

Integrin $\alpha 5/\beta 1$ Mediates Fibronectin-dependent Epithelial Cell Proliferation through Epidermal Growth Factor Receptor Activation

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Human integrin $\alpha 5$ was transfected into the integrin $\alpha 5/\beta 1$ -negative intestinal epithelial cell line Caco-2 to study EGF receptor (EGFR) and integrin $\alpha 5/\beta 1$ signaling interactions involved in epithelial cell proliferation. On uncoated or fibronectin-coated plastic, the integrin $\alpha 5$ and control (vector only) transfectants grew at similar rates. In the presence of the EGFR antagonistic mAb 225, the integrin $\alpha 5$ transfectants and controls were significantly growth inhibited on plastic. However, when cultured on fibronectin, the integrin $\alpha 5$ transfectants were not growth inhibited by mAb 225. The reversal of mAb 225-mediated growth inhibition on fibronectin for the integrin $\alpha 5$ transfectants correlated with activation of the EGFR, activation of MAPK, and expression of proliferating cell nuclear antigen. EGFR kinase activity was necessary for both MAPK activation and integrin $\alpha 5/\beta 1$ -mediated cell proliferation. Although EGFR activation occurred when either the integrin $\alpha 5$ -transfected or control cells were cultured on fibronectin, coprecipitation of the EGFR with SHC could be demonstrated only in the integrin $\alpha 5$ -transfected cells. These results suggest that integrin $\alpha 5/\beta 1$ mediates fibronectin-induced epithelial cell proliferation through activation of the EGFR.

INTRODUCTION

Epithelial cells receive important cues from the environment through soluble growth factors and insoluble extracellular matrix proteins. The receptors chiefly responsible for this binding are the growth factor receptors and integrins, respectively. The signaling triggered by these receptors effect changes in critical cell functions as diverse as proliferation, differentiation, and survival (Pignatelli and Bodmer, 1989; Streuli *et al.*, 1991; Roskelley *et al.*, 1994; Sastry *et al.*, 1996; Giancotti, 1997; Somasiri and Roskelley, 1999). With regard to cell cycle progression and proliferation, coordinated input from both growth factor receptors and integrins is necessary (Clark and Brugge, 1995; Zhu and Assoian, 1995, 1996; Wary *et al.*, 1996, 1998; Schwartz and Baron, 1999).

How growth factor and integrin signal transduction pathways are actually integrated in controlling cell functions is not well understood. Most studies have used mesenchymal cells and boluses of exogenous growth factors to stimulate growth factor receptor activity. Such acute conditions are not usually found in normal tissue. Epithelial cells are usually regulated by autocrine growth factor loops (Ferriola *et al.*, 1991, 1992; Bishop *et al.*, 1995; Damstrup *et al.*, 1999). Autocrine growth factor activation of the EGF receptor

(EGFR) is best described as a steady-state system as it approximates normal cell physiology in vivo (Wiley and Cunningham, 1981). Epithelial cells would rarely be exposed in vivo to acute and large concentrations of growth factors. In addition, the exposure of cells to boluses of EGF family growth factors usually results in only transient activation of the EGFR. The use of epithelial cells is clinically relevant in that they are the frequent targets of diseases, such as adenocarcinoma, in which aberrant growth is a characteristic finding.

Both integrins and the EGFR activate common members of the RAS-ERK signal transduction pathway (Pages *et al.*, 1993; Chen *et al.*, 1994; Lange-Carter and Johnson, 1994; Kelleher *et al.*, 1995; Morino *et al.*, 1995; Zhu and Assoian, 1995, 1996; Miyamoto *et al.*, 1996). Growth factor-induced cell proliferation is mediated by the MAPKs, also known as extracellular signal-regulated kinases (ERKs) (Pages *et al.*, 1993; Aliaga *et al.*, 1999). Although integrins and the EGFR can activate ERK independently, the emerging picture is that ERK activation must exceed a threshold to drive cell proliferation. Exceeding this threshold requires input from both integrins and growth factor receptors (Zhu and Assoian, 1995, 1996; Schwartz and Baron, 1999). How integrin and growth factor receptor signaling are integrated proximal to ERK is not well understood.

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At present, there are three known mechanisms by which integrins can activate ERKs, and all three mechanisms involve RAS as the activator of downstream MAPKs. The first mechanism is through the activation of Fyn by Shc, which is initially recruited by activated integrins via caveolin (Wary *et al.*, 1998). Interestingly, although integrins $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 5$, and αV interact with caveolin, only $\alpha 1/\beta 1$, $\alpha 5/\beta 1$, or αV can recruit Shc and activate Fyn (Wary *et al.*, 1996, 1998). Shc then recruits Grb2 and SOS, the latter of which activates the RAS-ERK pathway. The second mechanism of ERK activation is through integrin-mediated focal adhesion kinase activation, which results in the recruitment of Grb2 (Schlaepfer *et al.*, 1994, 1998; Hanks and Polte, 1997), which in turn recruits SOS and consequently leads to RAS activation. The third mechanism is integrin-mediated EGFR activation (Moro *et al.*, 1998; Li *et al.*, 1999), which also causes activation of Shc, Grb2, and RAS.

Epithelial cells express a large repertoire of various integrin receptors, and the redundancy of specific extracellular matrix proteins bound by these integrins complicates investigation. However, integrin $\alpha 5/\beta 1$ possesses high-affinity binding only to fibronectin (Hemler, 1990). The Caco-2 intestinal epithelial cell line used lacks detectable expression of the classic fibronectin receptor, integrin $\alpha 5/\beta 1$, and EGFR expression and function have been characterized extensively in this cell line (Hidalgo *et al.*, 1989; Bishop and Wen, 1994; Bishop *et al.*, 1995; Tong *et al.*, 1998; Kuwada *et al.*, 1999). Although adding EGF family ligands, such as EGF, does not greatly stimulate Caco-2 cell proliferation, interrupting autocrine EGF family growth factor activation of the EGFR can diminish cell proliferation significantly (Damstrup *et al.*, 1999; Kuwada *et al.*, 1999). Caco-2 cells were transfected with integrin $\alpha 5$ to study signaling interactions between integrin $\alpha 5/\beta 1$ and the EGFR that are involved in the control of epithelial cell proliferation.

We asked specifically whether integrin $\alpha 5/\beta 1$ -mediated EGFR activation occurs in epithelial cells and what role this plays in cell proliferation. We found that integrin $\alpha 5/\beta 1$ mediates fibronectin-induced EGFR activation, which leads to EGFR-mediated activation of MAPKs and cell proliferation.

MATERIALS AND METHODS

Antibodies and Kinase Inhibitors

Antibodies used were integrin $\alpha 2$ mAb (Becton-Dickinson, Franklin Lakes, NJ); integrin $\alpha 3$ mAb (Becton-Dickinson); integrin αV mAb, phosphoserine mAb, EGFR mAb, rabbit anti-mouse immunoglobulin G (Zymed, San Francisco, CA); integrin $\alpha 5$ subunit (mAb 16, Becton-Dickinson; and polyclonal antibody, Chemicon); PY-20 mAb, phospho-p44/42 MAPK E10 mAb, Shc polyclonal antibody, Grb2 mAb, ERK1 mAb, MEK1, proliferating cell nuclear antigen (PCNA) mAb (Transduction Laboratories, Lexington, KY); and alkaline phosphatase-conjugated goat anti-rabbit antibody (Jackson Immunoresearch, West Grove, PA). The MEK1 inhibitor PD 98059 (Calbiochem, La Jolla, CA) and the EGFR kinase inhibitor Compound 32 (PD 153035; Calbiochem) were solubilized in DMSO.

Integrin $\alpha 5$ Gene Construct

The human integrin $\alpha 5$ gene (in the plasmid pECE) was obtained as a generous gift from Dr. Erkki Ruoslahti. The *Sall*-*Xba*I fragment encompassing the integrin $\alpha 5$ gene was cloned into the *Xho*I and

*Xba*I sites of the vector pcDNA3 (Invitrogen, Carlsbad, CA), which has a G418 resistance gene.

Cell Lines, Tissue Culture, and Transfection

Caco-2 cells were obtained as a gift from Dr. Robert J. Coffey, Jr., and maintained in DMEM (ICN Biomedical, Costa Mesa, CA) supplemented with fibronectin-free 10% FBS (Hyclone, Logan, UT), glutamine, penicillin, and streptomycin in a 37°C humidified tissue culture incubator with a 5% CO₂ environment. The cells were cultured on uncoated or fibronectin-coated (10 μ g/ml; Sigma Chemical, St. Louis, MO) 35-mm bacterial plastic dishes (Falcon), 35-mm tissue culture dishes (Greiner), and 96-well tissue culture plastic dishes (Greiner). When mAb 225 (Imclone) and integrin mAb (Becton-Dickinson) were used, they were mixed with dispersed cells to a final concentration of 10 and 5 μ g/ml, respectively, just before cell plating. Compound 32 (PD 153035), solubilized in DMSO, was added to cell culture medium to achieve a final concentration of 4 μ M (controls received an equal concentration of DMSO alone).

Caco-2 cells were transfected with the pcDNA3 vector alone or with the human integrin $\alpha 5$ -pcDNA3 construct by a calcium phosphate method. Caco-2 cells between passages 90 and 95 were grown to ~70% confluence on 100-mm dishes. Ten to 40 μ g of plasmid DNA preparations (Wizard Preparations, Promega, Madison, WI) were added to sterile 1× TE buffer to a total volume of 500 μ L. Fifty microliters of sterile 2.5 M CaCl₂ in 10 mM HEPES, pH 7.2 was added to the plasmid DNA and mixed thoroughly. The calcium-DNA solution was then dripped into 0.5 ml of sterile 2× HEBS buffer with vigorous mixing every three drops. The mixture was incubated at room temperature for 30 min. The entire mixture was then added to the 10 ml of culture medium bathing the cells. The medium was aspirated off the next day, the cells were washed twice with PBS-EDTA, and fresh medium was added. Forty-eight hours after the transfection procedure, the cells were dispersed in trypsin and replated in 10 ml of medium containing 1.5 mg/ml G418 (LD curves demonstrated that a G418 final concentration of at least 1.2 mg/ml was needed to kill all Caco-2 cells in culture). Approximately 25–30 cell colonies were obtained, and the individual clones were removed with trypsin with the use of cloning cylinders (Bellco). The individual colonies were plated on 12-well plates in medium containing 1.5 mg/ml G418. Once confluent colonies were obtained in 12-well plates, the cells were replated on 10-cm dishes. During all steps, the cell culture medium was exchanged every 2 d.

For the soft agar proliferation assay, a 1.6% low-melting-point agar solution (in water) was microwaved until boiling and then cooled in a 37°C water bath. The agar solution was mixed 1:1 with 2× medium, and 1 ml of this solution was plated in 35-mm tissue culture plastic dishes. A total of 20,000 cells were added to a solution containing 2× medium and 1× medium in a 1:2 ratio (3 ml total volume per dish). Then, 1 ml of liquid 1.6% agar solution (at 37°C) was added to the cell mixture, which was vortexed gently to disperse the cells. One milliliter of the cell mixture was layered onto the bottom of the dish and left at room temperature until the agar solidified. The cells were placed in a 37°C incubator with 5% CO₂ and photographed each day.

Expression of Integrins by Caco-2 Cells

Flow cytometry and immunoblotting were used to evaluate cells for the expression of various integrins. For flow cytometric analysis of integrin expression, cells were dispersed in 0.25% trypsin in PBS-EDTA and washed twice in PBS-EDTA. The cells were then mixed at 37°C with integrin $\alpha 5$ mAb (Becton-Dickinson) at a dilution of 1:500 in blocking buffer A (1% radioimmunoassay-grade BSA [Bio-Rad, Richmond, CA] in PBS) for 60 min. The cells were washed twice in blocking buffer A, mixed with a 1:1000 dilution of FITC-labeled rabbit anti-mouse mAb (Jackson Immunoresearch) in blocking buffer A, and incubated at 37°C for 30 min. The cells were washed twice in PBS-EDTA and assayed with a flow cytometer

(Becton-Dickinson). Integrin expression was determined to be the percentage of FITC-positive cells. The gate setting was determined by the fluorescence intensity of the same cells stained with FITC-conjugated secondary antibody only.

For immunodetection of integrin $\alpha 5$ expression, cells were cultured for 2 d on plastic dishes and then prepared according to the immunoprecipitation protocol described below. For immunoprecipitation, 2 μg of integrin $\alpha 5$ mAb (Becton-Dickinson) was added to each tube of lysate, and the primary antibody used for immunodetection was integrin $\alpha 5$ polyclonal antibody (Chemicon) used at a 1:1000 dilution in blocking buffer B.

Immunoprecipitation and Immunodetection

For immunoprecipitation, cells were lysed in 4°C lysis buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM MgCl_2 , 1 mM EGTA, 100 mM NaF, 10 mM Na_2PO_4 , 1 mM Na_3VO_4 , 10% glycerol, 1% Triton X-100, and 1 $\mu\text{g}/\text{ml}$ each of aprotinin, leupeptin, chymostatin, and pepstatin) and clarified at 12,000 rpm at 4°C for 15 min. The lysates were then normalized for protein concentration to a total volume of 1 ml in lysis buffer. Antibody (1–2 μg) was added to each tube of lysate, which was then incubated on a rocker at 4°C for 2 h. The antibodies were immunoprecipitated with 50 μl of a slurry of protein A/G-Sepharose beads (Calbiochem) for 1 h on a rocker at 4°C. The beads were washed twice with lysis buffer and then boiled in sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 2% β -mercaptoethanol, 10 $\mu\text{g}/\text{ml}$ bromophenol blue) for 3 min. The beads were pelleted by brief centrifugation, and the supernatants were loaded on SDS-PAGE gels (7.5 or 10% acrylamide). The proteins were transferred to nitrocellulose and then incubated in blocking buffer B (1% BSA [Bio-Rad], 100 mM Tris-Cl, pH 7.4, 0.9% NaCl, 0.1% Nonidet) overnight at 4°C.

For immunodetection, the blocked blots were probed with primary antibody for 2 h at 4°C on a rocker. The blots were washed twice for 10 min each in blocking buffer B and then incubated with a 1:2000 dilution of a rabbit anti-mouse secondary antibody (for monoclonal primary antibodies). After two washes in blocking buffer B, the blots were finally incubated either with a 1:5000 dilution of alkaline phosphatase-conjugated rabbit anti-mouse antibodies (Jackson ImmunoResearch) or 50 ng/ml ^{125}I -protein A in blocking buffer B. Alkaline phosphatase was detected by a colorimetric reaction with the use of a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium kit (Zymed). ^{125}I -protein A-labeled blots were detected with the use of a Molecular Imager FX (Bio-Rad).

Cell Proliferation Assay

Cells were dispersed and plated at 40,000 cells per well in 96-well dishes. At various days in culture, the cells were gently washed twice with 100 μl per well of ice-cold blocking buffer A and twice with 100 μl per well of ice-cold PBS. The cells were fixed for 10 min in 100% ice-cold methanol (100 μl per well) and then allowed to air-dry. The cells were stained with 100 μl per well of 0.1% crystal violet in water for 10 min and then washed gently four times with double-distilled water and four times with PBS. The plates were then air-dried completely. The stained cells were solubilized in 1% sodium deoxycholate, and the plates were read at 590 nm in a spectrophotometer. The absorption at 590 nm is proportional to the number of attached cells (our unpublished results).

Signal Transduction

Cells were incubated in cell culture medium supplemented with mAb 225 (10 $\mu\text{g}/\text{ml}$ final concentration), mAb integrin $\alpha 5$ (Becton-Dickinson; 5 $\mu\text{g}/\text{ml}$ final concentration), PD 98059 (20–100 μM final concentration), or Compound 32 (4 μM final concentration) for various numbers of days. The degree of activation for immunoprecipitated EGFR, Shc, and Grb2 proteins was determined by immunodetection of antiphosphotyrosine with PY-20 mAb (1:2000 dilu-

tion in blocking buffer B; see Immunoprecipitation and Immunodetection for details). Activation of ERK1 and ERK2 was determined by immunodetection of cell lysates with phospho-MAPK E10 mAb (1:2000 dilution in blocking buffer B). Activation of MEK1 was determined by immunodetection of MEK1 immunoprecipitates with phosphoserine mAb (1:1000 dilution in blocking buffer B). Loading control experiments were performed by immunodetection of immunoprecipitates or lysates with EGFR mAb (1:500 dilution in blocking buffer B), Shc polyclonal antibody (1:5000 dilution in blocking buffer B), Grb2 mAb (1:2000 dilution in blocking buffer B), ERK1 mAb (1:2000 dilution in blocking buffer B), and MEK1 mAb (1:1000 dilution in blocking buffer B). PCNA expression was determined by immunodetection of cell lysates with PCNA mAb (1:5000 dilution in blocking buffer B).

RESULTS

Expression of Integrins in Caco-2 Cells

Caco-2 cells expressed integrins $\alpha 2$, $\alpha 3$, $\alpha 4$, $\beta 1$, $\beta 3$, $\beta 4$, and αV , but not $\alpha 5$, by indirect immunofluorescence microscopy (our unpublished results). Flow cytometry with two different mAbs raised against the human $\alpha 5$ integrin subunit failed to detect $\alpha 5$ integrin subunit expression in Caco-2 cells (Figure 1A, left panel), whereas another colonic epithelial cell line, SW-620, was positive for integrin $\alpha 5$ expression by flow cytometry (Figure 1A, right panel).

Caco-2 cells were transfected with the human integrin $\alpha 5$ subunit or vector alone (controls). Two stable integrin $\alpha 5$ transfectants (A105 and A9) and two stable control transfectants (B6 and B7) were isolated after selection in G418 and used in all subsequent experiments. Integrin $\alpha 5$ expression was confirmed in the transfectants by flow cytometry (Figure 1B) and Western blot analysis of integrin $\alpha 5$ immunoprecipitates (Figure 1C). Because other integrin receptor subunits are involved in fibronectin binding ($\alpha 3/\beta 1$, $\alpha 4/\beta 1$, and αV), the expression of integrin $\alpha 3$, $\alpha 4$, and αV was compared between the integrin $\alpha 5$ transfectants and control cells by flow cytometry (Figure 1D). The integrin $\alpha 5$ transfectants and control cells expressed the integrin $\alpha 3$, $\alpha 4$, and αV subunits at similar levels except for clone B6, which expressed lower levels of integrin αV , which probably represents minor clonal variation.

Fibronectin Overcomes Inhibition of EGFR-mediated Cell Proliferation

Autocrine growth factor signaling by the EGFR has been shown to be important in Caco-2 cell proliferation. We have shown that exogenously applied EGF family growth factors have little effect on Caco-2 cell proliferation. This may be because the cells already express EGF family ligands (amphiregulin, TGF α , EGF, and HB-EGF). (Damstrup *et al.*, 1999; Kuwada *et al.*, 1999). However, incubating Caco-2 cells with EGFR-blocking antibodies significantly reduces cell proliferation by interrupting autocrine growth factor binding (Damstrup *et al.*, 1999; Kuwada *et al.*, 1999).

The Caco-2 integrin $\alpha 5$ transfectants grew at similar rates as the control cells on plastic (Figure 2, A and B) or fibronectin-coated plastic (Figure 2, C and D). In the absence of exogenous fibronectin, both the integrin $\alpha 5$ transfectants (Figure 2A) and control cells (Figure 2B) demonstrated significant inhibition of cell proliferation in the presence of 10 $\mu\text{g}/\text{ml}$ of the antagonistic EGFR mAb 225, which inhibits

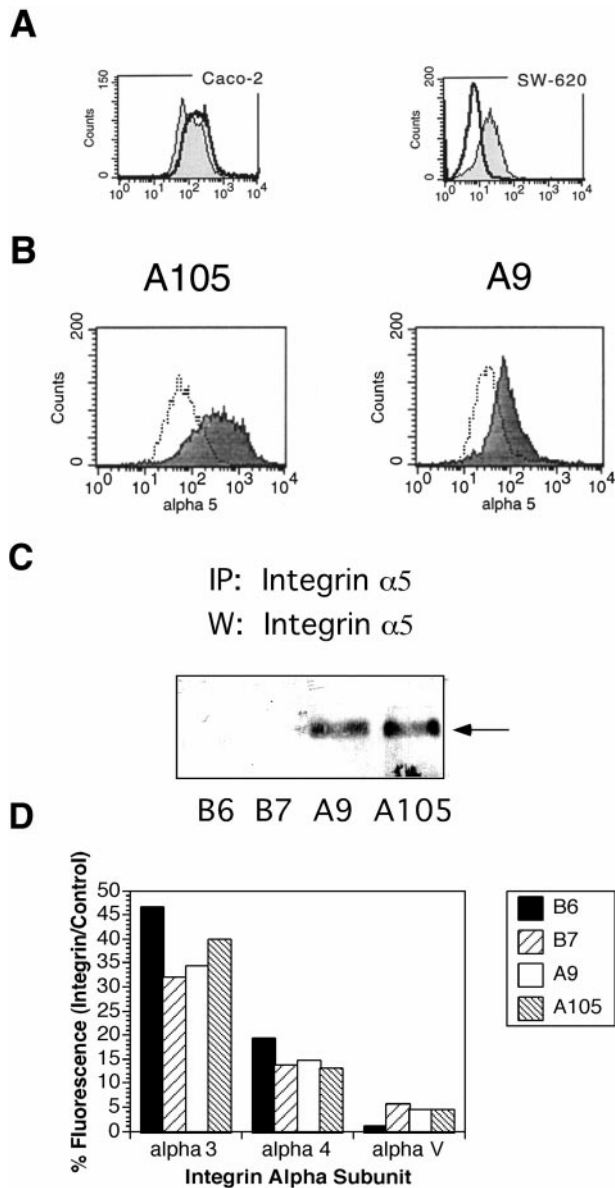


Figure 1. (A) Flow cytometry of Caco-2 and SW-620 cells demonstrating integrin $\alpha 5$ expression (gray areas). (B) Flow cytometry of Caco-2 cell lines, A105 and A9, stably transfected with integrin $\alpha 5$ (gray areas). The cells were stained with an integrin $\alpha 5$ mAb and FITC-conjugated goat anti-mouse antibodies or with FITC-labeled goat anti-mouse antibodies alone (controls; unshaded areas). The ordinate represents the number of cells counted (in thousands) and the abscissa the fluorescein intensity. (C) Immunoblots of integrin $\alpha 5$ immunoprecipitates from Caco-2 cells stably transfected with vector alone (B6 and B7) and integrin $\alpha 5$. A single ~ 120 -kDa band (arrow), corresponding to the heavy chain of integrin $\alpha 5$, was obtained under reducing conditions. (D) Integrin $\alpha 3$, $\alpha 4$, and αV subunit expression in the control and integrin $\alpha 5$ -transfected cells determined by flow cytometry. The transfectants were stained with integrin $\alpha 3$, $\alpha 4$, and αV mAbs followed by FITC-conjugated goat anti-mouse antibodies. For each integrin subunit, the fluorescence intensity was expressed as a percentage of the controls (stained with FITC-conjugated goat anti-mouse antibodies alone).

ligand binding to the EGFR, resulting in diminished EGFR tyrosine kinase activity (Gill *et al.*, 1984). On fibronectin-coated plastic, however, the integrin $\alpha 5$ transfectants grew at nearly the same rate in the absence or presence of mAb 225 (Figure 2C). In contrast, the control cells were growth inhibited to a similar degree by mAb 225 on uncoated or fibronectin-coated plastic (Figure 2, compare B and D). In addition, mAb 225 inhibited fibronectin-induced expression of the cell cycle protein PCNA in the control cells but not in the integrin $\alpha 5$ -transfected cells at d 2 in culture (Figure 3). Thus, the inhibition of autocrine EGFR-mediated cell cycle progression and proliferation was overcome in the integrin $\alpha 5/\beta 1$ -expressing cells in the presence of exogenous fibronectin. This ability of the integrin $\alpha 5/\beta 1$ -expressing cells to overcome EGFR inhibition appeared to occur early during the 7-d cell proliferation studies when the Caco-2 cells were subconfluent. This may reflect more of a role for integrin $\alpha 5/\beta 1$ -mediated cell proliferation when Caco-2 cells are rapidly proliferating and not yet fully differentiated.

To determine whether EGFR kinase activity was essential to the ability of the integrin $\alpha 5$ -transfected cells to overcome mAb 225-mediated growth inhibition on fibronectin, we performed parallel proliferation studies with the use of the tyrosine kinase inhibitor tyrphostin Compound 32. Compound 32 directly inhibits EGFR tyrosine kinase activity by competing for the ATP-binding site. Compound 32, used at a final concentration of 4 μ M, caused a significant inhibition of cell proliferation for the integrin $\alpha 5$ -transfected (Figure 4A) and control (Figure 4B) cells on plastic and inhibited EGFR autophosphorylation (our unpublished results). Unlike the case for mAb 225, culturing the integrin $\alpha 5$ -transfected cells on fibronectin-coated plastic could not reverse the growth inhibition caused by compound 32 (Figure 4A). These results suggested that EGFR kinase activity was necessary for the integrin $\alpha 5$ -transfected cells to overcome mAb 225-mediated growth inhibition in the presence of fibronectin. This implied that fibronectin might induce EGFR activation.

Integrin $\alpha 5/\beta 1$ -mediated Activation of the EGFR

Recent reports showed that the EGFR could be activated by integrin-mediated fibronectin binding (Moro *et al.*, 1998), even in the absence of growth factors (Li *et al.*, 1999). Therefore, we examined whether the EGFR was activated by adhesion to fibronectin in the integrin $\alpha 5$ -transfected and control cells. We found that the EGFR was activated in both the control and integrin $\alpha 5$ -transfected cells when adhered to fibronectin but not to plastic (Figure 5A).

The EGFR can activate Shc and Grb2, signaling proteins important in EGFR-mediated cell proliferation (Rozakis-Adcock *et al.*, 1993; Sasaoka *et al.*, 1994). Thus, we examined their levels of tyrosine phosphorylation in the control and integrin $\alpha 5$ -transfected cells (Figure 5, B and C). The levels of tyrosine phosphorylation of Shc and Grb2 were similar between the integrin $\alpha 5$ transfectants and controls.

Although the levels of tyrosine phosphorylation of total cellular Shc protein were similar under the various conditions, only a small proportion of total cellular Shc may be recruited by activated EGFR. Therefore, the levels of EGFR protein associated with Shc were studied in the control and integrin $\alpha 5$ -transfected cells. The control and integrin $\alpha 5$ transfectants were grown on either uncoated or fibronectin-coated plastic for 2 d and then lysed. The presence of EGFR

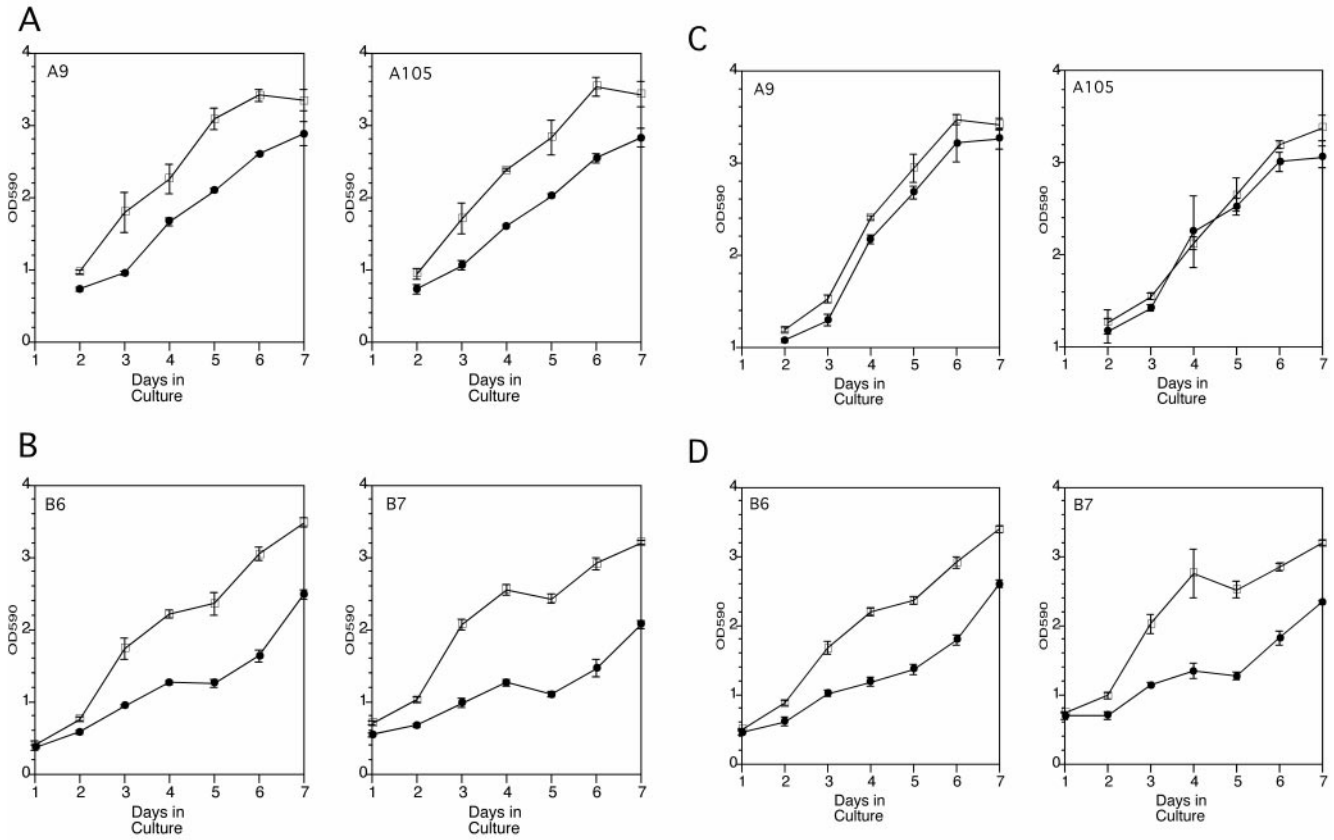


Figure 2. Proliferation assays of control and integrin $\alpha 5$ -transfected cells on plastic in the presence (●) or absence (□) of mAb 225. (A) Integrin $\alpha 5$ -transfected cells cultured on plastic. (B) Control cells cultured on plastic. (C) Integrin $\alpha 5$ -transfected cells cultured on fibronectin-coated plastic. (D) Control cells cultured on fibronectin-coated plastic. Cell counts were estimated with the use of a crystal violet staining method. The ordinate represents the optical density at 590 nm and the abscissa the number of days in culture. Cells were plated at 50,000 cells per well in 96-well plates at day 0. Each data point represents the average \pm SEM of triplicate experiments.

protein, tyrosine phosphorylated EGFR, and Shc protein was detected in Shc immunoprecipitates. Although Shc was immunoprecipitated from all the cell lysates under the various conditions at equal levels (Figure 6A, left panel), EGFR protein coprecipitated with Shc only in the integrin $\alpha 5$ -

transfected cells cultured on fibronectin-coated plastic (Figure 6B, right panel). Furthermore, EGFR protein that coprecipitated with Shc in the integrin $\alpha 5$ -transfected cells was tyrosine phosphorylated (Figure 6C, right panel), which is consistent with previous reports that Shc binds only to activated EGFR (Pelicci *et al.*, 1992; Ruff-Jamison *et al.*, 1993; Sasaoka *et al.*, 1994). Thus, the interaction of Shc with the EGFR was exclusive to the integrin $\alpha 5$ transfectants that were cultured on fibronectin.

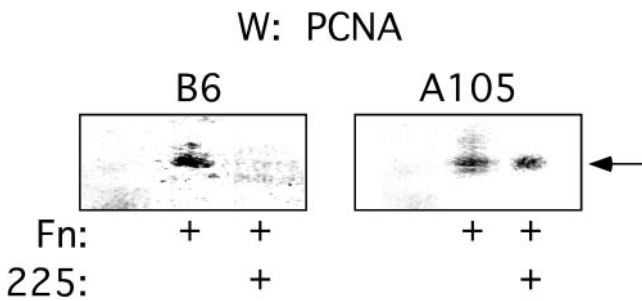


Figure 3. Immunoblot of PCNA performed on lysates of control and integrin $\alpha 5$ -transfected cells cultured for 2 d on uncoated or fibronectin (Fn)-coated plastic in the absence or presence of mAb 225 (10 μ g/ml). All lanes represent equal total protein concentrations.

Integrin $\alpha 5/\beta 1$ and EGFR-mediated MAPK Activation

Activated EGFR and integrin $\alpha 5/\beta 1$ can recruit both Shc and Grb2. Grb2 then recruits SOS, which activates RAS and leads to the recruitment of Raf to the cell membrane. Raf then activates MEK1, which in turn activates ERK1 and ERK2. Because MAPKs are important mediators of growth factor-induced cell proliferation (Pages *et al.*, 1993; Cowley *et al.*, 1994), we examined the ability of integrin $\alpha 5/\beta 1$ to activate ERK1 and ERK2.

Integrin $\alpha 5$ -transfected and control cells were cultured on uncoated or fibronectin-coated plastic dishes in the absence

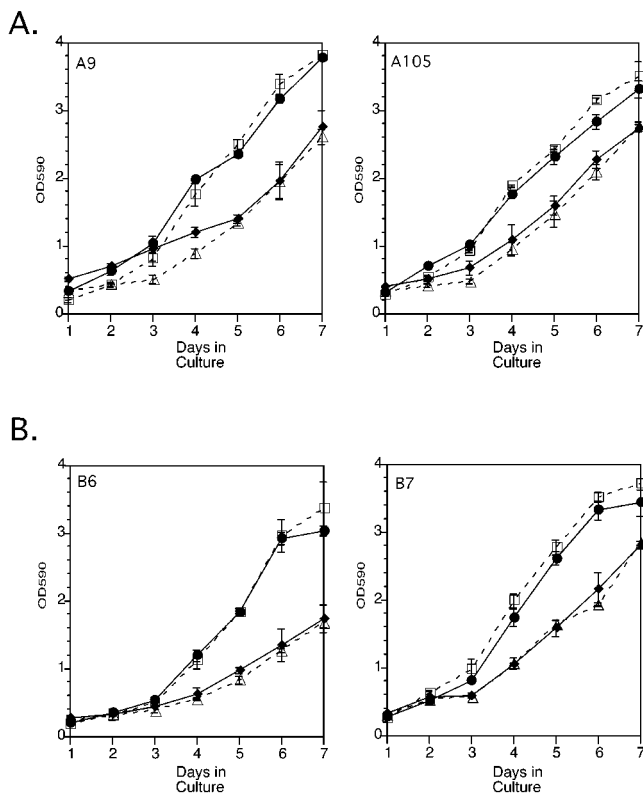


Figure 4. Proliferation assays of control and integrin $\alpha 5$ -transfected cells cultured on uncoated or fibronectin-coated plastic in the presence or absence of 4 μM Compound 32. (A) Integrin $\alpha 5$ -transfected cells cultured on plastic without (\square) and with (\triangle) Compound 32 or on fibronectin without (\bullet) and with (\blacklozenge) Compound 32. (B) Control cells cultured on plastic without (\square) and with (\triangle) Compound 32 or on fibronectin without (\bullet) and with (\blacklozenge) Compound 32. Each data point represents the mean \pm SEM for experiments performed in triplicate.

or presence of mAb 225 for 8 d. At various times from 2 to 8 d in culture, the cells were lysed and Western blots with an activated MAPK mAb were performed. Between 2 and 5 d in culture, significant differences in constitutive MAPK activation were seen between integrin $\alpha 5$ -transfected and control cells. When the control cells were cultured on uncoated or fibronectin-coated plastic, the activation of ERK1 and ERK2 was significantly inhibited in the presence of mAb 225 (Figure 7A, top left panel). Constitutive ERK1 and ERK2 activation in the integrin $\alpha 5$ transfectants grown on plastic was inhibited by mAb 225 (Figure 7A, top right panel). However, when the integrin $\alpha 5$ -transfected cells were cultured on fibronectin-coated plastic, ERK1 and ERK2 constitutive activation levels were not decreased by the presence of mAb 225 (Figure 7A, top right panel).

Similarly, constitutive ERK1 and ERK2 activation was inhibited by mAb 225 in the control cell line B7, but not in the integrin $\alpha 5$ -transfected cell line A9, when they were cultured on fibronectin (Figure 7B). When the integrin $\alpha 5$ -transfected and control cells were cultured on fibronectin in the presence of mAb 225 and an antifunctional integrin $\alpha 5$ mAb, ERK1 and ERK2 activation was unchanged in the

control cells but was inhibited in the integrin $\alpha 5$ transfectants (our unpublished results). This demonstrated that the integrin $\alpha 5/\beta 1$ -fibronectin interaction was specifically responsible for the activation of ERK1 and ERK2 in the integrin $\alpha 5$ transfectants in the presence of mAb 225.

Because MEK1 activates ERK1 and ERK2, we examined MEK1 activation. Activated (serine-phosphorylated) MEK1 was detected when the control cells (Figure 7C, top left panel) and integrin $\alpha 5$ transfectants (Figure 7C, top right panel) were cultured on uncoated or fibronectin-coated plastic. However, when the cells were cultured on fibronectin-coated plastic in the presence of mAb 225, activated MEK1 was not detected in the control cells (Figure 7C, top left panel) but was detected in the integrin $\alpha 5$ transfectants (Figure 7C, top right panel).

To determine whether MAPK activation was important to cell proliferation, the integrin $\alpha 5$ transfectants and controls were grown on fibronectin-coated or uncoated plastic in the presence or absence of the MEK inhibitor PD98059 for 8 d. In the presence of 50 or 100 μM PD98059, the growth of the control cells and integrin $\alpha 5$ transfectants was significantly inhibited on plastic and fibronectin (our unpublished results). Treatment of the control cells and integrin $\alpha 5$ transfectants with PD98059 (20 μM) significantly decreased ERK1 and ERK2 activation (our unpublished results). These results demonstrated the overall importance of ERK1 and ERK2 activation to the proliferation of the integrin $\alpha 5$ transfectants and control cells.

To determine whether EGFR kinase activity was necessary for ERK1 and ERK2 activation, the integrin $\alpha 5$ -transfected and control cells were grown in the presence of Compound 32 for 48 h, after which ERK1 and ERK2 activation were determined. For both the integrin $\alpha 5$ -transfected and control cells, Compound 32 resulted in the near-total inhibition of activated ERK1 and ERK2 in the presence or absence of fibronectin (our unpublished results).

These data suggested that ERK1 and ERK2 activation was dependent on the following: 1) EGFR kinase activity in both the integrin $\alpha 5$ -transfected and control cells, and 2) autocrine EGFR activation in the control cells but not in the integrin $\alpha 5$ -transfected cells.

Because focal adhesion kinase (FAK) could potentially mediate integrin activation of MAPK, FAK tyrosine phosphorylation was also studied in the transfectants. No significant differences in FAK tyrosine phosphorylation were seen for the control or integrin $\alpha 5$ -expressing cells cultured on plastic or fibronectin-coated plastic in the absence or presence of mAb 225 (our unpublished results).

Anchorage-independent Proliferation

A previous study showed that the expression of integrin $\alpha 5$ in the HT-29 colon cell line resulted in growth inhibition in the absence of exogenous fibronectin (Varner *et al.*, 1995). There was no growth arrest in the integrin $\alpha 5$ -transfected versus control Caco-2 cell lines in the absence of fibronectin. This may have been due to the fact that Caco-2 cells express fibronectin (Figure 8A) and HT-29 cells reportedly do not. Our results seemed paradoxical in that although the integrin $\alpha 5$ -transfected cells expressed endogenous fibronectin, the reversal of mAb 225-mediated growth inhibition could occur only in the presence of exogenous fibronectin. Most of the endogenous fibronectin protein recovered

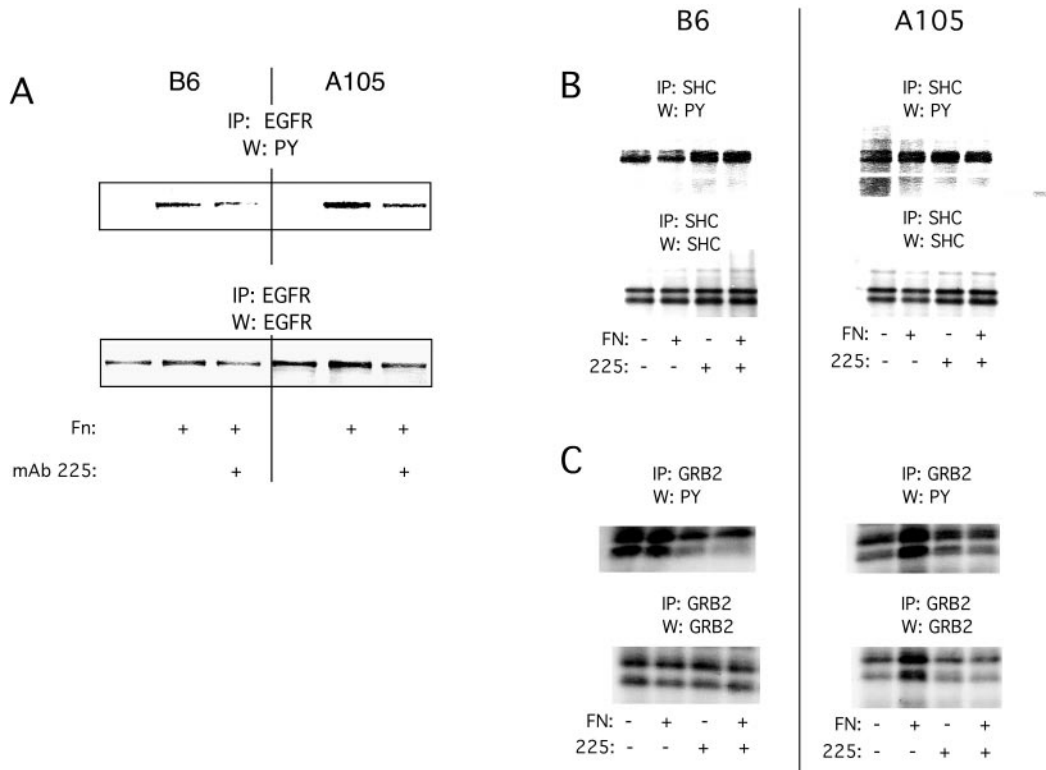


Figure 5. Control (B6) and integrin $\alpha 5$ -transfected (A105) cells were cultured for 2 d on uncoated or fibronectin-coated plastic in the presence or absence of mAb 225 (10 μ g/ml). Immunoprecipitates of EGFR, Shc, and Grb2 were obtained from lysates normalized for total protein and resolved by SDS-PAGE. Immunoblotting was then performed. (A) Phosphotyrosine (top panels) and EGFR (bottom panels) immunoblots of EGFR immunoprecipitates. (B) Phosphotyrosine (top panels) and Shc (bottom panels) immunoblots of Shc immunoprecipitates. (C) Phosphotyrosine (top panels) and Grb2 (bottom panels) immunoblots of Grb2 immunoprecipitates.

from the Caco-2 transfectants was associated with the cells themselves (our unpublished results). The exogenous fibronectin was presented to the cells on a fixed, rigid, and planar surface. It has been reported that exogenous fibronectin results in more robust cell adhesion in human epithelial cells (Wang *et al.*, 1999). Furthermore, recent studies have shown that extracellular matrix substrates can elicit completely different cellular responses

depending on whether they are presented to cells in a rigid or malleable form (Bissell and Guzelian, 1980; Bissell *et al.*, 1987; Ben-Ze'ev *et al.*, 1988; Garcia *et al.*, 1999). This may be due to the ability of a rigid extracellular matrix to provide forces of tension to cellular structures, which can effect specific signal transduction activity (Miyamoto *et al.*, 1995, 1998). Fibronectin deposited on the cell surface or presented to the cell in its malleable form, on the other

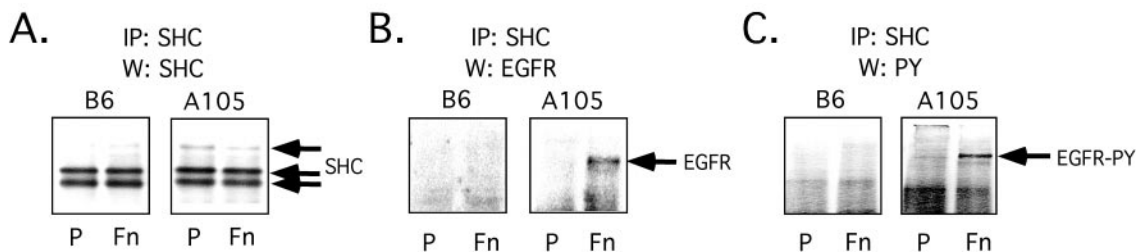


Figure 6. Shc (A), EGFR (B), and phosphotyrosine (C) immunoblots were performed on Shc immunoprecipitates from control and integrin $\alpha 5$ -transfected cells grown for 2 d on uncoated (P) or fibronectin-coated (Fn) plastic. Shc immunoprecipitation was performed on the different lysates that had been normalized for total protein. Each of the immunoprecipitates was then divided into three aliquots, which were used to generate the different immunoblots in A, B, and C. Panel A shows that equal amounts of Shc were immunoprecipitated from the different cell lysates.

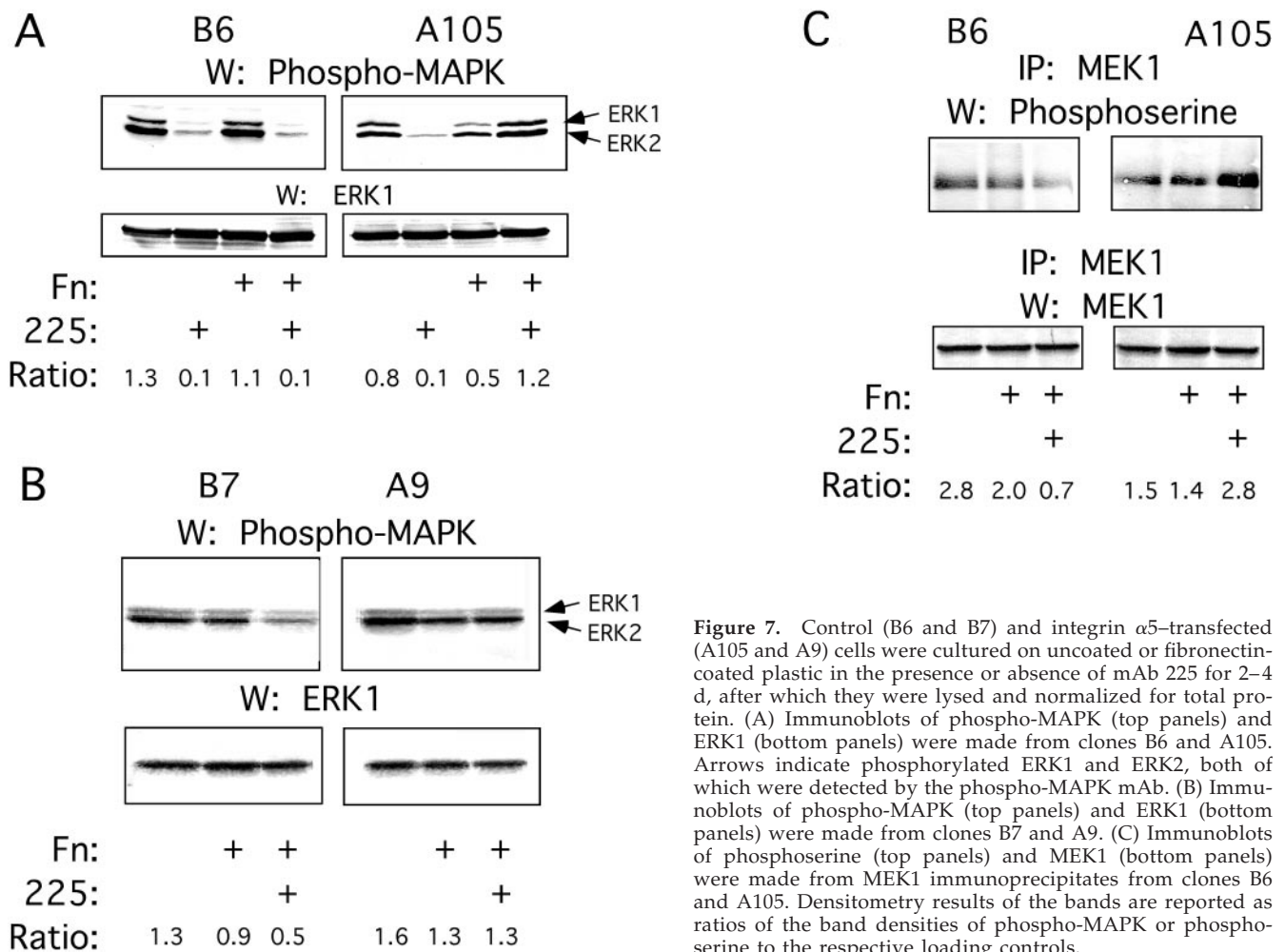


Figure 7. Control (B6 and B7) and integrin $\alpha 5$ -transfected (A105 and A9) cells were cultured on uncoated or fibronectin-coated plastic in the presence or absence of mAb 225 for 2–4 d, after which they were lysed and normalized for total protein. (A) Immunoblots of phospho-MAPK (top panels) and ERK1 (bottom panels) were made from clones B6 and A105. Arrows indicate phosphorylated ERK1 and ERK2, both of which were detected by the phospho-MAPK mAb. (B) Immunoblots of phospho-MAPK (top panels) and ERK1 (bottom panels) were made from clones B7 and A9. (C) Immunoblots of phosphoserine (top panels) and MEK1 (bottom panels) were made from MEK1 immunoprecipitates from clones B6 and A105. Densitometry results of the bands are reported as ratios of the band densities of phospho-MAPK or phosphoserine to the respective loading controls.

hand, cannot provide fixed points against which cellular structures can be anchored and tensioned.

To study this, we cultured the Caco-2 transfectants in soft agar in which any endogenous fibronectin could not be deposited as a rigid extracellular matrix. In soft agar, the integrin $\alpha 5$ -transfected cells were growth inhibited compared with the control cells (Figure 8B). These results are similar to those in HT-29 colonic epithelial cells expressing integrin $\alpha 5/\beta 1$ (Varner *et al.*, 1995). Interestingly, the shape of Caco-2 cells cultured in soft agar is round (Figure 8B), although they become columnar when cultured on a rigid surface (Hidalgo *et al.*, 1989). Cell shape is important in determining cell proliferation (Folkman and Moscona, 1978), and integrin-extracellular matrix interactions are important transducers of changes in cell shape (Ingber, 1990). It appears that although Caco-2 cells express fibronectin, only fibronectin presented to the cell as a rigid substrate can stimulate cell proliferation, presumably by both clustering and activating fibronectin receptors. Thus, Caco-2 cells transfected with integrin $\alpha 5$ are capable of depositing fibronectin on plastic, which may be sufficient to prevent integrin $\alpha 5/\beta 1$ -mediated growth inhibition in these cells.

DISCUSSION

Expression of human integrin $\alpha 5$ in Caco-2 cells resulted in significant differences in Caco-2 cell proliferation and signal transduction events. Fibronectin reversed the growth inhibition by mAb 225 for the integrin $\alpha 5$ -transfected cells but not the control cells. The reversal of mAb 225-mediated growth inhibition was associated with the activation of EGFR, MEK1, ERK1, and ERK2 and the expression of PCNA. Furthermore, the fibronectin-mediated activation of ERK1 and ERK2 was specific to integrin $\alpha 5/\beta 1$. Direct inhibition of EGFR tyrosine kinase activity by Compound 32 decreased cell proliferation and ERK1 and ERK2 activation. These effects of Compound 32 could not be reversed by fibronectin. These results suggested that EGFR tyrosine kinase activity was necessary for the fibronectin-induced and integrin $\alpha 5/\beta 1$ -mediated activation of ERK1 and ERK2 as well as cell proliferation. Furthermore, the inhibition of MAPK activity significantly decreased the proliferation of the control and integrin $\alpha 5$ -transfected Caco-2 cells.

Although tyrosine phosphorylation of the EGFR was seen in both the control and integrin $\alpha 5$ -transfected cells cultured

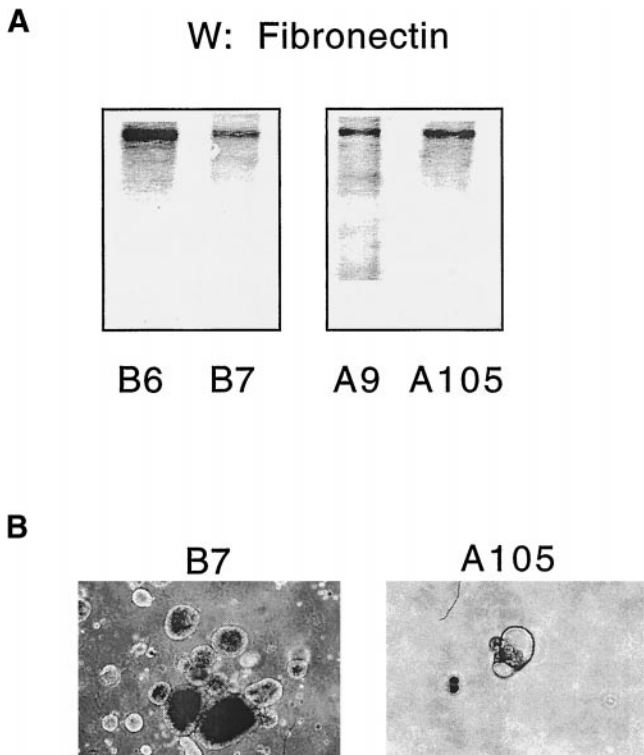


Figure 8. (A) Fibronectin immunoblot of control (B6 and B7) and integrin $\alpha 5$ -transfected (A9 and A105) cell lines cultured on plastic for 4 d. (B) Photomicrographs ($\times 40$) of control (B7) and $\alpha 5$ -transfected (A105) cell lines grown in soft agar for 6 d. Cells were seeded at the same density on d 1, and the assays were performed in triplicate.

on fibronectin, the EGFR coprecipitated with Shc only in the integrin $\alpha 5$ transfectants and in the presence of fibronectin. This suggests that the clustering of integrin $\alpha 5 / \beta 1$ receptors by fibronectin resulted in the access of activated EGFR to Shc. Integrins $\alpha 1 / \beta 1$ and $\alpha 5 / \beta 1$, but not other $\beta 1$ -containing integrin receptors, are coupled to the Ras-ERK pathway through Shc (Wary *et al.*, 1996, 1998). Integrin clustering by fibronectin and integrin $\alpha 5$ antibodies leads to the formation of large signaling complexes containing many substrates of the EGFR, including Grb2 and Shc (Mainiero *et al.*, 1995, 1997; Miyamoto *et al.*, 1995, 1998; Wary *et al.*, 1996, 1998). In addition, integrins can cluster and coprecipitate with EGFRs (Miyamoto *et al.*, 1996; Moro *et al.*, 1998), and we were able to coprecipitate the EGFR with integrin $\alpha 5$ from our integrin $\alpha 5$ -transfected cells as well (our unpublished results). Although the activated EGFR can recruit both Grb2 and Shc, the latter appears to be the preferred substrate through which the EGFR activates SOS and then RAS (Sasaoka *et al.*, 1994). Thus, fibronectin not only activates the EGFR but may result in the spatial juxtaposition of Shc with activated EGFRs. This suggests that integrins may play an important spatial regulatory role in controlling interactions between the EGFR and certain substrates. Finally, on fibronectin, ERK1, ERK2, and MEK1 were constitutively activated in the control and integrin $\alpha 5$ -transfected cells but were inhibited by mAb 225 only in the control cells. This suggests that the

activation of ERK1 and ERK2 may have occurred through somewhat different mechanisms in the two cell types.

Two recent studies that support our results demonstrated activation of the EGFR itself by integrin ligation. In one study, B82L fibroblasts migrated in response to EGF only when the cells were adhered to fibronectin (Li *et al.*, 1999). Furthermore, migration of these cells was dependent on integrin-mediated activation of an intact EGFR in a ligand (growth factor)-independent manner, thus placing the EGFR downstream of integrins for migratory signaling. In another study, when NIH 3T3 cells transfected with the human EGFR were adhered to fibronectin, activation of EGFR, Shc, and ERK1 occurred (Moro *et al.*, 1998). This integrin-mediated activation of the EGFR occurred in the absence of growth factors and was necessary for cell survival and entry into S phase of the cell cycle in the presence of exogenous EGF or serum. Treatment of the cells with the EGFR-specific tyrosine kinase inhibitor AG1478 or cotransfection with a dominant-negative mutant EGFR nearly abolished activation of EGFR, Shc, and ERK1 in response to adhesion to fibronectin. This again placed the EGFR downstream of integrins in integrin-mediated ERK1 activation. However, integrin-mediated activation of the EGFR alone was insufficient to drive cell proliferation in the absence of exogenous growth factors, suggesting that integrins potentiated growth factor-mediated activation of ERK1.

A previous study showed that the expression of integrin $\alpha 5$ in the HT-29 colon cell line resulted in growth inhibition in the absence of exogenous fibronectin (Varner *et al.*, 1995). There was no growth arrest in the integrin $\alpha 5$ -transfected versus control Caco-2 cell lines in the absence of fibronectin. This may have been due to the fact that Caco-2 cells express endogenous fibronectin and HT-29 cells do not.

The mechanism by which integrin-mediated EGFR activation occurs is currently unknown. It is possible that EGFR activation occurs through a ligand-dependent process such as intracrine EGFR activation (Kennedy *et al.*, 1993; Cao *et al.*, 1995). However, intracrine EGFR activation has been described in only a few cell types, and a growth factor-independent mechanism is more attractive because integrin-mediated EGFR activation occurred in cells devoid of any detectable EGF family growth factor expression (Moro *et al.*, 1998; Li *et al.*, 1999). Another potential mechanism is integrin-induced activation of the EGFR through heterodimerization with another member of the erbB family of receptors (Gamett *et al.*, 1997; Graus-Porta *et al.*, 1997). Caco-2 cells express erbB-2 receptors (our unpublished data), and heterodimerization of the EGFR with erbB-2 could lead to the transactivation of the EGFR even in the absence of EGFR ligand-induced activation (Gamett *et al.*, 1997; Worthylake *et al.*, 1999). In addition, certain colon cancer cells have been reported to express heregulins that stimulate cell proliferation (Vadlamudi *et al.*, 1999). Heregulins can bind to erbB-3 and erbB-4 receptors, which can in turn activate erbB-2, allowing it to heterodimerize with and activate the EGFR. Whether integrins can induce heregulin expression or its binding to erbB3 or erbB4 is unknown. Furthermore, there are no reports yet supporting integrin-stimulated heterodimerization of erbB receptors. Further studies will be necessary to determine whether integrins can cause the activation of erbB-2, thus leading to heterodimerization and activation of the EGFR.

In summary, our results show that the EGFR-RAS-MAPK pathway can be activated downstream of integrin $\alpha 5/\beta 1$ and stimulate epithelial cell proliferation. This adds to a growing body of literature supporting the view that the EGFR-RAS-MAPK pathway can be used as an important signaling pathway by other receptors to control or modulate critical cell functions attributed to EGRF-mediated signaling (Daub *et al.*, 1996; Rosen and Greenberg, 1996; Zwick *et al.*, 1997; Li *et al.*, 1998). Thus, the EGFR appears to play an important role in integrating environmental cues as diverse as cytokines, UV light, and extracellular matrix proteins (Sachsenmaier *et al.*, 1994; Daub *et al.*, 1996, 1997; Moro *et al.*, 1998; Li *et al.*, 1999) to achieve regulation of cell migration, survival, and proliferation. The fine level of integration and orchestration of signaling performed by the EGFR is underscored by the tumorigenic effects caused by the aberrant expression and/or functioning of proteins at all levels of the EGFR-Ras-ERK signal transduction cascade (Gerosa *et al.*, 1989; Ishitoya *et al.*, 1989; Cook *et al.*, 1992; Itakura *et al.*, 1994; Mansour *et al.*, 1994; Rajkumar and Gullick, 1994; Hirono *et al.*, 1995; Normanno *et al.*, 1995; Huang *et al.*, 1996; Stumm *et al.*, 1996; Bucci *et al.*, 1997; Kuttan and Bhakthan, 1997; Sekine *et al.*, 1998).

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