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Integrin $\alpha5\beta1$ Expression Negatively Regulates Cell Growth: Reversal by Attachment to Fibronectin

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Cells selected for overexpression of the integrin $\alpha5\beta1$ show decreased proliferation and loss of the transformed phenotype. We provide evidence that de novo expression of the integrin $\alpha5\beta1$ in HT29 colon carcinoma cells results in the growth arrest of these cells as characterized by reduced DNA synthesis and cellular proliferation in vitro. In fact, expression of integrin $\alpha5\beta1$ on these cells induces the transcription of growth arrest specific gene 1 (*gas*-1), a gene product known to induce cellular quiescence, but blocks transcription of the immediate early genes c-*fos*, c-*jun*, and *jun* B. In vivo, the $\alpha5\beta1$ transfectants display dramatically reduced tumorigenicity as well as a highly differentiated phenotype when compared with their pSVneo-transfected counterparts. Surprisingly, ligation of $\alpha5\beta1$ on these cells by cell attachment to a fibronectin substrate not only reverses the growth inhibition and *gas*-1 gene induction but activates immediate early gene transcription. These findings demonstrate that integrin $\alpha5\beta1$ expression in the absence of attachment to fibronectin activates a signaling pathway leading to decreased cellular proliferation and that ligation of this receptor with fibronectin reverses this signal, thereby contributing to the proliferation of transformed cells.

INTRODUCTION

Regulation of cellular proliferation and the cell cycle is a complex phenomena that incorporates the coordinated activities of cyclins (Nurse, 1990; Ohtsubo and Roberts, 1993), serine/threonine kinases (Kikkawa *et al.*, 1989; Norbury and Nurse, 1989; Boulton *et al.*, 1991; Koff *et al.*, 1993), growth factor receptor tyrosine kinases (Yarden and Ullrich, 1988; Ullrich and Schlessinger, 1990), *src* family tyrosine kinases (Hanks *et al.*, 1988; Cantley *et al.*, 1991), transcription factors (Cole, 1986; Rollins and Stiles, 1989; Blackwood and Eisenman, 1991; Hofbauer and Denhardt, 1991; Lewin, 1991; Smith and Prochownik, 1992), and effector molecules such as phosphatidylinositol triphosphate and calcium (Berridge, 1984, 1993). Normal cells can be induced to withdraw from the cell cycle and to become quiescent by maintenance in anchorage-independent conditions (Dike and Farmer, 1988). They are dependent on anchorage not only for growth (Dike and Farmer, 1988) but also for survival (Meredith *et al.*, 1993; Brooks *et al.*, 1994; Montgomery *et al.*, 1994). In contrast to normal cells, tumor cells and virally transformed cells are characterized by anchorage-independent growth.

Anchorage-independent growth likely results from an uncoupling of cell cycle dependence on signals transduced by attachment to the substratum (Guadagno *et al.*, 1993). Loss of the cell's normal adhesive factors may be a cause or a consequence of this alteration in cell cycle regulation. The adhesion protein fibronectin was originally characterized as "Large External Transformation Sensitive" protein (LETS), as it is lost from the surface of transformed cells (Vaheri and Ruoslahti, 1975; Mautner and Hynes, 1977). Loss of binding of fibronectin to the cell surfaces of some transformed cells (Wagner *et al.*, 1981) was later suggested to result either from a transformation-associated loss of surface expression of integrin $\alpha5\beta1$, the fibronectin receptor (Plantefaber and Hynes, 1989), or from an inactivation of the integrin $\alpha5\beta1$ via a phosphorylation event (Hirst *et al.*, 1986). That the expression and function of the integrin $\alpha5\beta1$ can actively regulate proliferation was demonstrated by a series of

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studies of tumor variants that displayed altered $\alpha5\beta1$ expression. Variants of MG63 osteosarcoma cells (Dedhar *et al.*, 1987, 1989) and K562 erythroleukemia cells (Symington, 1990) selected for increased ability to attach to fibronectin exhibited a fivefold up-regulation of $\alpha5\beta1$ expression and displayed significantly reduced growth in assays of anchorage-independent growth and tumorigenicity. Additionally, Chinese hamster ovary cells expressing 30-fold normal levels of $\alpha5\beta1$ by gene transfection displayed completely suppressed tumorigenicity and partially suppressed in vitro growth (Giancotti and Ruoslahti, 1990). In addition, total loss of integrin $\alpha5\beta1$ expression in Chinese hamster ovary cells enhances tumorigenicity (Schreiner *et al.*, 1991b). Thus, integrin $\alpha5\beta1$ expression can suppress growth in cells that overexpress it.

The mechanism by which $\alpha5\beta1$ suppresses growth remains unknown. It is known, however, that upon ligation with fibronectin, $\alpha5\beta1$ transduces signals across the membrane (Hynes, 1992; Juliano and Haskill, 1993). Among the integrin-generated signals identified to date are increases in intracellular pH (Schwartz *et al.*, 1989–1991; Schwartz and Lechene, 1992), increases in intracellular calcium (Pelletier *et al.*, 1992; Schwartz, 1992; Leavesley *et al.*, 1993), and increases in inositol lipid synthesis (McNamee *et al.*, 1993), tyrosine phosphorylation of a 125-kDa tyrosine kinase associated with focal contacts, pp125 FAK (Kornberg *et al.*, 1991; Guan and Shalloway, 1992), and activation of p34/cdc2 (Symington, 1993) and cyclin A (Guadagno *et al.*, 1993). We examined the mechanism by which $\alpha5\beta1$ negatively regulates proliferation with HT29 colon carcinoma cells transfected with the cDNA for the integrin $\alpha5$ subunit. We demonstrate that in the absence of a fibronectin substrate, $\alpha5\beta1$ transduces signals that negatively regulate cellular proliferation. This growth suppression is associated with an up-regulation of the growth arrest–inducing gene, *gas*-1 (Del Sal *et al.*, 1992, 1994) and a down-regulation of the immediate early genes c-*fos*, c-*jun*, and *jun* B.

MATERIALS AND METHODS

Transfection and Cell Culture

HT29 colon carcinoma cells were obtained from the American Type Culture Collection (Bethesda, MD) and were routinely cultured in DMEM (high glucose formulation), 10% fetal bovine serum, and gentamicin (50 μg/ml). One million HT29 cells in monolayers were washed three times with warmed DMEM without serum, and overlaid for 12 h with a 1:1 (w/w) suspension of lipofectin (Life Technologies, Gaithersburg, MD) and 40 μg of the vector pECE$\alpha5$ and 4 μg of pSVneo that had been previously linearized by digestion with the restriction enzyme *Pvu*II. The cells were cultured in complete medium for an additional 48 h, and thereafter in complete medium containing 1 mg/ml of the antibiotic G418. Antibiotic-resistant colonies were selected and clonally expanded over a 2-mo period. $\alpha5$ positive colonies were identified by flow cytometric analyses and verified by immunoprecipitation analyses. Colonies displaying identical levels of surface $\alpha5\beta1$ exhibited identical properties in all experiments. The expression vector pECE$\alpha5$ was a gift from Dr. Erkki Ruoslahti, La Jolla Cancer Research Foundation, La Jolla, CA.

Flow Cytometry

HT29 cells were harvested from tissue culture flasks with a brief trypsin-EDTA treatment, resuspended, and washed three times in cold medium containing 1% bovine serum albumin (BSA). Five hundred thousand cells were incubated for 30 min on ice in 10 μg of isotype-matched control antibodies or B1E5 (anti-$\alpha5$), AIIB2 (anti-$\beta1$), GoH3 (anti-$\alpha6$), P1H5 (anti-$\alpha2$), or P1B5 (anti-$\alpha3$) antibodies. Cells were washed three times in DMEM containing 1% BSA and incubated in fluorescein-labeled secondary antibodies for 30 min on ice. Cells were then washed three times with phosphate-buffered saline (PBS), fixed in 0.5% paraformaldehyde in PBS and analyzed by flow cytometry. Anti-human $\alpha5$ (B1E5) and anti-human $\beta1$ (AIIB2) were gifts from Dr. Carolyn Damsky, University of California at San Francisco; anti-human $\alpha2$ (P1H5) and anti-human $\alpha3$ (P1B5) were gifts from Dr. William Carter, University of Washington at Seattle; anti-human $\alpha6$ (GoH3) hybridoma supernatants were a gift from Dr. Arnould Sonnenberg, Netherlands Cancer Research Center; and anti-human αv (LM142) and $\alpha v\beta3$ (LM609) were from Dr. David Cheresh, Scripps Research Institute, La Jolla, CA.

Inhibition of Adhesion Assays

The wells of 96-well microtiter plates were coated with 10 μg/ml of fibronectin (Collaborative Biomedical Research, Becton Dickinson Labware, Bedford, MA) for 2 h at 37°C. The wells were then blocked by incubation in 3% BSA in PBS. Cells were removed from tissue culture dishes by incubation in versene, washed three times in DMEM containing 3% BSA, 2 mM $CaCl_2$, and 2 mM $MgCl_2$ at 5×10^5 cells/ml. Equal volumes of cells and antibody or peptide solutions at 2× final concentration were added to microtiter plate wells and cells were allowed to adhere for 1 h at 37°C. Wells were then washed four times with warmed adhesion buffer and rinsed once with PBS. Cells were fixed for 15 min in 0.5% paraformaldehyde in PBS, and were stained with a solution of 0.5% crystal violet in 20% methanol for 1 h at room temperature. Stained cell layers were washed with water and bound dye was solubilized with 1% sodium dodecyl sulfate in water. Absorbance was determined at 540 nm. Data is represented as percentage of total cells bound in the absence of any inhibitor. Antibodies used to inhibit adhesion are described under "Flow Cytometry." Goat anti-integrin $\alpha5\beta1$ antiserum gp 140 was developed in the laboratory of R.L. Juliano.

Adhesion Assays

The wells of 96-well microtiter plates were coated with serial dilutions of a 100 μg/ml solution of matrix protein (fibronectin, collagen, vitronectin, or laminin) for 2 h at 37°C and blocked in 3% BSA in PBS. Cells were removed from tissue culture dishes by incubation for a brief period in versene, then washed three times by centrifugation and resuspension in DMEM containing 3% bovine serum albumin, 2 mM $CaCl_2$, and 2 mM $MgCl_2$ at 5×10^5 cells/ml. Cells were added to wells of the coated plates and allowed to adhere for 60 min at 37°C. Unbound cells were removed by washing four times with warmed adhesion buffer and plates were processed as described for inhibition of adhesion assays.

Tumorigenicity Assays

Aliquots of 5×10^5 or 5×10^6 cells of HT29 parental cells, pSVneo-transfected clone 1, and $\alpha5$-expressing clones 34 and 30 were injected subcutaneously into the flanks of 10 female athymic nude mice per clone. Tumor dimensions were assessed with the aid of calipers every third day and animals were killed as tumors reached an approximate size equivalent to 1000 mm^3. Average tumor volumes = $(\text{width}^2 \times \text{length})/2$ in mm^3 were determined. The statis-

tical significance of the difference in growth between pSVneo-transfectants and $\alpha5$ transfectants was evaluated on the basis of Student's t-test.

In Vitro Growth Assays

$\alpha5$-positive and -negative pSVneo transfectants were seeded at 5×10^4 cells/ml in DMEM supplemented either with serum or, in the absence of serum, with insulin, transferrin, and selenium (ITS premix) in triplicate wells of 12-well tissue culture dishes. Some dishes were coated with matrix protein (25 μg/ml fibronectin or vitronectin in PBS for 1 h at 37°C followed by blocking in 3% BSA in PBS). Cells were cultured for up to 8 days, renewing the medium every 3 days. Total cell number was determined by removal of cells from the dishes with trypsin-EDTA and counting in an electronic particle counter (Model 80H, Particle Data, Elmhurst, IL). The mean of triplicate determinations for each day of growth and standard deviations from the mean were determined.

Thymidine Incorporation Determinations

The wells of a 96-well microtiter culture plate were coated either with no matrix protein or with 10 μg/ml solutions of fibronectin or polyL-lysine for 2 h at 37°C followed by incubation overnight at 4°C in 3% BSA in PBS. PSVneo-transfectant clone 1 and $\alpha5$-positive transfectant clone 30 were resuspended at 10^6 cells/ml after removal from tissue culture flasks with versene and washing three times in warmed serum-free DMEM medium containing 3% BSA, 2 mM $MgCl_2$, and 2 mM $CaCl_2$. Cells were resuspended in warmed serum-free DMEM containing 2 mM $CaCl_2$, 2 mM $MgCl_2$, ITS premix, and 10 μCi [^{3}H]thymidine/milliliter of culture medium. One hundred thousand cells (100 μl) were aliquoted into triplicate wells per condition and cultured at 37°C for 24 h. The culture medium was removed, cells were washed three times by centrifugation with culture medium, and acid perceptible counts were determined.

Reverse Transcriptase Polymerase Chain Reaction (PCR) Analyses

Aliquots (10^6 cells) of $\alpha5$ positive and pSVneo transfectants were removed from culture dishes, washed, and seeded in 1 ml of DMEM supplemented with serum or with insulin, selenium, and transferrin premix in wells of 12-well culture plates for 3, 7, 9, 12, 17, 24, 48, or 72 h before harvesting. Cells that had been maintained in suspension for these times were also cultured for 1–24 h on tissue culture wells that had been coated with 100 μg/ml of fibronectin, polyL-lysine, collagen, or vitronectin essentially as described for adhesion assays. RNA was isolated according to the method of Chomczynski and Sacchi (1987). First strand cDNA was synthesized by incubating up to 2 μg of RNA in 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 1.25 mM each of deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxythymidine triphosphate (dTTP), and deoxyguanidine triphosphate (dGTP), 10 pmol random hexamer primers, and 25 U of AMV reverse transcriptase in a final volume of 20 μl for 60 min at 42°C followed by 10 min at 65°C. cDNA was diluted to 100 μl with Tris-EDTA. PCR amplification of gene products was performed using 2.5 μl of first strand cDNA in a final volume of 50 μl of 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 1.25 mM each of dATP, dCTP, dTTP, and dGTP, and 0.5 μl of TAQ polymerase with 50 pmol each of forward and reverse primers for 35 cycles in a thermocycling apparatus with annealing at 56°C. Twenty percent of the PCR products was resolved by electrophoresis on 2% Tris-borate-EDTA agarose gels. Each PCR analysis was performed in duplicate on identical samples under nonsaturating conditions; each experiment was repeated at least three times. Amounts of starting material were standardized on the basis of amounts of actin product by performing successive rounds of PCR, adjusting the concentration of starting cDNA until equal amounts of actin product were obtained for each sample to be compared. The appropriate dilutions used to obtain equal amounts of actin product were used to compare PCR products for other PCR primers. The use of actin as an internal standard for PCR is well documented (Schmitt *et al.*, 1991; Lewis *et al.*, 1993). PCR primers for the growth factor messages PDGF, TNF-α, TGF-β, TGF-α, and EGF were purchased from Clontech Laboratories (Palo Alto, CA). PCR primers as follows:
for growth arrest–specific gene-1: 5′ **TGCGAATCGGTCAAAGAGAAC** 3′ and 5′ **AGCAACAGCAGCAGCAAACAG** 3′
for c-*jun*: 5′ **GGAAACGACCTTCTATGACGATGCCCTCAA** 3′ and 5′ **GAACCCCTCCTGCTCATCTGTCACGTTCTT** 3′
for c-*fos*: 5′ **GAATAAGATGGCTGCAGCCAAATGCCGCAA** 3′ and 5′ **CAGTCAGATCAAGGGAAGCCACAGACATCT** 3′
for c-*myc*: 5′ **TGTCTCAGGACTCTGACACTGTCCAACTTG** 3′ and 5′ **CTGGCAAAAGGTCAGAGTCTGGATCACCTT** 3′
for *jun* B: 5′ **CCAGTCCTTCCACCTCGACGACGTTTACAAG** 3′ and 5′ **GACTAAGTGCGTGTTTCTTTTCCACAGTAC** 3′
for histone: 5′**GAGGAAAGGGCGGAAAAGGCTTAGGCAAAG** 3′ and 5′ **CAAAAAGGGCCTTTGGGATGGAAACGTGCA** 3′
for p53: 5′ **TACAAGCAGTCACAGCACATGACGGAGGTT** 3′ and 5′ **AGTTGTAGTGGATGGTGGTACAGTCAGAG** 3′
for Harvey *ras*: 5′ **ACCATCCAGCTGATCCAGAACCATTTTGTC** 3′ and 5′ **CTGTACTGGTGGATGTCCTCAAAAGACTTG** 3′

PCR primers for gas-1 were designed with the aid of a sequence analysis program, primers for actin were the gift of Dr. Steve Haskill, and primer sequences for the other transcripts were taken from Irving *et al.* (1992). PCR product sizes were: *gas*-1 (322 bp), actin (150 bp), c-*fos* (236 bp), p53 (220 bp), c-*jun* (325 bp), *jun* B (257 bp), c-*myc* (187 bp), Hras (233 bp), and histone H4 (347 bp).

RESULTS

Expression of $\alpha5\beta1$ on HT29 Colon Carcinoma

Previous studies have demonstrated that overexpression of the integrin $\alpha5\beta1$ suppresses the proliferation of transformed cells (Dedhar, 1987, 1989; Giancotti and Ruoslahti, 1990; Symington, 1990). To examine the mechanism by which the integrin $\alpha5\beta1$ regulates proliferation, integrin $\alpha5\beta1$ negative colon carcinoma cells were co-transfected with linearized eukaryotic expression constructs containing the intact cDNA for the human integrin $\alpha5$ subunit (pECEα_5) and the neomycin resistance gene (pSVneo). Parental HT29 human colon carcinoma cells (from the Duke's stage I adenocarcinoma HT29 cell line) do not express the integrin $\alpha5\beta1$ (Schreiner *et al.*, 1991a) as assessed by flow cytometry (Figure 1A) and immunoprecipitation analyses (our unpublished observations).

Like parental HT29 cells, HT29 cells transfected only with the neomycin resistance gene (pSVneo transfectants) do not express the integrin $\alpha5\beta1$ (Figure 1B) and do not adhere to a fibronectin substratum (Figure 2A). After transfection of HT29 cells with the $\alpha5$ gene, $\alpha5\beta1$ surface expression could be demonstrated by flow cytometric analyses (Figure 1B) and immuno-precipitation analyses (our unpublished observations). Surface expression of $\alpha5\beta1$ did not significantly alter the expression of other integrin subunits, as no significant change in surface expression levels of other integrin subunits ($\beta1$, $\alpha2$, $\alpha3$, and $\alpha6$) were noted by FACS (Figure 1C) or ($\beta1$, $\alpha2$, $\alpha3$, $\alpha6$, αv, and $\beta5$) immunoprecipitation analyses (our unpublished observa-

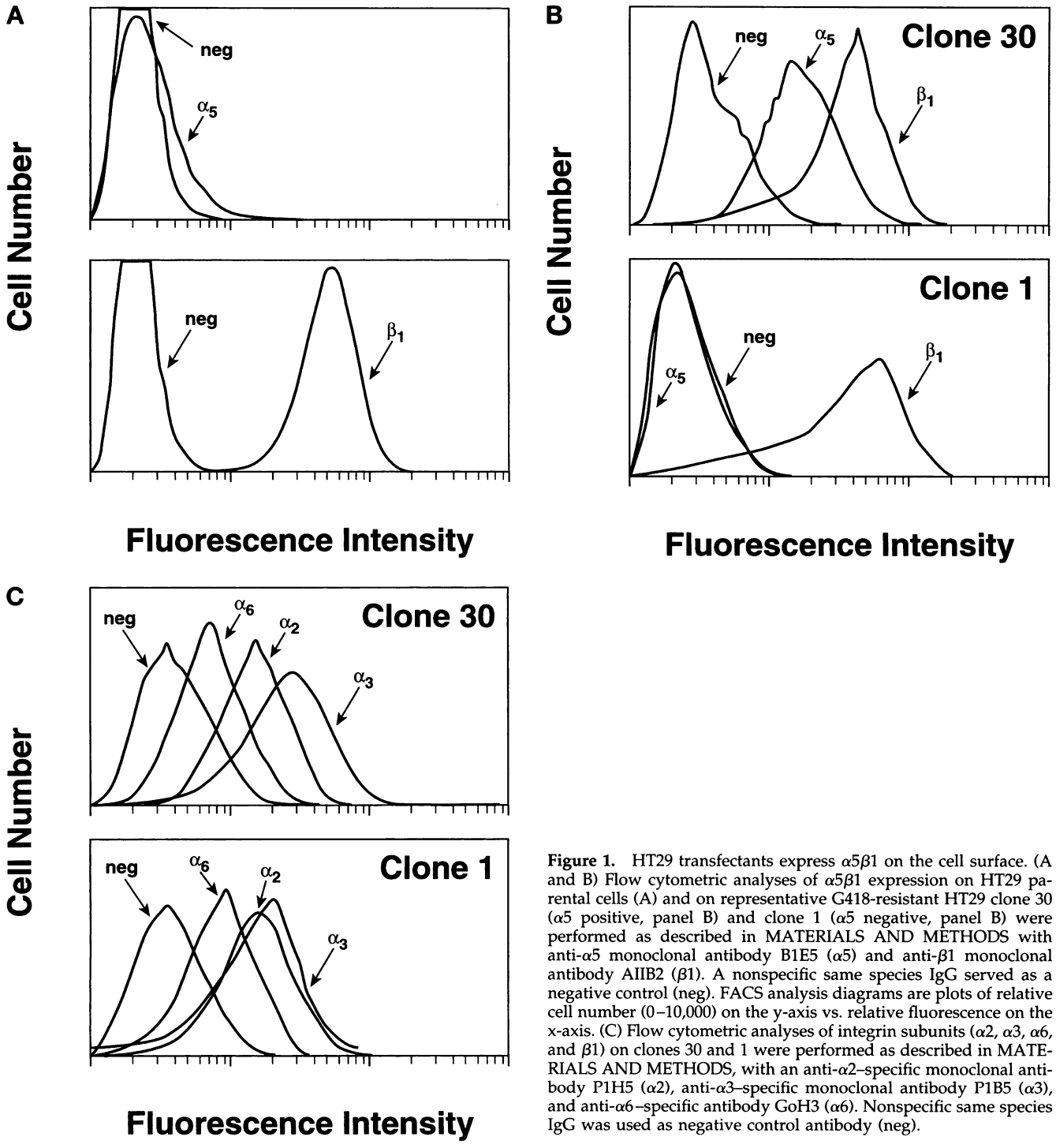

Figure 1. HT29 transfectants express $\alpha5\beta1$ on the cell surface. (A and B) Flow cytometric analyses of $\alpha5\beta1$ expression on HT29 parental cells (A) and on representative G418-resistant HT29 clone 30 ($\alpha5$ positive, panel B) and clone 1 ($\alpha5$ negative, panel B) were performed as described in MATERIALS AND METHODS with anti-$\alpha5$ monoclonal antibody B1E5 ($\alpha5$) and anti-$\beta1$ monoclonal antibody AIIB2 ($\beta1$). A nonspecific same species IgG served as a negative control (neg). FACS analysis diagrams are plots of relative cell number (0–10,000) on the y-axis vs. relative fluorescence on the x-axis. (C) Flow cytometric analyses of integrin subunits ($\alpha2$, $\alpha3$, $\alpha6$, and $\beta1$) on clones 30 and 1 were performed as described in MATERIALS AND METHODS, with an anti-$\alpha2$–specific monoclonal antibody P1H5 ($\alpha2$), anti-$\alpha3$–specific monoclonal antibody P1B5 ($\alpha3$), and anti-$\alpha6$–specific antibody GoH3 ($\alpha6$). Nonspecific same species IgG was used as negative control antibody (neg).

tions). These results indicate that transfection of HT29 cells with α_5 does not deplete the $\beta1$ subunit intracellular pool such that it alters surface expression or function of other α subunits.

The de novo expression of the integrin $\alpha5$ subunit in HT29 colon carcinoma cells enables cells to adhere to a fibronectin substrate (Figure 2A). Parental and pSV-neo-transfected HT29 cells are unable to adhere to fibronectin (Schreiner *et al.*, 1991a; Figure 2A). The $\alpha_{5\beta1}$ integrin positive transfectants, however, exhibit significant adhesion to fibronectin (Figure 2A). No changes were observed in adhesion to other substrates, such as

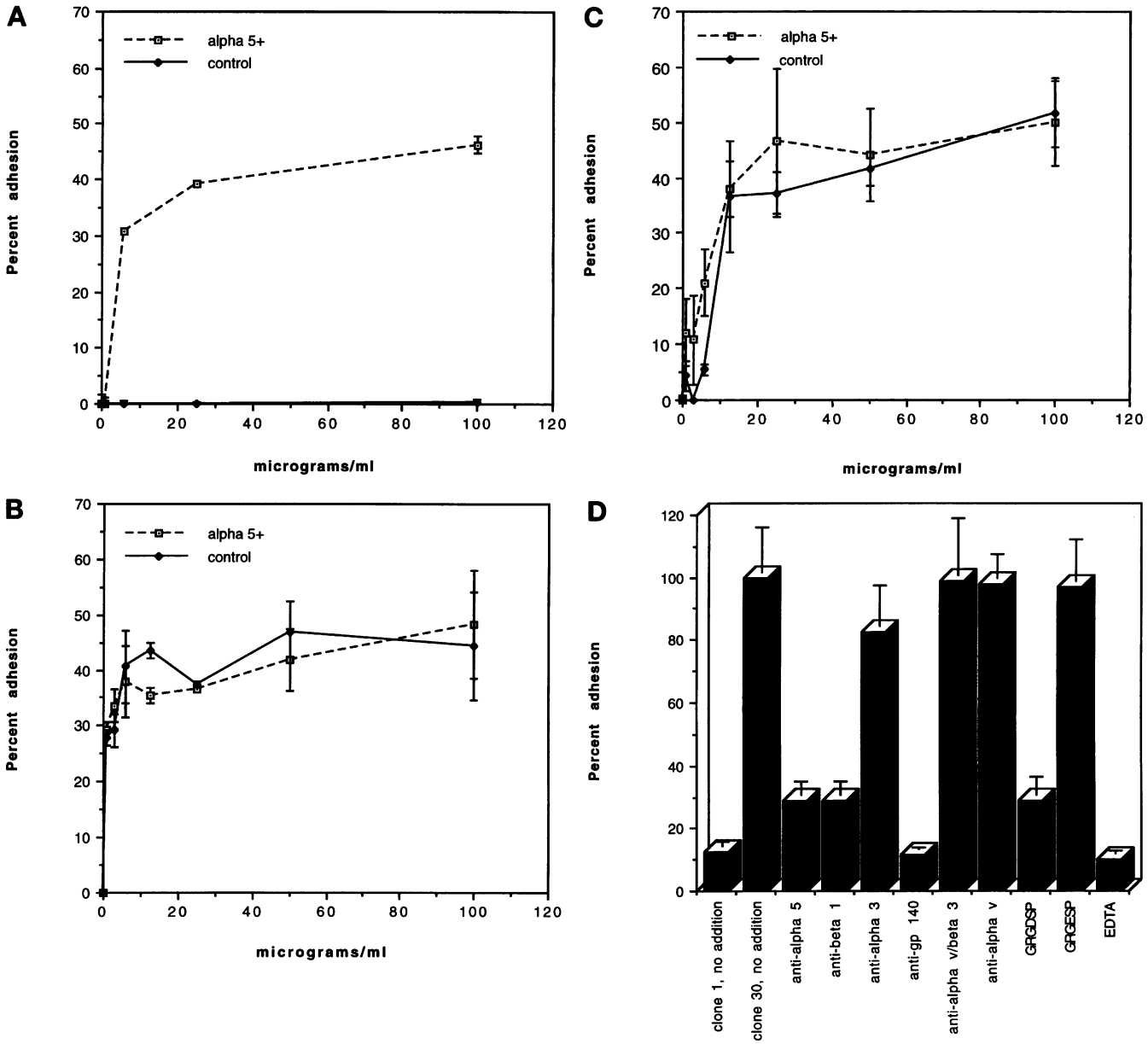

Figure 2. HT29 transfectants adhere to fibronectin via α5β1. HT29 transfectants (5×10^4 cells) were allowed to adhere for 30 min to the wells of a microtiter plate coated with serial dilutions of a starting solution of 100 μg/ml of fibronectin (A), collagen (B), or laminin (C). Percent of adhesion was determined as described in MATERIALS AND METHODS and was graphed vs. concentration of ligand. (D) HT29 transfectants (5×10^4 cells) were allowed to adhere for 30 min to the wells of a microtiter plate coated with 10 μg/ml of fibronectin in the absence or presence of 10 μg/ml of anti-α5 monoclonal antibody B1E5, anti-β1 monoclonal antibody AIIB2, anti-α3 monoclonal antibody P1B5, anti-αvβ3 monoclonal antibody LM609, anti-αv monoclonal antibody LM142, anti-α5β1 polyclonal antibody gp-140, 2.5 mM GRGDSP or 2.5 mM GRGESP, or 10 mM EDTA. Percent of adhesion was assessed as described in MATERIALS AND METHODS.

collagen, laminin (Figure 2, B and C), and vitronectin (our unpublished observations), indicating that the presence of the fibronectin receptor does not perturb normal HT29 integrin-mediated adhesion to other extracellular ligands. This de novo adhesion of α5 HT29 transfectants to fibronectin can be attributed entirely to the newly expressed $\alpha_5\beta_1$ integrin because adhesion of these cells to fibronectin was completely inhibited by the divalent cation chelator, EDTA (Figure 2D), as well as significantly by function-blocking anti-β_1 and anti-α_5 antibodies (70% inhibition). Moreover, a polyclonal anti-α5β1 antibody completely blocked adhesion to fibronectin. Several function-blocking monoclonal antibodies directed against other integrin

subunits ($\alpha 2$, $\alpha 3$, $\alpha 6$, $\alpha v\beta 3$, and αv) and isotype-matched control antibodies failed to inhibit adhesion of these cells to fibronectin. GRGDSP, the smallest fibronectin peptide sequence recognized by integrin receptors for fibronectin, but not the control peptide GRGESP, also significantly inhibited (by 70%) adhesion to fibronectin. Adhesion to fibronectin in colon carcinoma cells is thus strictly a property of the ectopically expressed integrin $\alpha 5\beta 1$.

Inhibition of Tumorigenicity

Previous studies have suggested that $\alpha 5\beta 1$ expression on cells is associated with reduced tumorigenicity. To examine whether de novo expression of $\alpha 5\beta 1$ on HT29 cells impacted their tumorigenicity, pSVneo and $\alpha 5$ transfectants were injected into nude mice. As shown in Figure 3, expression of $\alpha 5\beta 1$ on the cell surface led to complete suppression of tumor formation when 5×10^5 colon carcinoma cells were inoculated subcutaneously into athymic nude mice (Figure 3A). When mice were inoculated with 10-fold higher doses (5×10^6) of pSVneo transfectant clone 1 and $\alpha 5$-positive transfectant clones 34 and 30, $\alpha 5$-positive transfectants formed tumors, but at a significantly slower rate than did pSVneo transfectants (Figure 3B). Doubling times for the α_5 tumors from clones 30 and 34 are at least twice those of control transfectant and wild-type tumors (Table 1). Similar results were obtained in each of several identical tumorigenicity experiments. Flow cytometric analyses of cells explanted from tumors indicated that their integrin phenotypes had not changed while growing as tumors (our unpublished observations).

The $\alpha 5$-positive tumors that did grow were not only significantly smaller, but also exhibited a more differentiated phenotype than those of the pSVneo transfectants (Figure 3C). When sections of tumors were stained with Alcian blue and counterstained with hematoxylin, tumors from the $\alpha 5$-positive clones 30 and 34, but not from the pSVneo transfectant clone 1, exhibited significant numbers of Alcian blue–stained deposits, which is indicative of increased mucin or proteoglycan deposition (Figure 3, arrows), a characteristic of differentiated colon epithelia (Huet *et al.*, 1987; Lesuffleur *et al.*, 1991; McNeal *et al.*, 1991). All $\alpha 5$-positive tumors had similar morphologies to those shown here for clones 30 and 34. Thus, $\alpha 5$ expression leads to significant in vivo growth suppression in these carcinoma cells and may also lead to increased differentiation as evidenced by enhanced mucin secretion and altered morphology.

In Vitro Proliferation Is Dependent on Adhesion to Fibronectin

To determine whether $\alpha 5\beta 1$ expression also alters the in vitro proliferation of HT29 cells, we compared the in vitro proliferative rates of $\alpha 5\beta 1$ transfectants and pSVneo transfectants under a variety of growth conditions. When cultured in the presence of serum for 7 days, $\alpha 5$-positive transfectants initially exhibit a growth lag compared with pSVneo transfectants (Figure 4A), but eventually double at the same rate (29.5–32 h) as the pSVneo transfectants (Table 1). The growth rate of parental HT29 cells under these conditions is identical to that of pSVneo transfectants (our unpublished observations). $\alpha 5$ transfectants, however, reproducibly reach saturation inhibition of growth at a two- to threefold lower density than do control transfectants (Figure 4A). However, when cultured in the

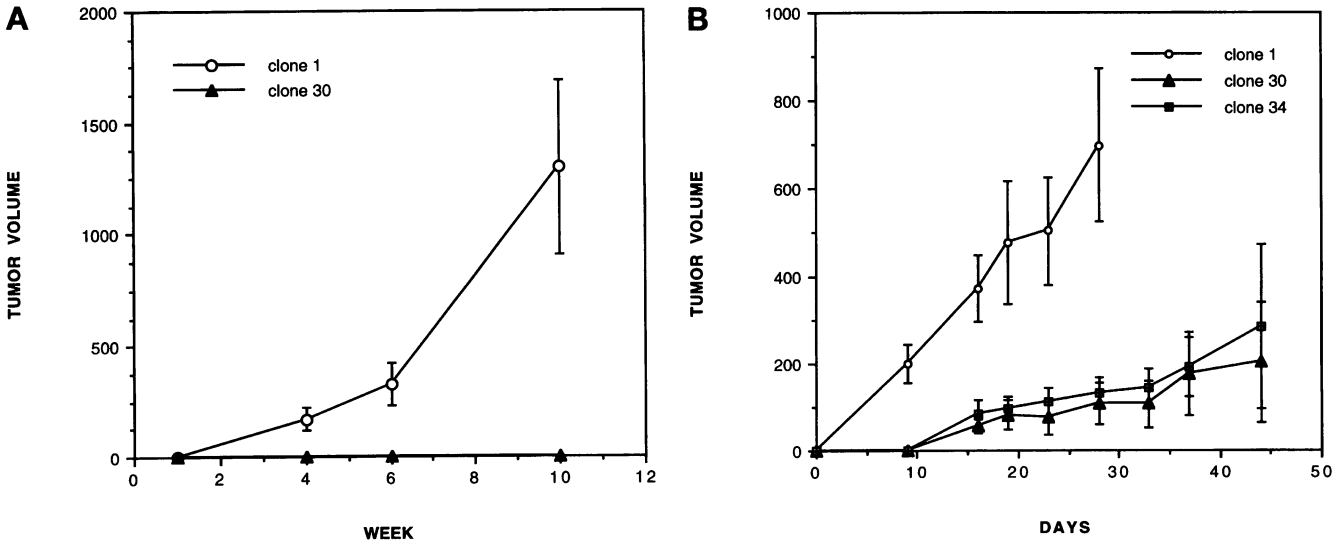

Figure 3.

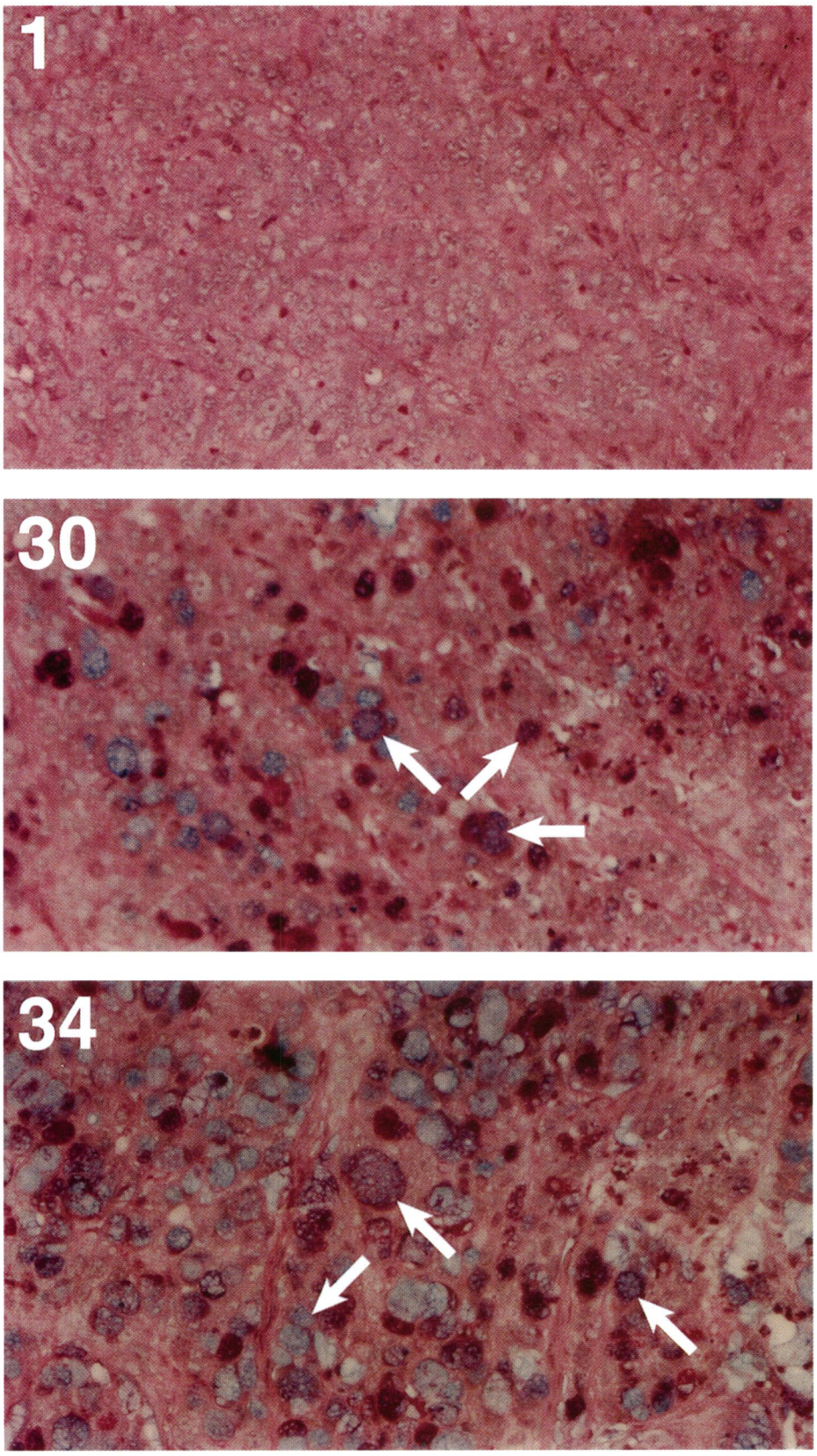

Figure 3 cont. Tumor growth and morphology is altered in integrin α5β1-positive HT29 transfectants. (A) Athymic nude mice were inoculated with aliquots of 5×10^5 cells of HT29 α5-negative clone 1, and α5-positive clone 30. Tumor dimensions were measured at regular intervals over 10 wk and average tumor volume was calculated as described in MATERIALS AND METHODS. Each point represents the mean of tumor growth in 10 mice each. (B) Athymic nude mice were inoculated with aliquots of 5×10^6 cells of HT29 α5-negative clone 1, and α5-positive clones 30 and 34. Tumor dimensions were measured at regular intervals over 6 wk and average tumor volume was calculated as described in MATERIALS AND METHODS. Each point represents the mean of tumor growth in 10 mice each. (C) Endpoint tumors (1 cm^3 in volume) from clones 1, 30, and 34 were fixed, sectioned, and stained with Alcian blue and hematoxylin. Representative sections of slides were photographed at 20× magnification. Arrows indicate Alcian blue-reactive inclusions.

Table 1. In vivo and in vitro doubling times

Clone	In vivo	In vitro: Serum	No serum	No serum, FN	No serum, VN
Parental	8d ± 1d	30 h ± 2h	48h ± 2h	—	—
1	8d ± 1d	29.5h ± 2h	48h ± 3h	46h ± 2h	48h ± 2h
34	17d ± 1d	32 h ± 3h	75h ± 3h	48h ± 2h	>72h ± 2h
30	17d ± 1d	32 h ± 1h	72h ± 2h	48h ± 2h	>72h ± 2h

Mean tumor volume doubling times were determined for the HT29 parental cell line and for transfectant tumors in in vivo growth studies in athymic nude mice over the course of a 60-day period, as described in Figure 3B. Mean cell number doubling times for the HT29 parental cell line and transfectants in in vitro growth studies in the presence of serum, in the absence of serum, and in the absence of serum on a fibronectin and a vitronectin substratum were determined over the course of 8 days in culture.

absence of serum in a defined medium containing insulin, transferrin, and selenium supplements, α5β1-positive transfectants double at a significantly slower rate (72–75 h) than do pSVneo transfectants (48 h) (see Figure 4B and Table 1). Under these conditions, α5-positive transfectants but not pSVneo transfectants eventually cease to proliferate during an 8-day period. Neither cell type adheres to the substratum during culture in serum-free media, suggesting that the cells do not secrete fibronectin or other matrix proteins.

Cell growth was examined after cells were allowed to attach to fibronectin and other matrix proteins. As shown in Table 1, adhesion to a fibronectin-coated substratum but not to other substrates reverses the growth inhibition displayed by α5-positive transfectants. These transfectants can be induced to proliferate at the same rate as pSVneo transfectants by adhesion to fibronectin. This effect appears to be specific for α5β1 because adhesion to vitronectin fails to reverse the growth inhibition (Table 1). Only α5-positive transfectants attached, spread, and grew as monolayers on fibronectin, whereas both α5-positive and -negative cells grew as monolayers on vitronectin (but at different rates). Immunohistochemical staining as well as immunoprecipitation analyses of tumors and cells in culture (our unpublished observations) for fibronectin expression indicates that neither pSVneo-transfectants nor α5-positive transfectants express or secrete any fibronectin. Thus, in the absence of an

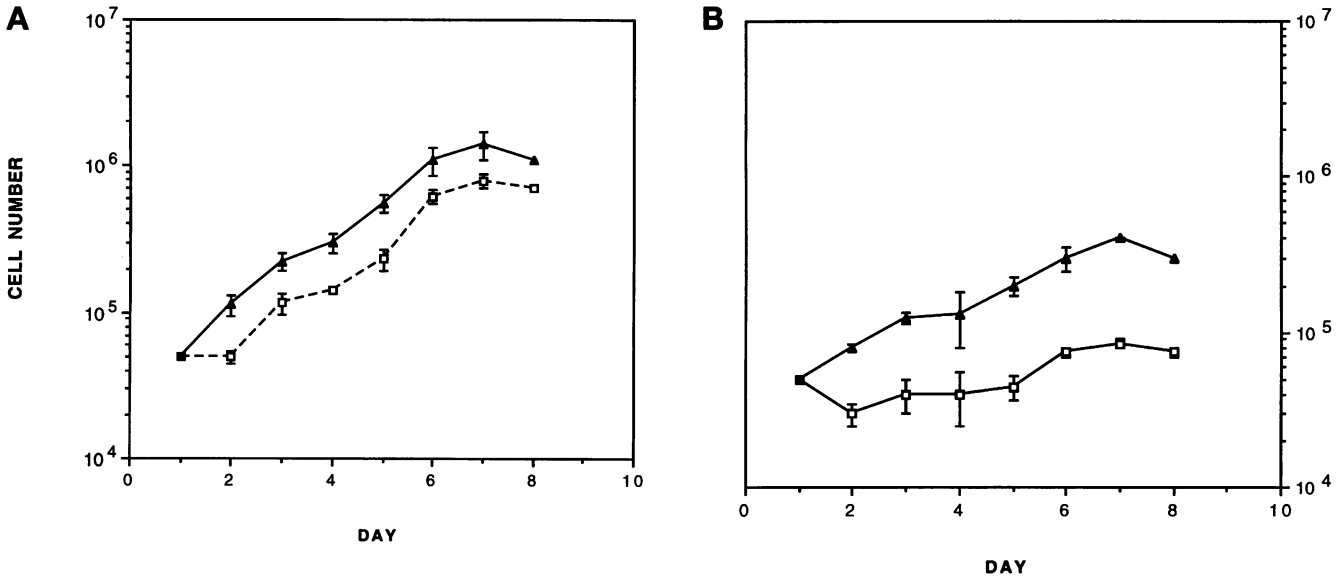

Figure 4. Integrin α5β1 expression inhibits in vitro growth only in the absence of attachment to fibronectin. α5-negative clone 1 and α5-positive clone 30 cells were seeded at equal densities (5×10^4 cells/ml) in triplicate wells of 12-well tissue culture dishes in the presence of serum (A) or in serum-free medium supplemented only with insulin, selenium, and transferrin on tissue culture plates blocked with BSA (B). Total cell number was determined over a 6- to 8-day period by removal of cells from the dishes with trypsin-EDTA followed by counting in an electronic particle counter.

exogenously added fibronectin substrate, the α5 transfectants remain unattached to fibronectin.

The reduced proliferation of α5-positive cells in the absence of a fibronectin substrate may be a consequence of a failure of the cell to progress through the cell cycle to S phase. HT29 α5β1-positive transfectants are unable to incorporate significant amounts of tritiated thymidine in serum-free culture compared with pSVneo transfectants (Figure 5). Only 20% of the amount of thymidine incorporated by pSVneo transfectants is incorporated by α5-positive transfectants in the absence of α5β1-mediated adhesion (Figure 5, ITS medium and polyL-lysine). As reflected by the in vitro growth studies, α5-positive cells are stimulated to reverse this block in cell cycle progression by adhesion to fibronectin but not by adhesion to other substrates. α5-positive transfectants allowed to adhere to fibronectin in the absence of serum incorporate nearly the same levels of thymidine incorporated by pSVneo-transfected cells in all serum-free culture conditions (Figure 5, fibronectin).

α5β1 Expression Is Associated with Enhanced Gas-1 Transcription and Down-regulation of Immediate Early Gene Transcription

To account for the effects of α5β1 expression and ligation on cell growth, we examined a number of transcriptional events known to be associated with cellular proliferation. Reverse transcriptase PCR analysis was performed to monitor potential alterations in gene expression patterns between α5β1 and pSVneo transfectants. All analyses were performed on samples adjusted to yield equal amounts of an actin PCR product under nonsaturating conditions from at least three different experiments. Identical patterns of expression were observed for each of the α5-positive clones studied.

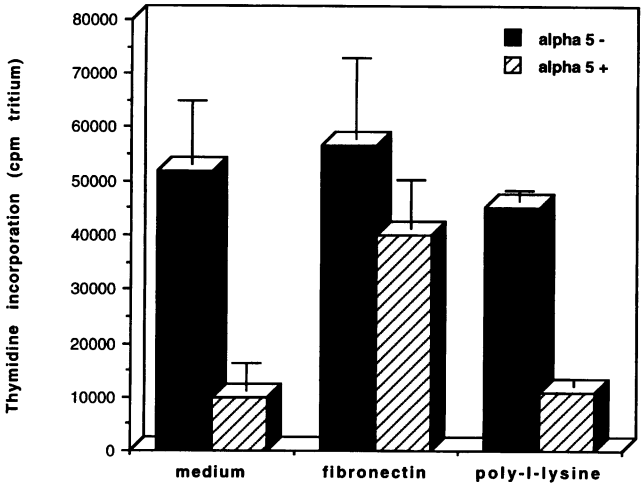

Figure 5. Thymidine incorporation by HT29 α5 transfectants is inhibited in the absence of attachment to fibronectin. PSVneo and α5-positive transfectants were maintained in culture in complete medium, in suspension culture in medium supplemented only with insulin, selenium, and transferrin (ITS medium), or in medium supplemented with insulin, transferrin, and selenium on tissue culture plastic that had been coated with 25 μg/ml fibronectin (fibronectin) or polyL-lysine (poly-l-lysine) in the presence of 10 μg/ml tritiated thymidine at 5×10^4 cells/ml for 24 h and acid precipitable counts were determined.

Expression of the integrin α5β1 did not alter the pattern of expression of growth factors secreted by HT29 cells when cultured in the presence or absence of serum (Figure 6). Levels of TGF-α, TGF-β, EGF, PDGF, and TNF-α messages remain unchanged. In contrast, expression of α5β1 in the absence of attachment to a fibronectin substrate on HT29 cells (i.e., during culture in the absence of interaction with fibronectin substratum) induces expression of mRNA for a growth arrest–associated protein, growth arrest–specific gene-1 (*gas*-1; Del Sal *et al.*, 1992, 1994). *Gas*-1 was first identified in serum-starved fibroblasts as a growth arrest–inducible transcript that is regulated independently from the immediate early genes c-*fos* and c-*jun*. *Gas*-1 was also shown to induce growth arrest when introduced into either normal or transformed cells (Del Sal *et al.*, 1992, 1994). After 12 or more hours of culture in the absence of adhesion, α5-positive cells, but not α5-negative cells, begin to transcribe *gas*-1 and continue to transcribe it for at least 72 h, the latest time point examined (Figure 7). These results suggest that expression of α5β1 in the absence of attachment induces expression of a gene product, *gas*-1, that has been shown to contribute directly to long-term withdrawal from the cell cycle.

In addition to the enhanced *gas*-1 expression, α5-positive cells but not pSVneo transfectants down-regulate c-*fos*, c-*jun*, and *jun* B transcription when cells are cultured in vitro in the absence of serum for up to 36 h (Figure 8A). Actin, H-*ras*, p53, histone, and c-*myc* transcription remain unchanged under these conditions. Expression of c-*fos* and c-*jun* is significantly down-regulated and *jun* B expression is completely inhibited (Figure 8A).

C-*fos*, c-*jun*, and *jun* B expression are rapidly down-regulated in a time course of response that is distinct from *gas*-1 expression. For example, c-*fos*, but not c-*myc*, expression is down-regulated within 3 h of removing α5-positive cells from contact with the substratum and remains down-regulated for up to 72 h, the latest time point examined. Only α5-positive cells display a c-*fos* transcriptional response to the induction of suspension culture (Figure 8B). Thus, α5β1 expression in the unligated state is associated not only with an increased transcription of *gas*-1, but also with decreased transcription of immediate early genes regulating proliferation.

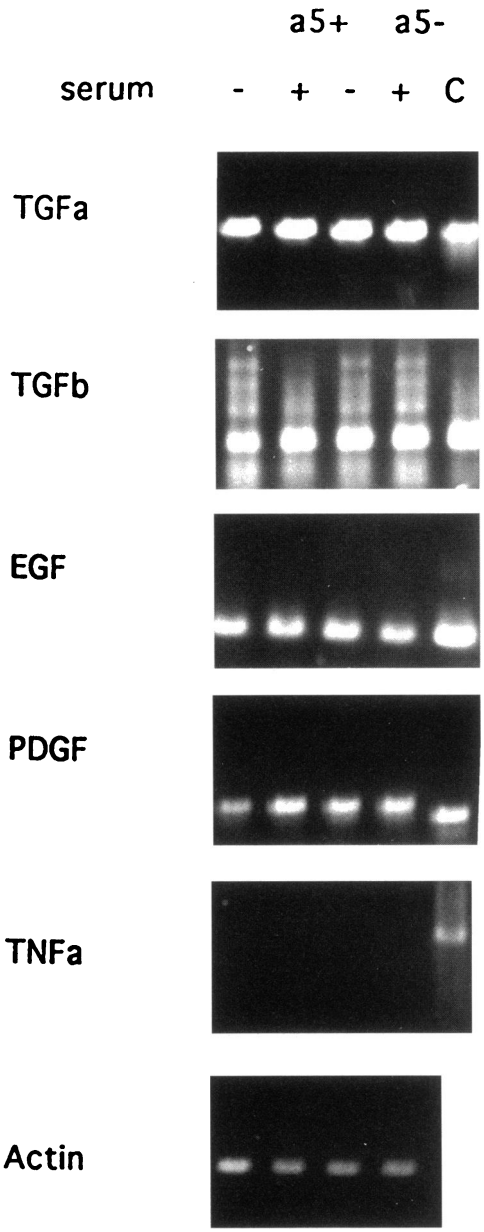

Figure 6. Expression of growth factors is unaltered in α5-positive transfectants. PSVneo (α5-) and α5-positive (α5+) transfectants were maintained in serum or serum-free culture over a period of 24 h. RNA was isolated and first strand cDNA was synthesized. PCR amplification of gene products was performed with primers specific for TGF-α, TGF-β, PDGF, EGF, and TNF-α at 56°C after standardization of cDNA levels for actin message levels. Results were analyzed by electrophoresis on TBE 2% agarose gels with molecular weight markers (1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72 bp) and ethidium bromide-stained products were photographed.

Adhesion to Fibronectin Reverses Gene Expression Changes

To examine the effect of ligation of α5β1 with fibronectin on the transcriptional regulation of *gas*-1 and immediate early genes, α5β1 cells were allowed to adhere to fibronectin or other substrates and gene expression was examined. As shown in Figure 9A, the decrease in c-*fos* expression levels can be reversed and returned to the levels exhibited by pSVneo transfectants by adhesion to fibronectin, but not to other adhesive substrates. α5-positive cells up-regulate c-*fos* expression within 1 h of adhesion to fibronectin, whereas expression of other gene products (actin, histone H4, and c-*myc*) remains unchanged and identical to the levels expressed by pSVneo transfectants. In addition, *gas*-1 expression is down-regulated by adhesion to fibronectin, but only after 3 h of adhesion (Figure 9B). Adhesion to other substrates such as polyL-lysine or other matrix proteins does not induce these alterations in gene expression (Figure 9C). Thus, integrin α5β1 is capable of generating both positive and negative signals depending on the adhesive state of the cell.

These results indicate that ectopic expression of α5β1 in HT29 colon carcinoma cells alters gene expression events that directly impact cellular proliferation. The integrin α5β1–mediated growth inhibition that we describe here and that others have previously described (Giancotti and Ruoslahti, 1990) appears to be mediated by the unattached integrin and can be reversed only through ligation of the integrin by adhesion to fibronectin.

DISCUSSION

Regulation of cellular proliferation is a complex phenomenon, which requires the precise and timely interplay of a variety of signaling and transcription events. The roles of growth factors and their receptors in the regulation of the cell cycle have been the subjects of much scrutiny (Ullrich and Schlessinger, 1990; Hofbauer and Denhardt, 1991). In contrast, the contribution of anchorage to the substratum to the regulation of proliferation is not as well understood (Juliano and Haskill, 1993). As members of several families of cell adhesion receptors (integrins, cadherins, and Ig superfamily CAMs) are increasingly studied, their roles in the life cycle of cells are being elucidated. In the studies presented here, we have shown that the integrin α5β1 exerts a dominant negative effect on cellular proliferation. The same integrin, however, permits proliferation upon cell attachment to a fibronectin substrate. These findings provide the first evidence that both ligated and unligated integrin transduce signals that differentially regulate transcription.

HT29 colon carcinoma cells normally do not express the integrin α5β1 and do not adhere to fibronectin. Transfection of these cells with the gene for the integrin α5 subunit allowed us to examine the role of this integrin and its interactions with the extracellular matrix ligand fibronectin in the regulation of prolifera-

tion. Surface expression of α5β1 enabled cells to adhere to a fibronectin substrate without apparently altering the expression or function of other integrins. Although monoclonal antibodies and RGD peptides significantly blocked the adhesion of the α5 transfectants to fibronectin (70%), complete inhibition was not achieved. It is possible that the levels of added blocking antibody or peptide were not sufficient in these experiments to completely inhibit adhesion, because an anti-α5β1 polyclonal antibody did completely inhibit adhesion. Alternatively, it is also possible that other fibronectin receptors such as αvβ1 or αvβ6 have been induced or activated through integrin-crosstalk (Blystone *et al.*, 1994) in the α5-transfected cells and may contribute to the de novo adhesion to fibronectin.

In addition to exhibiting de novo adhesion to fibronectin, the α5β1 transfectants, but not pSVneo transfectants, became dependent for proliferation on anchorage to fibronectin, apparently via their integrin α5β1. α5β1 transfectants were unable to proliferate as well as their pSVneo transfected counterparts in vivo or in vitro in the absence of attachment to fibronectin. In fact, the temporary growth lag exhibited by HT29 α5-positive transfectants after they are placed into serum culture may result from a temporary withdrawal from the cell cycle that is induced by their removal from the fibronectin substratum that is present in serum culture. Incorporation of soluble fibronectin at physiologically relevant concentrations (25 μg/ml, approximately the amount of fibronectin or vitronectin present in a 10% serum culture medium) into the serum-free growth medium fails to reverse the growth inhibition displayed by α5-positive or pSVneo transfectants (Varner and Juliano, unpublished results). Although soluble fibronectin has been shown to interact with the fibronectin receptor, it does so poorly, requiring very high (5–10 mg/ml), nonphysiologically relevant concentrations of fibronectin (Yamada and Kennedy, 1984). These results suggest that interaction of the integrin α5β1 with a fibronectin substratum is needed to permit the α5-positive cells to proliferate. Although the α5β1 on the surface of suspended cells is not occupied by fibronectin, it is possible that it is occupied by as yet unknown surface or secreted components that could participate in the α5β1-induced negative growth regulation.

The α5 transfectants proliferate in serum-free, insulin-supplemented media as well as their control-transfected counterparts only when cultured on a fibronectin substrate. Although many α5β1-positive cell types grow well on vitronectin and other matrices in serum or in defined media, the HT29 α5 transfectants do not grow well on vitronectin in insulin-supplemented, serum-free media. It is possible that other growth conditions (i.e., other growth factor supplements) would induce these cells to proliferate on vitronectin and other matrices. Recent studies suggest that specific growth factors collaborate with distinct integrins to

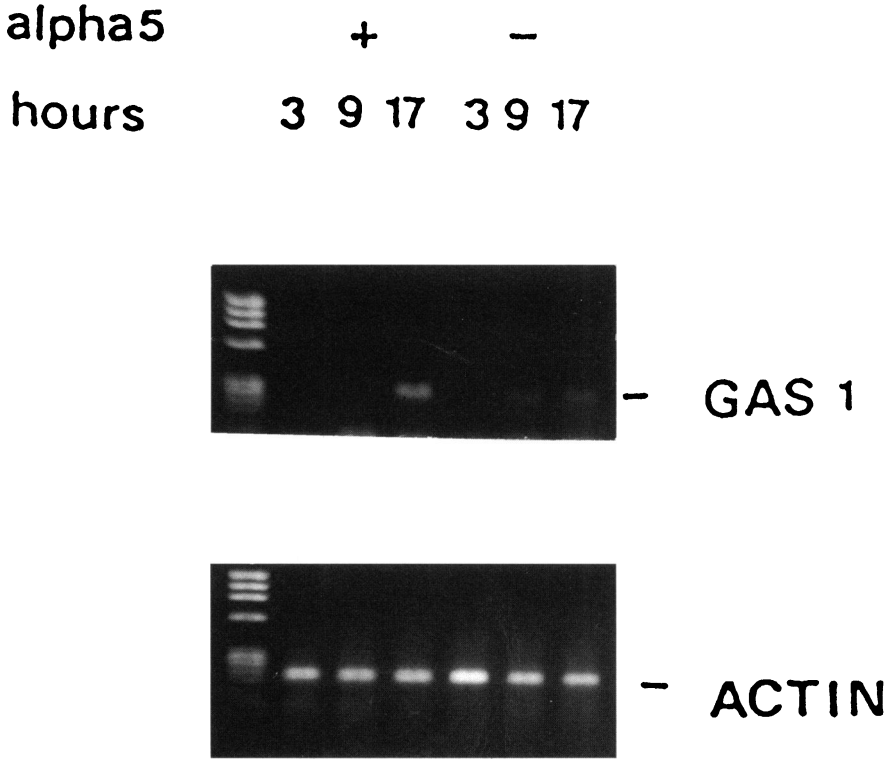

Figure 7. Growth arrest specific gene-1 is expressed α5 positive transfectants in the absence of attachment to fibronectin. PSVneo (α5−) and α5-positive (α5+) transfectants were maintained in suspension culture over a period of 24 h. RNA was isolated at time points of 3, 9, 17, and 24 h after initiation of suspension culture and first strand cDNA was synthesized. PCR amplification of gene products was performed with primers specific for *gas*-1 (322 bp) and actin (150 bp) at an annealing temperature of 56°C as described in MATERIALS AND METHODS. Results were analyzed by electrophoresis on 2% TBE agarose gels with molecular weight markers (1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72 bp) and ethidium bromide-stained products were photographed.

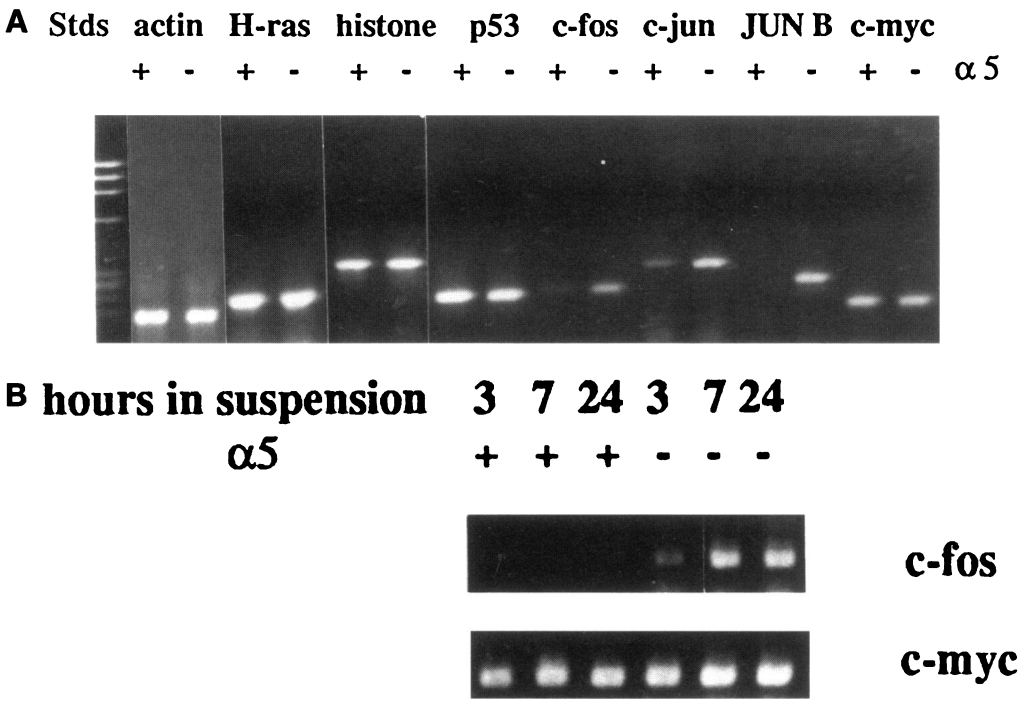

Figure 8. Immediate early gene transcription is down-regulated in α5-positive transfectants. (A) PSVneo (α5−) and α5-positive (α5+) transfectants were cultured in suspension for 24 h before isolation of RNA and synthesis of first strand cDNA as described in MATERIALS AND METHODS. PCR analyses for expression of gene products was performed at 56°C using primers specific for actin (150 bp), H-*ras* (233 bp), histone H4 (347 bp), p53 (220 bp), c-*fos* (236 bp), c-*myc* (187 bp), c-*jun* (325 bp), and JUN-B (257 bp) after standardization of cDNA levels for actin message levels. (B) Replicate samples of pSVneo (α5−) and α 5-positive (α5+) transfectants were cultured in suspension for up to 24 h before isolation of RNA at 3, 7, and 24 h after suspension initiation and synthesis of first strand cDNA as described in MATERIALS AND METHODS. PCR analyses for expression of gene products was performed at 56°C using primers specific for c-*fos* (product size of 236 bp) and c-*myc* (product size of 187 bp) after standardization of cDNA levels for actin message levels. Results of PCR analyses were analyzed after electrophoresis on 2% TBE agarose gels and ethidium bromide-stained products were photographed.

effect different biological behaviors (Klemke *et al.*, 1994).

Unlike the parental HT29 cells or pSVneo transfectants, the α5β1 transfectants were unable to form tumors efficiently in nude mice. No evidence of fibronectin expression or presence in any HT29 tumor was observed, suggesting that the receptor is unoccupied by fibronectin in the tumors (Varner and Juliano, unpublished results). Although colon carcinoma cells secrete many growth factors, including TGF-α, TGF-β, PDGF (Anzano *et al.*, 1989), and EGF, pSVneo and α5β1 transfectants exhibited no differences in expression levels of these growth factors. Differences in tumor formation thus cannot be attributed to intrinsic growth factor differences nor to a deficiency in vascularization because both α5β1-positive and control tumors appeared to be equally well vascularized. The α5β1 transfectants do not synthesize fibronectin in an in vitro or in vivo setting and they lack the capacity to organize fibronectin into a matrix (Varner and Juliano, unpublished results).

An examination of the downstream molecular mechanism by which this in vivo and in vitro inhibition of cellular proliferation occurs identified a series of transcriptional events that are associated with α5β1-dependent attachment to a fibronectin substrate. The most striking of these transcriptional events is the de novo transcription of *gas*-1 (del Sal *et al.*, 1992, 1994) by α5β1 transfectants when they are cultured in the absence of attachment to fibronectin. *Gas*-1 is a 46-kDa cell surface protein that is expressed de novo at the borders of contact inhibited and serum-starved cells. Ectopic expression of *gas*-1 induces growth arrest in normal as well as transformed cells. In addition to inducing the transcription of a growth arrest gene, failure to ligate α5β1 is associated with reduced transcription of the immediate early genes c-*fos*, c-*jun*, and *jun* B, but not of c-*myc*. The immediate early genes c-*fos*, c-*jun*, and *jun* B are transcribed in a burst of transcriptional activity upon stimulation of growth-arrested (adherent) normal cells with serum growth factors (Rollins and Stiles, 1989; Rivera and Greenberg, 1990). They are required for the transition from G0 to G1 (Nishikura and Murray, 1987; Smith and Prochownik, 1992) and hence for cellular proliferation (Riabowol *et al.*, 1988; Ryder and Nathans, 1988; Ryseck *et al.*, 1988; Herschman, 1991; Kovary and Bravo, 1991). In contrast, the immediate early gene c-*myc* is not required for the G0/G1 transition, but plays a role in regulating the G1 to S transition (Heikkla *et al.*, 1987). In fact, c-*myc* expression is present at measurable levels through the cell cycle (Evan *et al.*, 1992). Hence, as observed for the HT29 transfectants and control cells, c-*myc* is generally regulated separately from the immediate early genes c-*fos*, c-*jun*, and *jun* B.

Cellular adhesion has been associated with gene expression of immediate early genes, proteases, and cytokines in monocytes (Haskill *et al.*, 1988, 1991; Sporn *et al.*, 1989), synovial fibroblasts (Werb *et al.*, 1989), and T cells (Yamada *et al.*, 1991). When α5β1 transfected HT29 cells adhere to fibronectin, but not to other substrates such as vitronectin, collagen, or polyL-lysine, immediate early gene expression is induced while *gas*-1 transcription is suppressed. After adhesion to fibronectin, cells subsequently synthesize DNA and proliferate at nearly the same rate as the parental and pSVneo transfectant cell lines. These transcription and proliferation events are not mediated by mere cell attachment, as they are not induced by cell attachment to and spreading on vitronectin, collagen, or polyL-lysine. Although adhesion and integrin-clustering events have previously been shown to induce intracellular events leading to de novo gene expression, we have been able to demonstrate that simple α5β1 expression versus α5β1-mediated cell adhesion induces distinct patterns of gene expression.

Integrins generate intracellular signals in response to clustering by adhesion or by antibodies, which include synthesis of phosphatidylinositol phosphate, activation of protein kinase C, and tyrosine phosphorylation (for review, Juliano and Haskill, 1993). Integrin cytoplasmic tails interact directly with some cytoskeletal (Otey *et al.*, 1990) and signaling molecules (Otey *et*

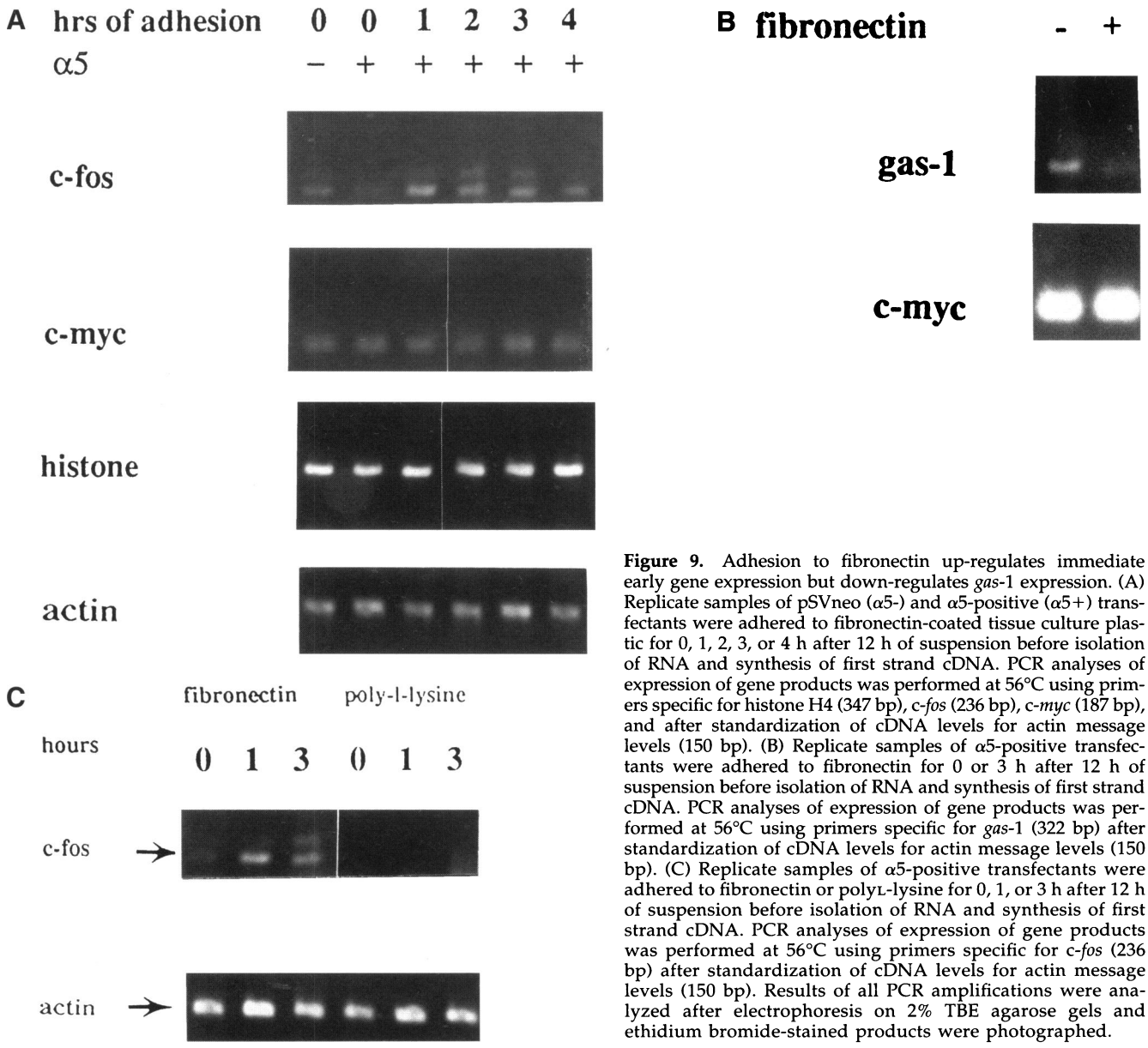

Figure 9. Adhesion to fibronectin up-regulates immediate early gene expression but down-regulates *gas*-1 expression. (A) Replicate samples of pSVneo (α5-) and α5-positive (α5+) transfectants were adhered to fibronectin-coated tissue culture plastic for 0, 1, 2, 3, or 4 h after 12 h of suspension before isolation of RNA and synthesis of first strand cDNA. PCR analyses of expression of gene products was performed at 56°C using primers specific for histone H4 (347 bp), c-*fos* (236 bp), c-*myc* (187 bp), and after standardization of cDNA levels for actin message levels (150 bp). (B) Replicate samples of α5-positive transfectants were adhered to fibronectin for 0 or 3 h after 12 h of suspension before isolation of RNA and synthesis of first strand cDNA. PCR analyses of expression of gene products was performed at 56°C using primers specific for *gas*-1 (322 bp) after standardization of cDNA levels for actin message levels (150 bp). (C) Replicate samples of α5-positive transfectants were adhered to fibronectin or polyL-lysine for 0, 1, or 3 h after 12 h of suspension before isolation of RNA and synthesis of first strand cDNA. PCR analyses of expression of gene products was performed at 56°C using primers specific for c-*fos* (236 bp) after standardization of cDNA levels for actin message levels (150 bp). Results of all PCR amplifications were analyzed after electrophoresis on 2% TBE agarose gels and ethidium bromide-stained products were photographed.

al., 1993). Ligation-induced alterations in integrin conformation may stimulate a cascade of signaling events that ultimately lead to transcription. $\alpha5\beta1$ exertion of a dominant negative effect on proliferation in colon carcinoma may indicate that the unligated integrin $\alpha5\beta1$ interacts directly with a key regulatory element of a preexisting signaling cascade, thus inhibiting its participation in signaling. Ligation by fibronectin may permit release of this signaling molecule and hence the cascade of events that lead to proliferation. It is important to note that the gene expression changes described here are not directly correlated with cell spreading or with focal contact formation because both $\alpha5$-positive and -negative cells adhere to and spread on vitronectin but their growth differential is not altered. Hence, although both alterations in gene expression and in cytoskeletal arrangement are though to be the result of signal transduction events, the gene transcription events we describe are dissociated from simple cell spreading due to the very different effects on proliferation displayed by different matrices.

The integrin $\alpha5\beta1$ has previously been implicated in tumor suppression (Dedhar *et al.*, 1987, 1989; Giancotti and Ruoslahti, 1990; Symington, 1990, 1993). In each of the previous experimental systems studied, overexpression of $\alpha5\beta1$ by 5- to 30-fold was required to suppress tumor growth. In the studies we describe here, levels of $\alpha5\beta1$ expression equivalent to those of other HT29 cell surface integrins were sufficient to suppress tumorigenicity and in vitro growth in the absence of fibronectin. In each of these studies, failure to ligate all of the tumor cell $\alpha5\beta1$ molecules with sufficient fibronectin may account for the in vivo tumor suppression. We therefore suggest that the ligation state of the integrin $\alpha5\beta1$ is capable of regulating the cell cycle by modulating a signaling pathway that is required to induce immediate early gene expression and suppression of the expression of the growth arrest inducing gene, *gas*-1.

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