $\beta 1$  blocking antibody P5D2 for 15 min at 4°C and were then plated onto the microtiter plates at a density of 50,000 cells/well in 200  $\mu$ l of serum-free DMEM containing 0.05% BSA. The plates were centrifuged (top side up) at 10 g for 5 min, and then incubated for one hour at 37°C in humidified 5% CO<sub>2</sub>. Non-adherent cells were removed by centrifugation top side down at 48 g for 5 min. The attached cells were fixed with 1% formaldehyde and stained with 0.5% crystal violet, and then the wells were 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 Labs., Hercules, CA). The data were expressed as the mean of the absorbance of triplicate wells. Measurements of absorbance were determined to be linearly related to input cell number over the range of 10,000–50,000 cells/well.

### Immunofluorescent Localization

Cells were harvested by trypsinization before reaching 75% confluence. 1–3  $\times 10^4$  cells were plated onto sterile acid-washed 12-mm-diam glass coverslips that were coated with fibronectin (10  $\mu$ g/ml) and blocked with 1% BSA in PBS. Coverslips were incubated for 4 h at 37°C in 5% CO<sub>2</sub>. Cells were rinsed briefly in PBS, fixed with 2% paraformaldehyde (Fisher Scientific, Pittsburgh, PA) in PBS (pH 7.4) for 10 min, incubated for 20 min in 0.1% Triton X-100 in PBS, and processed for immunofluorescence microscopy as follows: cell monolayers were blocked for 20 min in 1% BSA. Cells were incubated for 60 min in primary antibody; and then for 30 min in biotinylated sheep anti-mouse IgG (1:50); and finally for 30 min in streptavidin-fluorescein (1:100). Coverslips were briefly rinsed in distilled water and mounted on microscope slides using Fluoromount G (Fisher Scientific).

### Tumor Growth and Metastasis In Vivo

Luciferase-transfected SW480 cells in log phase growth were removed from tissue culture plates with 0.05% trypsin/EDTA, and cell counts and viability were assessed by counting cells stained with 0.4% trypan blue in a hemocytometer. Only cell preparations demonstrating greater than 95%

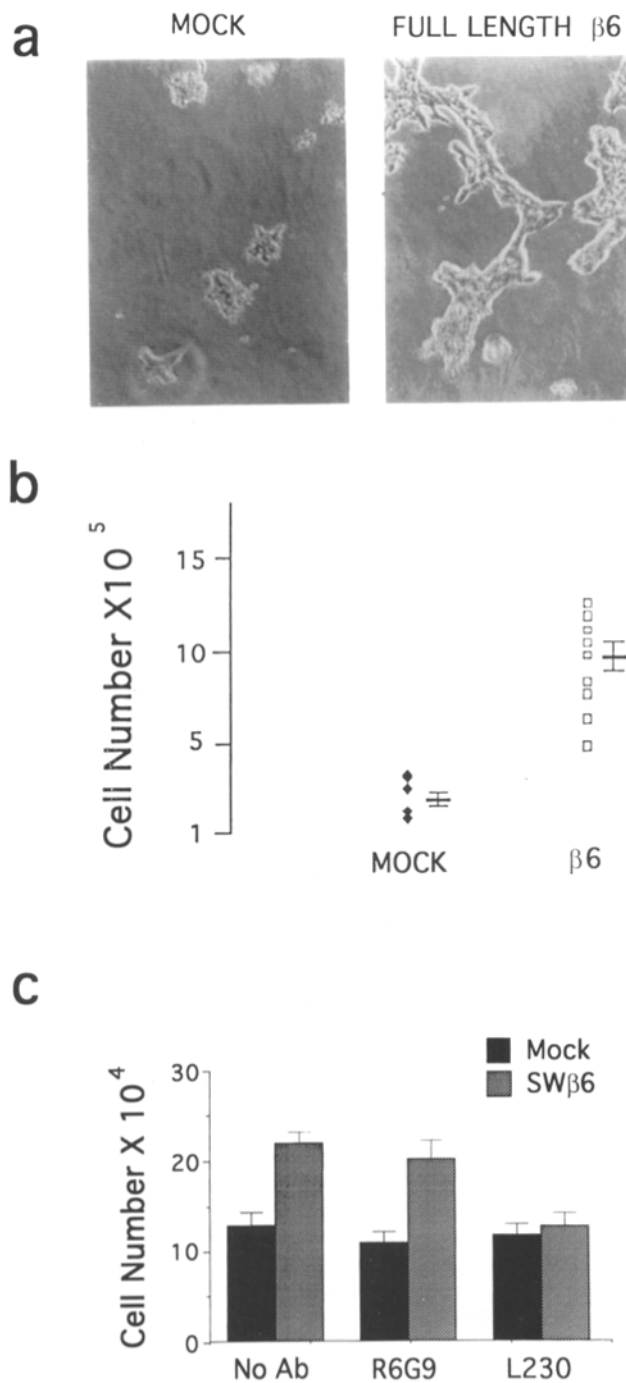
viability were used for injection into mice. Pelleted cells were suspended in BSS, and either  $10^6$  or  $5 \times 10^6$  cells in  $50 \mu\text{l}$  were injected through a 27-gauge needle into the subcutaneous region of the right flank of 8-wk-old Balb C/nude athymic male mice (Simonsen, S., San Francisco, CA). In additional experiments, tumor cells were either injected directly into the spleen, or into the wall of the cecum. Primary tumors, as well as liver, lung, heart, cecum, brain, and femur were removed after either 6 wk (for animals injected with  $5 \times 10^6$  cells) or 9 wk (for animals injected with  $10^6$  cells). The samples were frozen in liquid nitrogen, and the entire tissue was pulverized with a Bessmer tissue pulverizer (Fisher Scientific), and then incubated with 1 ml of Luciferase Assay lysis buffer (Promega) for one hour at room temperature. Lysates were centrifuged at 13,000 rpm for 20 min,  $10 \mu\text{l}$  of supernatant was added to  $100 \mu\text{l}$  of luciferin substrate, and luminescence was measured immediately in a luminometer (model 20e, Turner Designs, Sunnyvale, CA) equipped with a 0.25% neutral gray filter. Luciferase activity was determined to be linearly related to cell number over the range of  $10^3$ – $10^8$  cells/assay using this technique.

## Results

### Effects of $\beta 6$ Expression on Proliferation in Collagen Gels

To examine the effects of  $\alpha v\beta 6$  on epithelial cell behavior, we stably transfected the human colon carcinoma cell line, SW480, with full-length  $\beta 6$  cDNA. We have previously reported that transfection of these cells with  $\beta 6$  alone results in surface expression of  $\alpha v\beta 6$  and increased capacity to attach to fibronectin-coated culture dishes (Weinacker et al., 1994). Wild-type SW480 cells migrate poorly on fibronectin, and efficiently form colonies in soft agar. These properties were not affected by heterologous expression of  $\alpha v\beta 6$  (data not shown). However, in a three-dimensional collagen gel, non-transfected or vector only transfected cells grew poorly, whereas  $\beta 6$ -transfected cells formed large branching colonies (Fig. 1 a). This difference in colony morphology was accompanied by a significant difference in cell number. To confirm that this proliferative effect was a direct consequence of  $\beta 6$  expression, and not simply a function of clonal variation, we repeated these experiments with a series of mock and  $\beta 6$ -transfected clones. A total of 14 clones were established (five mock and nine  $\beta 6$  transfectants) and enhanced in vitro growth was observed for all nine  $\beta 6$  clones tested compared with the five mock-transfected clones (Fig. 1 b). Incorporation of the anti- $\alpha v$  blocking antibody L230 (Weinacker et al., 1994) into the collagen gel had no effect on the proliferation of mock-transfected SW480 cells, but abolished the increase in proliferation in  $\beta 6$ -transfected cells (Fig. 1 c), providing further evidence that the increased proliferation of  $\beta 6$  transfectants was a direct result of  $\alpha v\beta 6$  expression.

The nine  $\beta 6$ -transfected clones expressed similar amounts of  $\beta 6$  on their surface (expression varied by only threefold based on mean fluorescence intensity determined by fluorescence-activated cell analysis).  $\beta 6$  expression in these transfectants was similar to the level we have previously described for native expression in the pancreatic carcinoma cell line FG (Weinacker et al., 1994). Over this narrow range of expression, we were unable to detect any quantitative relationship between the level of  $\beta 6$  expression and the magnitude of the proliferative response. To determine whether heterologous expression of  $\beta 6$  affected the expression of other integrin subunits, we analyzed surface expression of  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 5$ ,  $\alpha 6$ ,  $\beta 1$ ,  $\beta 3$ ,  $\beta 5$ , and  $\beta 8$  in three mock and three  $\beta 6$ -transfected clones by fluorescence-activated cell analysis. As



**Figure 1.** Effect of  $\beta 6$  transfection on the ability of SW480 cells to proliferate within three-dimensional collagen type I gels. (a) Photomicrograph of mock and full-length  $\beta 6$  transfectants after 7 d in collagen gels. (b) Total cell counts for each of five mock clones and nine full-length  $\beta 6$  clones. Cells were removed from the gel by treatment with collagenase and counted in a hemocytometer. Bars to the right of each set of individual data points describe the mean  $\pm$  standard error. (c) Effect of blocking antibody against  $\alpha v$  (L230) on total cell counts of mock and  $\beta 6$ -transfected cells allowed to proliferate for 7 d in collagen gels. The non-blocking anti- $\beta 6$  antibody, R6G9, was used as a control (Weinacker et al., 1994). Shaded bars represent mean values of triplicate wells and lines above each bar represent the standard error of the mean. The results shown are representative of three similar experiments.

**Table I. Expression of Various Integrin Subunits (as Measured by Mean Fluorescence Intensity Using FACS) of Mock- and  $\beta$ -transfected SW480 Clones**

	$\alpha$ 2	$\alpha$ 3	$\alpha$ 5	$\alpha$ 6	$\beta$ 1	$\beta$ 3	$\beta$ 5	$\beta$ 6
MOCK								
C2	420	1301	187	159	1596	9	410	6
F3	517	1196	450	446	1928	9	961	9
F11	512	1229	274	407	1487	9	924	8
SW $\beta$ 6								
C7	700	910	353	353	1775	10	483	318
B2	511	910	290	290	1540	15	411	196
G2	440	1684	638	638	2073	11	412	522

shown in Table I, there was some clonal variation in expression of other integrins among transfected SW480 cell clones, but no consistent difference in expression of  $\alpha$ 2,  $\alpha$ 3,  $\alpha$ 5,  $\alpha$ 6, or  $\beta$ 1. None of the clones expressed significant amounts of  $\beta$ 3. There was a tendency for lower expression of  $\beta$ 5 in  $\beta$ 6-transfected clones, perhaps reflecting competition for endogenous  $\alpha$ v. This effect is not likely to explain the observed differences in proliferation, however, since mock clone C2

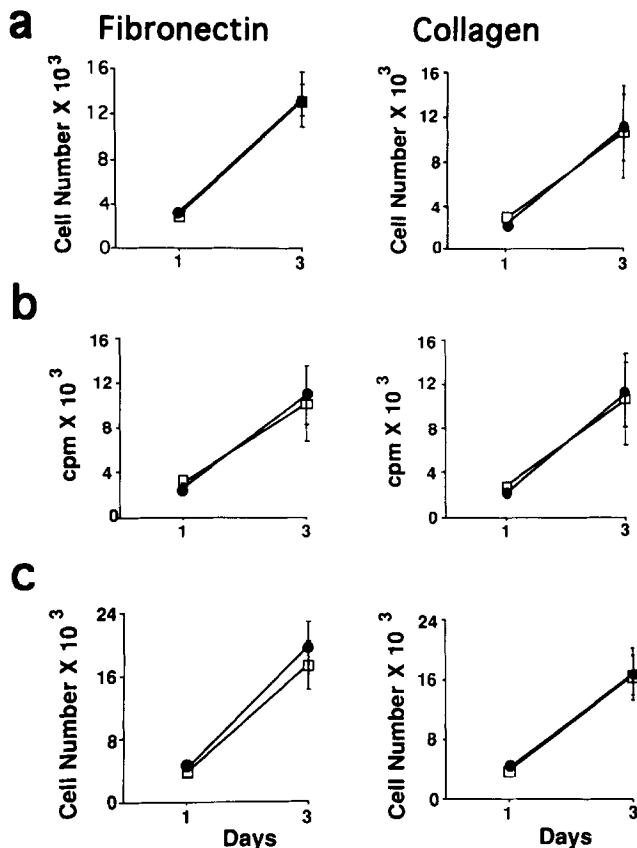
expressed the same level of  $\beta$ 5 as the three  $\beta$ 6 transfectants studied, but did not exhibit enhanced proliferation.

#### Effects of $\beta$ 6 Expression on Cell Proliferation in Two-Dimensional Culture

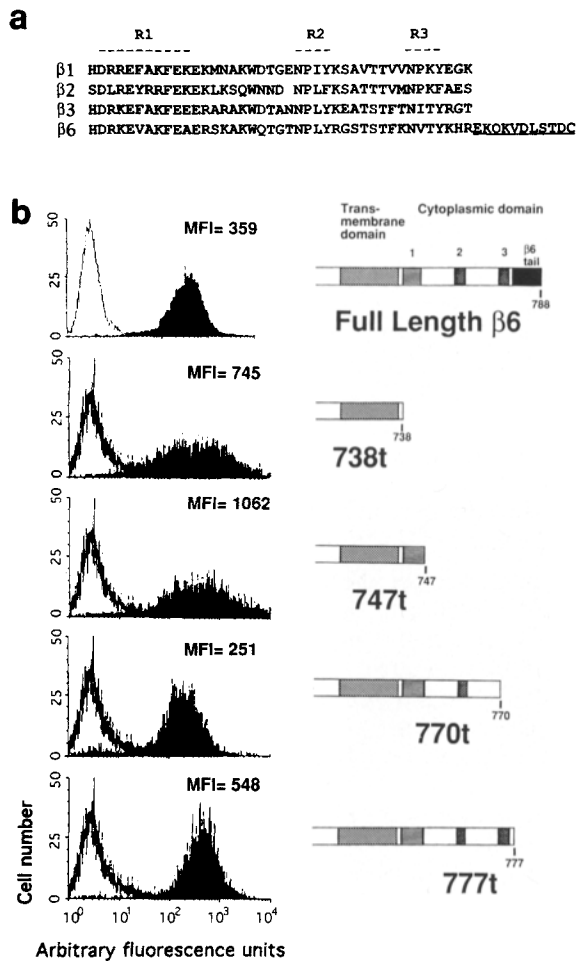
To determine whether the enhanced proliferation of  $\beta$ 6-transfected SW480 cells in collagen gels required a three-dimensional culture system, we examined the proliferation of three mock- and three  $\beta$ 6-transfected clones in standard tissue culture dishes coated with either fibronectin or collagen. Because proliferation in collagen gels required the presence of 5% FCS, we performed these assays in the presence or absence of serum. As shown in Fig. 2, mock- and  $\beta$ 6-transfected cells proliferated equally well on both substrates in the presence or absence of serum. Thus, the enhanced proliferation of  $\beta$ 6-transfected SW480 cells appears to require a three-dimensional culture system.

#### Effects of Cytoplasmic Domain Deletions on $\beta$ 6-mediated Proliferation in Collagen Gels

The effect of  $\beta$ 6 expression on SW480 cell proliferation raises the question of which structural feature(s) of the  $\alpha$ v $\beta$ 6 receptor are responsible for this effect. The most obvious candidate is the  $\beta$ 6 cytoplasmic domain, which is likely to be involved in transmitting any signals unique to  $\beta$ 6. Comparison of the  $\beta$ 6 cytoplasmic domain to the well-characterized cytoplasmic domains of  $\beta$ 1 and  $\beta$ 3 reveals that the  $\beta$ 6 cytoplasmic domain contains sequences that are known to be required for focal contact localization (*overlined* in Fig. 3 a). In addition, the  $\beta$ 6 cytoplasmic domain contains an 11-amino acid COOH-terminal extension that is unrelated to sequences found in other integrins. We speculated that this 11-amino acid extension might be necessary for the proliferation-stimulating effects of  $\beta$ 6. To test this hypothesis, we generated stably transfected SW480 cells expressing a truncated  $\beta$ 6 construct lacking these 11 amino acids. Three additional mutants with more extensive cytoplasmic domain deletions were also tested. Each of the mutants was well expressed on the cell surface as shown by surface staining with the anti- $\alpha$ v $\beta$ 6 antibody E7P6 (Fig. 3 b). Heterologous expression of these mutant versions of  $\beta$ 6, like expression of the full-length version, did not produce any systematic alteration in the expression of other integrins (Table II). As shown in Fig. 4 a, deletion of the 11-amino acid extension completely abolished the ability of  $\beta$ 6 to promote SW480 cell proliferation in collagen gels. As expected, more extensive or complete deletion of the  $\beta$ 6 cytoplasmic domain similarly abolished  $\beta$ 6-enhanced proliferation.



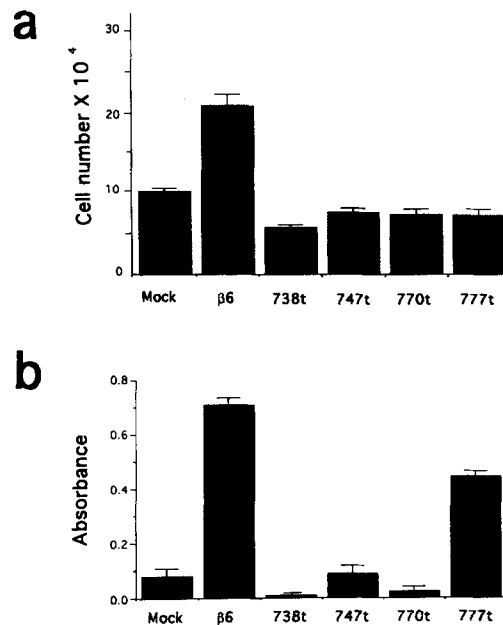
**Figure 2.** Proliferation of mock- (●) and  $\beta$ 6- (□) transfected SW480 cells in culture dishes coated with collagen or fibronectin. Cells were plated in the absence of serum (*a* and *b*) or in the presence of 5% FCS (*c*) on 6-well plates coated with 10  $\mu$ g/ml of either fibronectin (*left column*) or type I collagen (*right column*) and harvested 1 or 3 d after plating. *a* and *c* show mean ( $\pm$  standard error) data for cell counts from duplicate wells of three separate clones of each transfectant at each time point, and *b* shows mean ( $\pm$  standard error) values for [ $^3$ H]thymidine incorporation.



**Figure 3.** (a) Alignment of the cytoplasmic domains of the integrin  $\beta$  subunits,  $\beta 1$ ,  $\beta 2$ ,  $\beta 3$ , and  $\beta 6$  using the one letter amino acid code. Dashed lines above the sequences mark the three highly conserved regions (R1, R2, and R3) identified as important for localization of the  $\beta 1$  subunit to focal contacts (Reszka et al., 1992). The 11-amino acid COOH-terminal extension in  $\beta 6$  is underlined. (b) Flow cytometry analysis of SW480 cells transfected with truncated versions of the human  $\beta 6$  cytoplasmic domain. White peaks represent unstained cells. Black peaks represent cells transfected with the  $\beta 6$  truncations. The extent of the COOH-terminal truncations is shown schematically to the right of each graph. Values for mean fluorescence intensity (MFI) are shown above and to the right of each histogram.

#### Effects of Cytoplasmic Domain Deletions on $\alpha v\beta 6$ -mediated Cell Adhesion and on $\beta 6$ Localization to Focal Contacts

The effects of cytoplasmic domain deletions on cell proliferation suggest that the 11-amino acid extension of  $\beta 6$  could

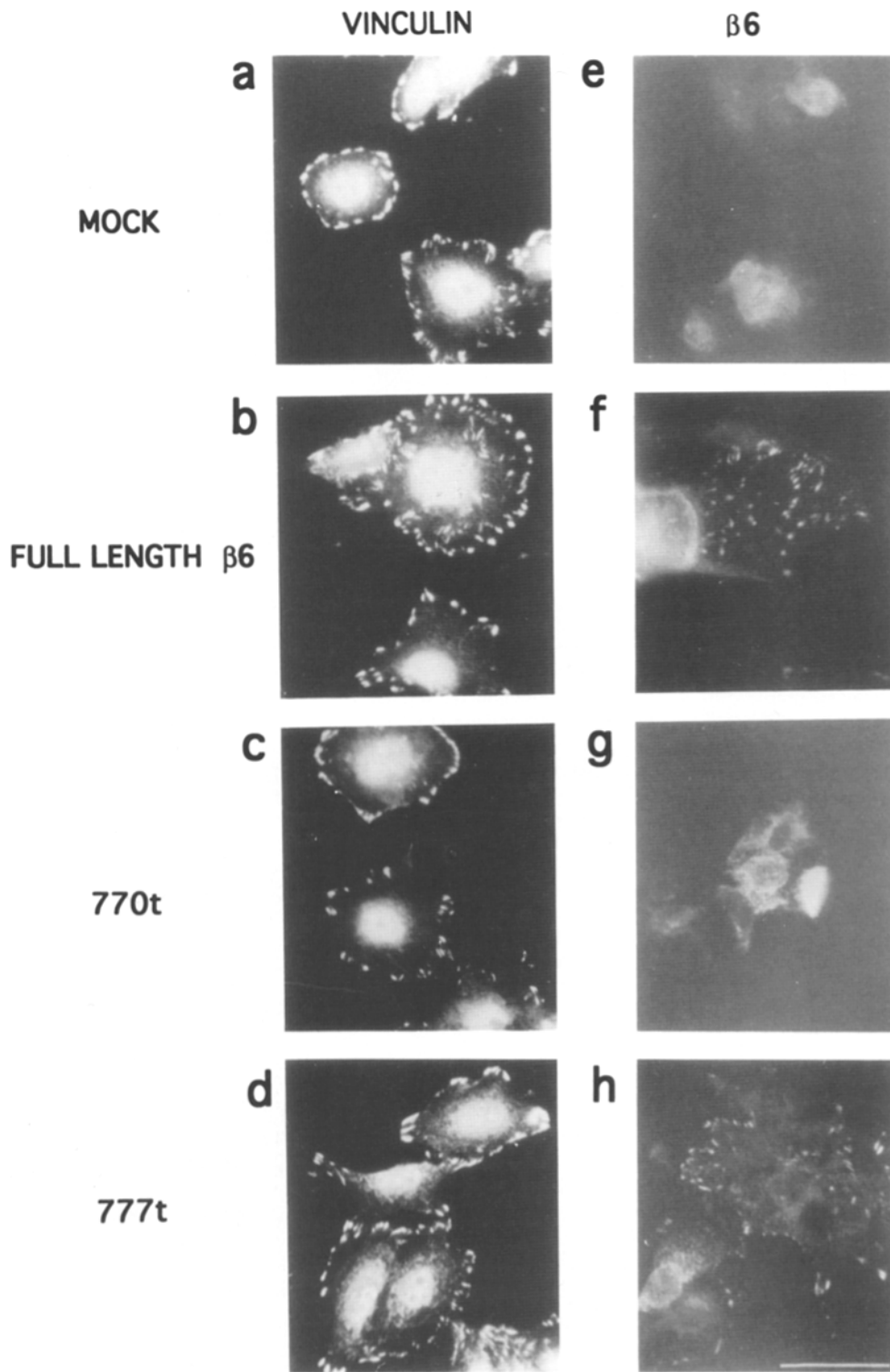


**Figure 4.** Effect of  $\beta 6$  cytoplasmic domain deletions on the ability of SW480 cells to proliferate within three-dimensional collagen type I gels (a), and on cell adhesion to fibronectin (b). (a) Triplicate gels were seeded with  $10^4$  cells for each of the transfectants and the gels dissolved seven days later with collagenase before estimating cell number in a hemocytometer. Shaded bars represent mean values from triplicate wells and lines above each bar represent standard errors. The data shown are representative of three similar experiments. (b) Mock- and  $\beta 6$ -transfected SW480 cells (full-length  $\beta 6$  and deletion mutants) were allowed to adhere to individual wells of 96-well microtiter plates which had been coated with fibronectin ( $3 \mu\text{g}/\text{ml}$ ).  $5 \times 10^4$  cells were seeded in the presence of  $\beta 1$  blocking antibody P5D2. Non-adherent cells were removed by centrifugation and the attached cells stained with crystal violet (Weinacker et al., 1994). Cell adhesion was quantitated by measuring the absorbance at 595 nm in a Microplate Reader. The data shown represent mean (+ standard error) values from triplicate wells from a single experiment.

be responsible for transmitting a proliferative signal unique to  $\beta 6$ . However, an alternative explanation would be that the 11-amino acid extension is required for the overall function of the cytoplasmic domain. To test this possibility, we analyzed the ability of the deletion mutants to perform other known functions of  $\beta 6$ , i.e., promotion of adhesion to fibronectin and localization to focal contacts. Removal of the 11-amino acid extension did not abolish the ability of  $\beta 6$  to promote adhesion to fibronectin, whereas more extensive deletions did (Fig. 4 b). Transfectants expressing the receptor missing the COOH-terminal 11 amino acids were also

**Table II.** Expression of Various Integrin Subunits (as Measured by Mean Fluorescence Intensity Using FACS) of SW480 Cells Transfected with Full-Length and Various Truncated  $\beta 6$

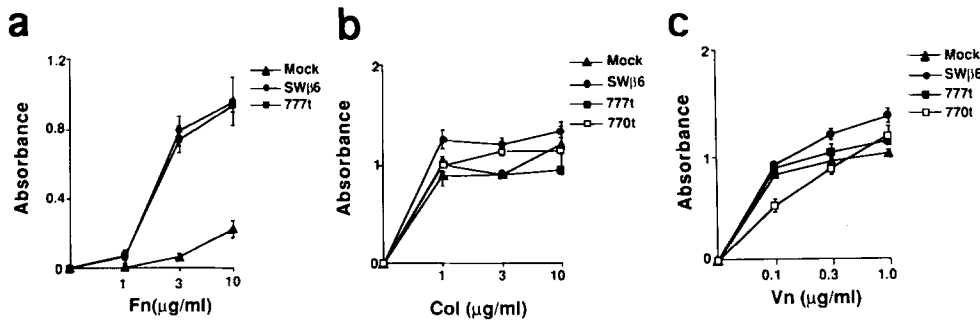
	$\alpha 2$	$\alpha 3$	$\alpha 5$	$\alpha 6$	$\beta 1$	$\beta 3$	$\beta 5$	$\beta 6$
SW $\beta 6$	540	1367	689	483	2191	7	732	359
738t	479	2158	326	300	2013	18	928	745
747t	605	1436	261	244	1846	23	594	1062
770t	348	1442	200	177	1664	10	207	251
777t	502	1677	269	246	1747	10	899	548



**Figure 5.** Effect of  $\beta 6$  cytoplasmic domain deletions on immunolocalization of the  $\beta 6$  subunit. Vinculin localization within focal adhesions is shown in *a-d* for (*a*) mock, (*b*) full-length  $\beta 6$ , (*c*) 770t, and (*d*) 777t. Localization of  $\beta 6$  to focal adhesions was seen only for full-length  $\beta 6$  and the receptor lacking the 11-amino acid COOH-terminal extension (777t) (*f* and *h*, respectively). There was no consistent difference in the spatial distribution of  $\beta 6$ -containing focal contacts between cells expressing full-length  $\beta 6$  or 777t from multiple coverslips stained on two different occasions. No  $\beta 6$  was seen in focal adhesions for mock transfectants and cells transfected with the truncation 770t (*e* and *g*, respectively).  $\beta 6$  also failed to localize to focal adhesions in cells transfected with the truncations 738t and 747t. Length of the white bar in *h* represents 20 microns.

able to spread normally on fibronectin in the presence of  $\beta 1$  blocking antibody (data not shown). Similarly, removal of the COOH-terminal 11 amino acids had no effect on localization of the receptor to focal contacts, but more extensive deletions completely abolished focal contact localization (Fig. 5). This finding is consistent with our recent observation that the COOH-terminal 11 amino acids are not required for localization of  $\beta 6$  to focal contacts in CHO cells

(Cone et al., 1994). These observations indicate that the portion of the  $\beta 6$  cytoplasmic domain that is homologous to  $\beta 1$  and to  $\beta 3$  is sufficient to support functions that are shared with  $\beta 1$  and  $\beta 3$  integrins, i.e., localization to focal contacts and promotion of cell adhesion and spreading. In contrast, a unique function of  $\beta 6$ —stimulation of cell proliferation—critically depends on the presence of the unique 11-amino acid extension.



**Figure 6.** Quantitative effects of expression of full-length or truncated  $\beta 6$  on adhesion of SW480 cells to fibronectin, vitronectin, and type I collagen. (a) SW480 cells transfected with pcDNAIneo, pcDNAIneo $\beta 6$ , or pcDNAIneo $\beta 6777t$  were allowed to adhere to individual wells of 96-well microtiter plates which had been coated with a range of concentrations of fibronectin.

5  $\times$  10<sup>4</sup> cells were seeded in the presence of  $\beta 1$  blocking antibody P5D2. The same three cell lines, as well as cells transfected with pcDNAIneo- $\beta 6770t$  were also allowed to attach to wells coated with a range of concentrations of type I collagen (b) or vitronectin (c). Non-adherent cells were removed by centrifugation and the attached cells stained with crystal violet (Weinacker et al., 1994). Cell adhesion was quantitated by measuring the absorbance at 595 nm in a Microplate Reader. The data shown represent mean ( $\pm$  standard error) values from the nine data points obtained from triplicate wells in three separate experiments in a and the three data points from triplicate wells in a single experiment in b and c.

To obtain more quantitative information about the relative ability of SW480 cells transfected with intact or truncated  $\beta 6$  cDNA to use  $\beta 6$  for attachment to fibronectin, we performed additional cell adhesion assays examining the attachment of pooled populations of SW480 cells transfected with pcDNAIneo alone, with pcDNAIneo $\beta 6$  or with pcDNAIneo $\beta 6777t$  to a range of concentrations of fibronectin. Each of these assays was performed on three separate occasions, and mean absorbance values were calculated from the nine data points obtained for each cell line at each concentration. As shown in Fig. 6 a, in the presence of  $\beta 1$ -blocking antibody, cells expressing full-length  $\beta 6$  or  $\beta 6777t$  bound equally well to all concentrations of fibronectin, whereas mock transfectants demonstrated little binding to any concentration. To determine whether heterologous expression of intact or truncated  $\beta 6$  altered the ability of SW480 cells to adhere to matrix proteins that are not ligands for  $\alpha v\beta 6$ , we also performed cell adhesion assays to type I collagen and to vitronectin with the same populations of SW480 cells, and with cells transfected with pcDNAIneo770t. As shown in Fig. 6 (b and c), all three types of transfectants attached well to type I collagen and to vitronectin, similar to the results we have previously reported for SW480 cells transfected with pcDNAIneo and pcDNAIneo $\beta 6$  (Weinacker et al., 1994).

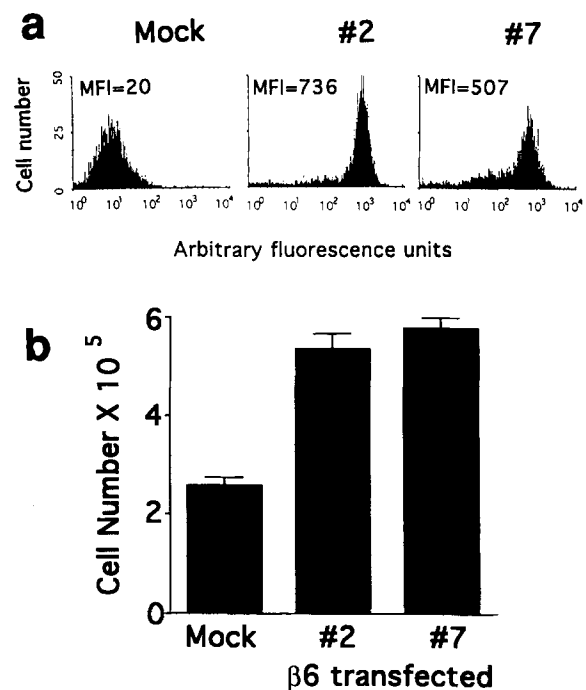
crease the growth and/or spread of tumors formed by SW480 cells in vivo, we stably transfected pools of mock-transfected SW480 cells and SW480 cells expressing either full-length or truncated  $\beta 6$  with a mammalian expression plasmid containing a full-length cDNA encoding the enzyme firefly luciferase. We then selected luciferase-transfected clones of

### Effects of $\beta 6$ Expression on the Proliferation of Embryonic Kidney Cells (293) in Collagen Gels

We were uncertain whether the proliferative effect of  $\beta 6$  was specific to colon carcinoma cells. We therefore stably transfected the human embryonic kidney cell line 293 with pcDNAIneo or pcDNAIneo $\beta 6$ , and selected one mock- and two  $\beta 6$ -transfected clones for further study. Mock-transfected cells do not express detectable amounts of  $\beta 6$ , whereas both  $\beta 6$ -transfected clones express levels of  $\beta 6$  comparable to those expressed by transfected SW480 cells (Fig. 7 a). Both  $\beta 6$ -transfected clones proliferated better in collagen gels than did the mock-transfected clone studied (Fig. 7 b), suggesting that this effect of  $\beta 6$  is not specific for SW480 cells, and can occur in non-tumor cells.

### In Vivo Proliferation of SW480 Cells in Nude Mice

To determine whether expression of  $\alpha v\beta 6$  would also in-



**Figure 7.** Proliferation of mock and  $\beta 6$ -transfected 293 cells in three-dimensional collagen gels. (a) Fluorescence-activated cell analysis histograms showing  $\beta 6$  expression (detected with monoclonal antibody E7P6) in one mock and two  $\beta 6$  transfected 293 cell clones (#2 and #7). Mean fluorescence intensity (MFI) for each clone is shown above each histogram. (b) Duplicate gels were seeded with 10<sup>4</sup> cells for each of the transfectants and the gels dissolved seven days later with collagenase before estimating cell number in a hemocytometer. Shaded bars are mean values of four data points from duplicate wells in two separate experiments, and lines above each bar represent standard errors.





studies have suggested that the  $\alpha v \beta 3$  integrin plays an important role during the invasive growth phase of malignant melanoma (Albelda et al., 1990; Boukerche et al., 1989; Felding-Habermann et al., 1992). In one of these studies, melanoma cells lacking  $\alpha v \beta 3$  were shown to produce smaller tumors in nude mice than their  $\alpha v \beta 3(+)$  counterparts (Felding-Habermann et al., 1992). Recently, the same group has shown that melanoma cells lacking  $\alpha v \beta 3$  undergo apoptosis in collagen gels in the absence of growth factors, but are protected from apoptosis by heterologous expression of  $\alpha v \beta 3$  (Montgomery et al., 1994). In that study, the authors suggested that proteinases secreted by the melanoma cells were degrading the collagen, and revealing a cryptic RGD site that was then recognized by  $\alpha v \beta 3$ . It is conceivable that the cells we studied (SW480 and 293 cells) are also capable of secreting proteinases that degrade collagen, and that  $\alpha v \beta 6$ , which also recognizes the RGD site in fibronectin (Busk et al., 1992), is thus interacting directly with degraded collagen. However, in contrast with transfected melanoma cells, we did not observe survival of transfected cells in collagen gels in the absence of serum (data not shown). Furthermore, when we transfected SW480 cells with full-length  $\beta 3$  cDNA, expression of  $\alpha v \beta 3$  did not enhance the proliferation of these cells (data not shown). Finally, since  $\beta 3$  does not contain the 11-amino acid sequence implicated in  $\beta 6$ -dependent growth regulation, it is likely that different mechanisms are involved in growth regulation by  $\alpha v \beta 6$  and  $\alpha v \beta 3$ .

In the present study, subcutaneously injected SW480 cells expressing full-length  $\beta 6$  demonstrated an increased capacity to proliferate in vivo. Because this observation was made with a single tumor cell line in a small number of mice, we cannot determine whether  $\alpha v \beta 6$  generally contributes to in vivo tumor growth.  $\beta 6$ -transfected SW480 cells did not form detectable metastases within 6–9 wk. Furthermore, injection of tumor cells directly into the spleen and implantation of tumor cells into the wall of the cecum did not produce significant numbers of metastases (data not shown). Thus, it will be necessary to examine the effects of  $\beta 6$  transfection on carcinoma cells with higher metastatic potential in order to determine the possible role of  $\alpha v \beta 6$  in tumor invasion, spread, or growth at metastatic sites.

The results of the present study do not allow us to directly determine whether the effects of  $\beta 6$  transfection on cell proliferation require the interaction of  $\alpha v \beta 6$  with specific ligand(s). Since SW480 cells themselves secrete large amounts of fibronectin, it was not possible to study the ability of these cells to proliferate in the absence of ligand for  $\alpha v \beta 6$ . Furthermore, fibronectin may not be the only ligand for this receptor. Recent studies have suggested that  $\alpha v \beta 6$  may also be a receptor for the extracellular matrix protein tenascin (Prieto et al., 1993, and unpublished data). Nonetheless, the notion that  $\alpha v \beta 6$ -ligand interaction is required for enhanced proliferation is supported by our finding that the blocking anti- $\alpha v$  antibody, L230, abolished the  $\beta 6$ -dependent increment in proliferation. Since the L230 antibody is known to block  $\alpha v \beta 6$ -mediated adhesion to fibronectin (Weinacker et al., 1994), it appears likely that this antibody inhibits proliferation by preventing  $\alpha v \beta 6$ -ligand interactions. Furthermore, we have recently constructed a mutant version of  $\beta 6$  that includes a single point mutation in an aspartic acid residue (Asp<sup>122</sup>) previously shown to be critical for interactions of  $\beta 1$  and  $\beta 3$  integrins with ligands

(Loftus et al., 1990; Takada et al., 1992). This mutant  $\beta 6$  forms a heterodimer with  $\alpha v$  that is well expressed on the cell surface, but is incapable of binding fibronectin (unpublished observations). SW480 cells expressing levels of this mutant receptor comparable to the levels of wild-type receptor in the  $\beta 6$ -transfectants described in this manuscript do not demonstrate enhanced proliferation in collagen gels (unpublished observation). It thus appears likely that ligand binding is important in the enhanced proliferation induced by  $\beta 6$ .

Our study implicates the COOH-terminal 11 amino acids of  $\beta 6$  in the generation of a signal that leads to increased proliferation of colon carcinoma cells. This 11-amino acid sequence is not required for  $\beta 6$ -mediated adhesion, cell spreading, or focal contact formation, suggesting that  $\beta 6$  enhances growth by directly generating cytoplasmic signals, and not by altering anchorage or shape of the cells. The molecular mechanisms underlying this effect remain to be elucidated. It is possible that the  $\beta 6$  COOH terminus directly interacts with a cytoplasmic signaling molecule. Alternatively, the  $\beta 6$  COOH terminus might modulate the function of another region of the  $\beta 6$  cytoplasmic domain, or of the  $\alpha v$  cytoplasmic domain, leading to altered signaling capacity. Finally, it is possible that removing the COOH-terminal 11 amino acids results in a change in conformation in some other region of  $\beta 6$  that is itself responsible for induction of the proliferative response. For example, removal of the COOH-terminal 11 amino acids could lead to a change in the conformation of the remaining conserved region proximal to the transmembrane domain. The transfectants we have generated should be an ideal system to examine these possibilities and to identify the downstream events involved in augmentation of proliferation by the  $\alpha v \beta 6$  integrin.

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