Differences in Sensitivity of Biological Functions Mediated by Epidermal Growth Factor Receptor Activation with Respect to Endogenous and Exogenous Ligands


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ABSTRACT

Despite constitutive expression of autocrine transforming growth factor-α (TGF-α) in growth factor independent colon carcinoma cells, the epidermal growth factor receptor (EGFr) is not saturated and can be further activated by exogenous EGFr ligand. Given that the activation of EGFr by exogenous growth factor has no further effect on DNA synthesis, the question arises as to what function this additional EGFr activation might have. We report that EGF induces integrin α2 expression, integrin-mediated adhesion and micromotility of HCT116 cells. The stimulatory effect of ligand on these biological functions is abrogated by treatment with AG1478 and EGFr blocking monoclonal antibody. This provides evidence that the biological responses are EGFr-mediated and EGFr is upstream of integrin α2 expression. Therefore, while exogenous EGF has no effect on DNA synthesis beyond that induced by autocrine TGF-α (at subsaturating levels of EGFr occupation) exogenous growth factor does induce integrin α2 expression, cell adhesion and micromotion. An important finding revealed by this study is the documentation of biological responses of EGFr-mediated functions including DNA synthesis, cell adhesion and micromotion, which differ in sensitivity with respect to different degrees of EGFr activation at the basal state and in response to exogenous ligand.
Introduction

Human colon carcinoma cell line HCT116 is aggressively tumorigenic, invasive, undifferentiated and growth factor independent (1-5). The HCT116 cell line is representative of growth factor-independent carcinoma cell lines (2,6). Constitutive expression of a full-length TGFα antisense cDNA has shown that the basis for the growth factor independence of these cells is the constitutive expression of TGF-α and, consequently, a low level constitutive activation of EGFr even when the cells are growth arrested in Go (6,7). We hypothesize that the relatively low level of EGFr activation resulting from autocrine TGF-α may be sufficient to fulfill a highly sensitive response(s) to EGFr signal transduction, but would require augmentation by exogenously activated EGFr to optimally enable less sensitive functions. In HCT116 cells DNA synthesis from the Go state is fully activated by autocrine TGF-α such that exogenous EGF (or other growth factors) has no effect on this EGFr function in spite of the availability of unoccupied EGFr and signal transduction intermediates such as ERK (2,7). Thus, the objective of this work was to identify whether there are any important functions mediated by the unoccupied EGFr in response to exogenous EGF and to determine whether this function is shared in part by autocrine TGF-α as well. We report that EGFr activation by exogenous ligand results in increased integrin expression, cell adhesion and cell micromotion with less sensitivity than that exhibited by the optimal mitogenic function stimulated through relatively low level receptor occupation generated by autocrine TGFα activity. In contrast, low level receptor occupation of EGFr by autocrine TGFα resulted in a relatively low level of basal integrin expression and biological function relative to that obtained with high levels of receptor occupation generated by exogenous EGF or TGFα.
Growth factors are important effectors of cell adhesion, cell motility and integrin expression although the underlying mechanisms are still unclear (8-10). It has been demonstrated that EGF can induce \( \beta_1 \) integrin mRNA expression in quiescent mouse 3T3 cells (11), but it was not determined whether EGF-induced changes in \( \beta_1 \) subunit mRNA expression led to changes in cell surface protein levels as well as functional alterations in cell adhesion. Fujii et al. showed that EGF induced HSC-1 human cutaneous squamous carcinoma cell interaction with type I collagen by up-regulation of integrin \( \alpha_2 \beta_1 \) but not by \( \alpha_3 \beta_1, \alpha_5 \beta_1 \) or \( \alpha_\gamma \beta_3 \) expression (12). Recently, Moro et al. showed that in normal human skin fibroblasts and FCV 304 endothelial cells, integrin-dependent EGFr activation was associated with cell survival and proliferation in response to ECM (13). These reports suggested EGFr activation of integrin expression might represent an additional function to that of mitogenesis by EGFr signaling in HCT116 cells.

EGFr-mediated control of integrin expression is important because integrins and their ligands have significant roles in tumor cell biology (14,15). For example, transformation of epithelial cells to the malignant state is often accompanied by quantitative changes in integrin expression which in turn may control cell proliferation and cell metastasis (16,17). Moreover, alteration of integrin expression can in turn lead to crosstalk with growth factor signaling by the insulin and the transforming growth factor \( \beta \) system (18,19).

We report that exogenous EGF up-regulates cell surface integrin-\( \alpha_2 \) expression, cell adhesion, and cell micromotion on ECM protein irrespective of its inability to induce DNA synthesis above that of the basal EGFr activation in these cells induced by autocrine TGF-\( \alpha \). However, autocrine TGF-\( \alpha \) is also responsible for basal levels of integrin \( \alpha_2 \) as disruption of
autocrine TGF-α signaling by EGFr antibodies or chemical inhibitors of EGFr activation inhibit basal cell adhesion, cell locomotion and integrin α2 expression. This indicates differences in sensitivity of EGFr responses in which the signal transduction pathway leading to DNA synthesis is fully saturated by a relatively low level of EGFr activation while the signal transduction pathway leading to integrin expression is not saturated by a low level of EGFr activation, instead showing increased response with increasing receptor saturation.
EXPERIMENTAL PROCEDURES

Materials - Collagen type IV (CN IV) and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Polyclonal antibody specific for integrin α2 subunit (Ab1936) was procured from Chemicon International Inc. (Temecula, CA) and monoclonal antibodies PIE6 (α2), PIB5 (α3), and PID6 (α5) were purchased from Life Technologies, (San Diego, CA). EGFr monoclonal blocking antibody, mAb528 and mAb225 was obtained from Oncogene Science, (Manhasset, N.Y.) and tyrphostin AG1478 was purchased from Calbiochem. Anti-EGFr (activated) and anti-actin monoclonal antibodies were purchased from Transduction Laboratories (San Diego, CA) and Santa Cruz Biotechnology (Santa Cruz, CA) respectively. McCoy’s 5A medium, transferrin and insulin were obtained from Sigma, whereas EGF was purchased from R&D Systems (Minneapolis, MN). Arrays of gold-film coated electrodes for cell motility experiments were purchased from Applied Biophysics Inc, New York.

Cell Culture and Adhesion Assay - HCT116 cells and TGF-α antisense mRNA expressing HCT116 clones 33 were previously established in tissue culture and extensively characterized (5,7). Cells were maintained at 37°C in a humidified incubator with 5% CO2 in chemically-defined serum-free medium consisting of McCoy’s 5A medium supplemented with 4 μg/ml transferrin (T) and 20 μg/ml insulin (I) either in the absence or presence of EGF (E) (10 ng/ml) depending upon experimental design.

For adhesion assays, 96-well tissue culture clusters were coated with CN IV by allowing 0-2.5 μg/ml of collagen in 0.25M acetic acid to bind to culture plates at room temperature
overnight, followed by blocking with 3% BSA for 3h at room temperature. Subsequently, the methylthiazole tetrazolium (MTT) procedure was followed as described previously (20,21).

For the inhibition of adhesion by specific antibodies, 96-well tissue culture clusters were coated with CN IV as described above. Mouse ascites anti-integrin α2, α3, and α5 subunit monoclonal antibodies, were added to the plates as 1:50 to 1:500 dilutions and cells were incubated in the presence or absence of antibody for 30 min at 37°C. Similarly, EGFr antibody was used to determine the effects of blocking autocrine TGF-α on cell adhesion. After trypsinization, cells were incubated at 37°C with AG1478 for 3 hours as an additional approach to determine autocrine TGF-α-mediated cell adhesion functions. Subsequently, adhesion assays were performed as given above.

*Cell Surface Radiolabeling and Immunoprecipitation -* The iodination of cell surface proteins was carried out using suspended cells. Cells at 80% confluency were washed twice with phosphate-buffered saline, suspended by scraping in Tris Buffer (125 mM NaCl, 5 mM KCl, 1 mM Tris, 1 mM EDTA, pH 7.4), centrifuged and resuspended in 0.5 ml buffer solution containing 125 mM NaCl, 5 mM KCl, 1 mM CaCl2, and 25 mM HEPES, pH 7.4. Cell surface iodination was carried out by addition of 1 mCi/ml of Na $^{125\text{I}}$ (Dupont, 17 Ci/mg), 0.2 mg/ml of lactoperoxidase (Sigma), and 0.001% H$_2$O$_2$ (Sigma) for 10 min at 4°C. The reaction was stopped by centrifugation, and cells were washed 4 times in the same buffer. The cell pellet was solubilized by vortexing in an ice-cold buffer consisting of 125 mM NaCl, 1 mM MgCl2, 25 mM Tris pH 7.5 and 100 mM n-octal-β-D-glycopyranoside (Sigma) for 30 min. The insoluble
material was removed by centrifugation at 13,000x g for 10 min. The supernatant protein content was determined by the Bio-Rad assay and radioactive incorporation was calculated.

Equal amounts of supernatant protein were treated with Triton X-100 (0.5% v/v) and BSA 0.5 mg/ml, precleared by incubation with 50 µl of packed protein A-agarose beads (Oncogene Science, Manhesset, NY) for 2 hours and centrifuged. Precleared supernatants containing equal amounts of protein from each sample were incubated with monoclonal antibodies against integrin α2 at 1:50 dilution for 2 hours at 4°C with constant rotation, followed by rabbit anti-mouse IgG (ICN, Costa Mesa, CA) at 1:20 dilution for 2h at 4°C. The use of equal amounts of protein from each sample insured that changes in integrin expression were selective rather than a reflection of overall changes in protein synthesis. Immune complexes were precipitated by protein A-agarose beads for 2 hours at 4°C, washed 4 times with 1% Triton X-100, 25 mM Tris and 1 mg/ml BSA and once with 150 mM NaCl and 25 mM Tris, at pH 7.4. Laemmli buffer was added and samples heated at 100°C for 3 min, and proteins analyzed by electrophoresis on 7.5% SDS-PAGE gel, followed by Coomassie blue staining, destaining, gel drying and autoradiography.

**Biotinylation and Western Blot Analysis**

- Subconfluent cultures of cells were treated with Joklik’s EDTA for 8 min at room temperature and subsequently cells were scraped into a tube and kept on ice. The culture dish was rinsed with cold PBS and cells pooled with the Joklik’s EDTA fraction. Cells were pelleted by centrifugation in a clinical centrifuge for 1-2 min at 800 g. The pellet was washed twice with cold PBS, and cells biotinylated in suspension with NHS-LC-Biotin (Pierce), 0.1 mg/ml in DMSO at room temperature for 1 hour. Cells were washed
three times with PBS and lysed in buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂ and 1% NP40) by shearing them through a 26 gauge needle and centrifuging at 16,000 g for 20 min at 4°C in a microcentrifuge. The supernatant was analyzed for protein content by the Bio-Rad protein assay. Equal amounts of protein from treated and untreated (control) cell lysates were incubated with streptavidin agarose (GIBCO) for 90 min at 4°C. Agarose beads were pelleted by centrifugation at 4°C and subsequently washed 5 times with lysis buffer containing phenylmethyl sulphonyl fluoride. The beads were boiled in 2x Laemmli buffer containing 4% β-mercaptoethanol for 10 min and supernatant filtered through Bio-Rad columns (22) and applied in 7.5% SDS-PAGE. The proteins were transferred to nitrocellulose membranes (Hybond) by electroblotting using a mini-Bio-Rad transblot apparatus. The membrane was blocked for at least 1 hour with 5% non-fat dry milk in Triton Tris buffered saline (TTBS) and subsequently incubated overnight at 4°C with appropriate primary antibody. After washing the membrane with TTBS, it was incubated for 1 hour at room temperature with HRP-conjugated rabbit or mouse secondary antibody. The membrane was washed and detection of specific binding was achieved by using enhanced chemiluminescence (ECL) reagent (Amersham).

**RNA Isolation and Analysis by RNase Protection Assay** - Total cellular RNA was isolated by lysing cells with guanidine isothiocyanate-EDTA and fractionating the resulting extract through a cesium trifluoro acetic acid gradient (23). Equivalent amounts (40 μg) of RNA samples were used in RNase protection assays. The α₂ subunit template was constructed by subcloning a 292 base pair EcoR V-Hinc II fragment of the human α₂ subunit cDNA into plasmid PBSK(-).
High specific activity $\alpha_2$ subunit riboprobe was synthesized by T7 RNA polymerase, whereas actin antisense probe was prepared by Sp6-RNA polymerase in presence of ($^{32}$P) UTP (3,000 Ci/mmol; Amersham). High specific activity $\alpha_3$, $\alpha_5$, and $\beta_1$ subunit riboprobes were synthesized by T3 polymerase in presence of ($^{32}$P) UTP. Normalization of sample loading was assessed as previously described (4) and quantitation of protected fragments was achieved by densitometry (Alpha Imager 2000).

Cell Motility Measurements by the Electrical Cell Impedance Sensor (ECIS) Technique - Cell motility was quantitated by the micromotion detection method using the ECIS technique (24-26). Cells were plated on small gold electrodes (area $5 \times 10^{-4}$ cm$^2$) etched by photolithographic procedures on the bottom of tissue culture wells (area $0.5$ cm$^2$) (Applied BioPhysics, Troy, NY). A 1 $\mu$A, 4000 Hz AC signal from a constant current source was applied between the small electrode and a much larger counter electrode, while the culture medium acted as an electrolyte. This signal was not large enough to disturb the cells or to change cell behavior (27). The voltage of the system was monitored by a lock-in amplifier (Stanford Research Systems, Model SR 830, Sunnyvale, CA) interfaced with a computer which controlled amplifier settings as well as stored the data collected by the amplifier. The in-phase and out-of-phase voltage across the electrode were recorded by the lock-in amplifier once every second for measuring micromotion and once every two minutes for measuring cell attachment. The ECIS software (Applied BioPhysics, Troy, NY) calculated the resistance and capacitance values of the electrode over this period of time. Attachment and movement of the cells on the electrode changed the flow of the current, resulting in fluctuations in the electrode resistance and capacitance. These cellular movements were called micromotion (25) and were a measure of the motile ability of the cell being...
measured. As the cells moved on the electrode, the sensitive nature of the lock-in amplifier detected the fluctuations in the resistance and capacitance values (24). These fluctuations were then statistically analyzed using the ECIS software to reveal the percentage variation in resistance, which in turn was a reflection of cellular micromotion on the electrode (25).
RESULTS

Determination of cell surface EGFr- A multipoint binding assay was performed on HCT116 cells grown in serum-free medium lacking EGF to measure the number of cell surface EGFr in the basal state without exogenous growth factor. Using a computer Scatchard analysis program (EBDA/LIGAND), it was determined that HCT116 cells have approximately $6.8 \times 10^4$ EGF cell surface receptors with an apparent dissociation constant (Kd) of approximately 10 nM and the Bmax was 110 fmol/10^6 cells. There is only one class of receptors based on Scatchard analysis.

Effect of ECM Concentration on Adhesion of HCT116 Cells Maintained in the Presence or Absence of EGF - HCT116 cells have been extensively characterized in previous studies. This work has shown that exogenous EGF (as well as other growth factors) does not influence proliferation or induction of DNA synthesis in the HCT116 colon carcinoma cell line due to autocrine activation of EGFr by TGFα (2,4-7,28,29). Despite saturation of mitogenesis by autocrine TGFα, additional EGFr activation and downstream signaling is observed upon addition of exogenous EGFr ligands. This raised the question as to the function of this response to exogenous EGFr ligand. We hypothesized EGFr activation resulted in the modulation of α integrin expression.

The maintenance of cells in serum-free chemically defined medium allowed us to determine the effects of the long-term (~5 days) presence or absence of EGF on cellular adhesion to tissue culture plates coated with basement membrane type IV collagen (0-2.5 μg/ml). HCT116 cells showed a 2 fold higher level of adhesion to this extracellular matrix (ECM) protein than on BSA coating in the absence of EGF. Maintenance of the cells in an EGF containing medium resulted in approximately a 6-fold increase in adhesion to CN IV (2.5 μg/ml) relative to adhesion in the
absence of EGF (Fig. 1A). In contrast, withdrawal of EGF from the culture medium for 48 hours resulted in lower attachment to CN IV (Fig. 1B). HCT116 cells adhere well to other ECM proteins fibronectin (FN) and laminin (LN) in the absence of EGF as they showed 2-fold higher levels of adhesion on FN and LN than on BSA coating (Fig. 1C). Maintenance on EGF containing medium resulted in more than a 4-fold increase in adhesion on LN and about a 3-fold increase in adhesion on FN (Fig. 1D) relative to adhesion in the absence of EGF. Thus, HCT116 cells exhibit differential adhesion with respect to specific ECM proteins. Higher cell adhesion was observed on CNIV than on LN and FN. It is also noteworthy that higher concentrations (10 μg/ml) of FN and LN were required for optimal cell adhesion as compared to lower concentrations of CNIV (2.5 μg/ml). Approximately, 2.5 μg (20 pmol) of CN IV are equivalent to 4.4 μg (20 pmol) of FN and 8.8 μg (20 pmol) of LN, whereas the molarities of the EGFr ligands used are equivalent. Thus, after adjustment for molar concentration, higher cell adhesion was observed on CN IV than on FN and LN. At 20 pM concentration of CN IV, cell adhesion in the presence of EGF is 5.5 fold over that in the absence of EGF. Moreover, adhesion to 20 pg CN IV is 2.25 fold higher than that of bovine serum albumin in the absence of EGF. In comparison to CN IV, the cell adhesion on 20 pM LN in the presence of EGF is approximately 3.5 fold over that in the absence of EGF. Adhesion to 20 pM FN is even less than that of LN. The role of TGFα as a promoter of cell adhesion in HCT116 cells was also determined (Fig. 1E). The effect of exogenously added TGFα (the ligand responsible for autocrine control of these cells) was essentially the same as that of EGF. Adhesion of HCT116 Cells to CN IV is Mediated Predominately by α2 Integrin - The specificity of α2 integrin in mediating adhesion of HCT116 cells was determined by treatment with specific
functional blocking antibodies to inhibit binding to CN IV. Monoclonal anti-α₂ integrin antibody was highly effective in preventing HCT116 cell adhesion to CN IV both in the presence and absence of EGF. Inhibitory levels ranged from 65-8% in the absence of EGF and 80-45% in the presence of EGF at antibody dilutions ranging from 1:50 - 1:500 (Fig. 2A). Antibody to the integrin α₅ subunit had no effect on HCT116 cell adhesion to CN IV. While, antibody to the integrin α₃ subunit was only slightly inhibitory (5-10% at 1:50 dilution) to HCT116 cell adhesion to CN IV. Integrin subunits α₃ and α₅ are the predominant cell adhesion receptors for laminin and fibronectin, respectively on these cells (Figs 2B, 2C).

Antibody to α₃ subunit inhibited binding of HCT116 cells to LN coated plates in a concentration dependent manner (Fig 2B). Inhibitory levels ranged from 80-15% in the absence of EGF and 90-40% in the presence of EGF at antibody dilutions ranging from 1:50 – 1:500. Although the integrin α₂ predominately mediates adhesion to CN, it has been shown that it is a receptor for LN on endothelial cells and some types of tumor cells (30,31). Consequently, it was not surprising that antibody to integrin α₂ subunit was also capable of inhibiting HCT116 cell adhesion to LN (Fig. 2B). However, inhibition by antibody to α₂ subunits was not as effective as anti-α₃ subunit. Antibody to the integrin α₅ had no effect on HCT116 cell adhesion to LN (Fig 2B) whereas the antibody was extremely effective in preventing adhesion to FN. Inhibitory levels ranged from 90-70% both in the presence and absence of EGF at dilutions ranging from 1:50-1:500 (Fig 2C). These results showed that cell adhesion to FN was primarily through integrin α₅. Neither antibody to the α₂ nor the α₃ subunit affected adhesion of HCT116 to FN.

Kinetics of Cell Adhesion to CN IV - The experiments described above were performed with cells continuously maintained in the presence or absence of EGF. Therefore, they could reflect a
steady-state situation in which cells may have made adaptations leading to differences in cell adhesion and integrin expression which were not related to control by EGFr activation. Thus it was necessary to determine whether short-term changes in EGF exposure could alter cell adhesion and integrin expression. Therefore, we determined the kinetic effects on cell adhesion of removal of EGF from the medium of HCT116 cells adapted to growth in the presence of the polypeptide as well as determining the kinetic effects of addition of EGF to the medium of HCT116 cells adapted to growth in EGF deficient medium. Subsequent characterizations were performed with CNIV and integrin $\alpha_2$ since this pair appeared to enhance adhesion to a greater extent than the other integrin-ECM combinations investigated.

Significant enhancement of adhesion to CN IV was observed within 6 hours of EGF addition and by 12 hours cell adhesion was increased by 2 fold rising to nearly 4-fold by 24 hours and 6-fold by 48 hours (Fig. 3). Withdrawal of EGF from the medium of EGF adapted HCT116 cells would be predicted to generate the opposite results. Within 6 hours of EGF removal from the EGF adapted cells, adhesion to CN IV was reduced by 15% and was further reduced by nearly 35% to 70% between 12 and 48 hours.

Kinetics of Cell Surface Integrin $\alpha_2$ Expression and Steady-State Levels of $\alpha_2$ mRNA- The results of integrin antibody blocking experiments were consistent with a role for EGF in controlling cellular adhesion to CN IV by controlling cell surface expression of the integrin $\alpha_2$ subunit. Therefore, we determined cell surface expression of integrins in colon carcinoma cells continuously maintained in the absence or presence of EGF in chemically defined tissue culture medium by immunoprecipitation of $^{125}$I-labeled cell surface integrins. As expected from integrin blocking antibody experiments, HCT116 cells showed cell surface expression of
integrin $\alpha_2$ subunit when maintained in the absence of EGF. Increased adhesion by HCT116 cells continuously maintained in EGF supplemented medium suggested that integrin expression would be increased under these conditions. The kinetics of cell adhesion to CN IV should also reflect changes in cell surface functional integrin$\alpha_2$ expression. Increased integrin $\alpha_2$ subunit expression was observed within 6 hours of EGF addition to EGF deficient cells. The 6 hour level increased by 3-fold at 12 hours and about 5-fold at 24 and 48 hours (Fig. 4 A; left upper panel). EGF withdrawal resulted in a significant reduction of cell surface $\alpha_2$ subunit expression within 12 hours with more than 50% reduction in expression by 24 hours. There was a continued decline in integrin $\alpha_2$ expression up to 48 hours post EGF withdrawal (Fig. 4A; left bottom panel). The quantitation of the kinetics of integrin $\alpha_2$ expression in the presence or absence of EGF are shown in Figs. 4A (right upper and lower panels, respectively). In contrast to integrin $\alpha_2$, the integrin $\alpha_1$ protein is not modulated by EGF, although it is expressed in HCT116 cells (Fig 4B), thus indicating the selectivity of EGF dependent changes in integrin expression by HCT116 cells. Furthermore, exogenous EGF did not have any effect on actin levels. Transforming growth factor-$\alpha$ was equivalent with EGF in terms of its ability to induce integrin expression (data not shown).

To determine the effect of EGF on the expression of integrin $\alpha_2$, total RNA (40 $\mu$g) was analyzed by RNase protection assays from HCT116 cells which were treated for different time periods with EGF. As shown in Fig. 5A, EGF enhances levels of integrin $\alpha_2$ mRNA (2-4 fold) in a temporal fashion over the course of 48 hours post addition of the growth factor. The EGF-mediated kinetics of integrin $\alpha_2$ mRNA expression were similar to those found for protein levels.
The quantitation of kinetics of integrin α₂ mRNA expression is shown in Fig. 5A (lower panel). Further, we have determined that EGF affects the expression of integrin α₃ (Fig 5B) and integrin α₅ genes (Fig 5C) but the increases in expression levels of these α subunits are lower than those on the expression of integrin α₂ gene. An RNase protection assay showing the induction by EGF on expression of integrin β₁ mRNA is shown in Fig 5D. The effects of exogenous EGF on cell surface integrin expression suggested that autocrine TGF-α may have a role in determining functional integrin expression and cell adhesion as well.

Antibody to the EGFr Blocks HCT116 Cell Adhesion to CN IV, Integrin α₂ Expression and EGFr Activation- The experiments described above indicated a role for exogenous EGF in α₂ integrin-mediated cell adhesion to CN IV via EGFr by HCT116 cells. HCT116 cells have an active autocrine TGF-α loop which is responsible for their growth factor independence (4,7). If this TGF-α also acted in an autocrine manner to affect cell adhesion it would be expected that addition of anti-EGFr antibody would block cell adhesion of cells maintained in the absence of exogenous EGF. **Fig. 6A** shows that the anti-EGFr antibody mAb528 was effective in blocking adhesion to CN IV by HCT116 cells maintained in the absence of EGF. A similar experiment was performed with cells maintained in the presence of EGF by using a 50-fold excess of EGFr antibody. Treatment of HCT116 cells under these conditions was effective in blocking 90% of the adhesion to all CN IV coated plates (**Fig. 6B**). HCT116 cells were treated with EGFr blocking mAb 528 and its effect on integrin α₂ levels was observed by Western blot analysis. **Figure 6C** shows that mAb 528 treated cells showed inhibition of expression of integrin α₂ (lane 1) relative to control HCT116 cells (lane 2). Loss of integrin α₂ expression and adhesion were directly correlated with reduced EGFr activation by the mAb.
TGFα Antisense Transfected Cells Show Attenuation of Cell Adhesion, Integrin α2 Expression and EGFr Activation- The results from above indicate that in addition to its effect on growth factor independent mitogenesis, autocrine TGF-α contributes to adhesion to CN IV by HCT116 cells. If TGF-α were acting in an autocrine manner to affect cell adhesion, it would be expected that anti-TGF-α transfected cells would inhibit integrin-mediated cell adhesion. TGF-α antisense transfected cells showed reduced adhesion to CN IV relative to control parental cells. The percentage of reduction in adhesion of the TGF-α antisense clone varied from 80-47% depending on the concentration of coated CN IV when compared to HCT116 cells, under identical conditions (Fig. 7A). These data are consistent with the effects of EGFr blocking antibody on CN IV-mediated adhesion by HCT116 cells. **Further, the reduction in cell adhesion of the TGFα antisense clone is correlated with a reduction in the expression of integrin α2 protein.** Lane 1 in Figure 7B shows levels of integrin α2 in HCT116 cells, whereas comparison with lane 3 shows reduced levels of integrin α2 in the TGFα antisense transfected cells. This indicated that autocrine TGFα plays a role in the steady state expression of integrin α2. The expression of integrin α2 was rescued by treating antisense cells with exogenous EGF (lane 4), indicating that reactivation of EGFr rescues the antisense effect. Lane 2 shows induction of integrin α2 protein by exogenous EGF in HCT116 cells. Actin levels were not altered. Expression of integrin α2 mRNAs paralleled the protein expression described above (data not shown).

Attachment of HCT116 Cells to Electrodes Precoated with Either BSA or CN IV as a Function of Time - To explore the biological role of increased cell adhesion and expression of integrin α2 by EGF, we determined the role of the EGF signaling pathway on HCT116 cell motility using
CN IV as a substrate. The ECIS technique (24-26) was used to quantitate cell motility. A small AC signal was applied across the gold electrode on which cells were plated, while the resistance and the capacitance of the electrode were measured over time. Within the first two hours at 37°C, there was a notable rise in resistance of the electrodes coated with CN IV, but not in those coated with BSA (Fig. 8). This increase in resistance in collagen-coated electrodes is due to initial cell attachment, pH and temperature changes which result in an increase in area covered by cells on the electrode (32). Of note, however, is that the CN IV-coated electrodes (E3-E5) displayed a marked fluctuation in resistance, whereas those electrodes coated with BSA were smooth (E1 and E2). These fluctuations in resistance represent the presence of viable attached and spread cells on these electrodes, and are caused by the small movements of these cells on the electrodes (25). These small cellular movements, termed micromotion (24), result in constant changes in cell-cell or cell-substrate interactions which accordingly changes the rate of current flow across the cell layer (25). In contrast, the BSA-coated electrodes did not display these fluctuations in resistance indicative of the absence of attached cells on these electrodes. It is noteworthy that cell attachment to extracellular matrix and cell motility on extracellular matrix are biologically related processes (33-36). If fewer cells are attached to the coated electrodes, the total voltage across the electrode is reduced. Consequently, the attachment curves will show a reduced gain as in Figure 8 (E1, E2 vs E3-E5). Micromotility is the additional fluctuation (as a percentage calculated by the ECIS software) that results from cell movement above the total voltage as shown in Figures 9-11.

Functional Blocking mAb to Integrin α2 Decreases Cell Micromotion in a Concentration Dependent Fashion. To establish that cell motility on CN IV was specifically integrin α2
mediated, cells were preincubated either with mouse IgG or with different concentrations of a function blocking mAb (clone PIE6) before recording micromotion. The percent variations in resistance observed were 3.192 (control IgG), 0.365 (mAb 1:50 dilution) and 1.105 (mAb 1:150 dilution). As shown in Fig. 9, the decrease in cell motility (resistance) of HCT116 cells by mAb at 1:50 dilution was 89% (middle panel), and at 1:150 dilution of mAb, the decrease in locomotion observed was 65% (bottom panel) as compared to control IgG treated cells (top panel).

**EGF Enhances CN IV-Induced Cell Motility Whereas Tyrphostin AG1478 Abrogates EGF Effects** – The role of EGFr activation in micromotion was then investigated using tyrphostin AG1478, which is a highly selective EGFr inhibitor (37). In untreated HCT116 cells, the percent variation in resistance measured over a period of about 70 minutes was found to be 4.130 (Fig. 10A). Addition of EGF (10 ng/ml) to the cell medium increased the fluctuations, indicating an increase in cell motility, such that the percent variation in resistance was now 7.103 (Fig. 10B). To ensure that this effect of EGF was indeed due to the stimulation of the EGFr, we then further treated these cells with AG1478 (10 μM). Addition of AG1478 to the EGF-stimulated cells abrogated the increase in cell motility caused by EGF (percent variation in resistance 3.119; Fig. 10C). The basal micromotion percent variation in resistance value (4.13) was slightly higher than micromotion observed in the presence of AG1478, probably reflecting autocrine TGF-α contributions to micromotion as well. These observations were confirmed by results from three experiments shown in Fig. 10D.

**HCT116 Cell Micromotion is Attenuated in TGFα Transfected Antisense Cells** – To define the role of autocrine TGFα in cell motility, micromotion of HCT116 cells was compared to that of
HCT116 antisense cells under identical conditions (Fig. 11). In the upper left panel, the percent variation in resistance (micromotion) of control HCT116 cells was determined to be 2.939 and, on addition of EGF, the resistance was increased to 5.370 (83% increase, upper panel; right). The lower left panel shows a percent variation in resistance of 1.741 in control antisense cells which is about 40% lower than that of parental HCT116 cells, thus showing that autocrine TGFα contributes to cell micromotion. Further, addition of EGF to antisense cells increases percent variation in resistance to 2.995 (lower right panel), thus showing that exogenous EGF is capable of rescuing micromotion in antisense cells. Taken together our results point to a significant role of autocrine TGFα in controlling steady state cell micromotion, cell adhesion and integrin α2 expression in addition to controlling mitogenesis.

DISCUSSION

Earlier we published a general profile of adhesion to distinct ECM proteins in the absence of growth factor by several colon cancer cell lines, including HCT116 cells (21). We have now characterized the role of EGFr activation (both endogenous and exogenous) on cell adhesion. HCT116 cells continuously maintained in EGF were compared to cells devoid of EGF for adhesion to CN IV. Maintenance of cells in EGF enhanced adhesion of HCT116 cells to the ECM protein (Fig. 1). The cell adhesion to CN IV was higher than on FN or LN, thus showing differential adhesion of HCT116 cells on ECM proteins. These results indicated that a known mitogen, EGF, could stimulate cell adhesion, but that the stimulation was unrelated to the mitogenicity of EGF since HCT116 cells do not respond to exogenous EGF with increased cell proliferation (2,4). The increase in cell adhesion and integrin production in response to EGF in
cells continuously maintained on the growth factor could be due to a steady-state situation in which cells may have made adaptations to culture conditions in different media. We investigated this by determining kinetic effects on cell adhesion to ECM protein following addition or withdrawal of EGF from cells which were adapted to grow in the absence or presence of EGF, respectively. The results indicated a relatively rapid modulation of cell adhesion in response to addition of EGF or TGFα to EGF-deficient cells. We demonstrated that EGF enhanced cell adhesion by stimulating expression of functional integrin α2. This was further confirmed by treating HCT116 cells with specific monoclonal antibodies to integrin α2 which blocked adhesion of HCT116 cells to CN IV in a concentration-dependent fashion. Inhibition of cell adhesion by antibodies was more effective on cells grown in EGF suggesting that a higher proportion of the binding to substrate protein in EGF maintained cells was due to integrin binding than in EGF devoid cells (Fig 2). Adhesion of HCT116 cells was significantly enhanced before 24 hours and continued to increase up to 48 hours after treatment with EGF (Fig. 3A). Removal of EGF from HCT116 cells had the opposite effect as adhesion levels decreased within 24 hours. Interestingly, removal of EGF did not affect adhesion as extensively as addition within the 48-hour period in which experiments were performed (Fig. 3B). It may be that autocrine TGF-α levels in these cells help counteract the immediate effects of EGF removal from the medium so that kinetic effects on cell adhesion are slowed. In addition, the half-life of integrin α2 may be quite long. Immunoprecipitation of integrin subunit α2 with specific antibodies indicated that HCT116 cells maintained in EGF expressed several fold higher amounts of cell surface integrin than cells devoid of EGF (Fig. 4A). Under these conditions, levels of actin and α1 proteins did not change (Fig 4B). Similarly, RNase protection assays revealed that exogenous
EGF up-regulated (2-4 fold) mRNA levels of integrin α2 within 48 hours (Fig. 5A). The increase in expression in integrin α3 and α5 mRNAs by EGF were relatively small. The kinetic changes of cell surface integrin subunit α2 expression reflect the differences in kinetics of cell adhesion observed with EGF addition or withdrawal.

We used different approaches to confirm cell adhesion and integrin expression as a less sensitive function of EGFr than mitogenesis. **These included the use of specific blocking antibodies, and TGF-α antisense transfected cells.** If the increase in adhesion of cells maintained in EGF was in fact due to EGF interaction with its receptor then antibodies which blocked the receptor should be capable of blocking adhesion. An EGFr antibody (designated mAb528) is known to recognize the EGF / TGF-α binding site, competes for EGF/TGF-α binding and blocks EGF/TGF-α induced receptor autophosphorylation (10, 38). Monoclonal antibody 528 was capable of inhibiting 90% of HCT116 cell adhesion to CN IV, abrogated expression of integrin α2 protein and inhibited activation of EGFr, thus confirming that EGF mediates cell adhesion and integrin α2 expression via receptor irrespective of its mitogenic properties (Fig. 6). These data confirmed that, changes in CN IV adhesion and integrin α2 expression in HCT116 cells were via the EGFr signaling pathway. The role of TGFα in cell adhesion and integrin expression is demonstrated by the use of TGFα antisense transfected cells (Figs. 7A and 7B). The antisense cells showed attenuated cell adhesion and integrin α2 expression. Expression of integrin α2 is rescued by exogenous EGF, thus exhibiting the specificity of the effect on EGFr by TGFα antisense.
The exact site(s) of the tyrosine residue(s) involved in the activation of the EGFr in integrin expression and cell adhesion remains to be determined. In general, the EGFr autophosphorylates at least five tyrosine residues in the cytoplasmic tail in response to EGF (39, 40). The stoichiometry of the tyrosine autophosphorylation sites of the EGFr in mammalian cells is not known. The hierarchy of autophosphorylation sites may offer different regulatory roles in the EGFr function. Epidermal growth factor receptor uniquely binds at multiple clustered tyrosine sites with adaptor proteins containing a single SH2 domain. It has been reported by Batzer et al. that both Grb 2 and Shc adaptor proteins have high affinity and low affinity binding sites on the EGFr (40). It was revealed that Grb 2 primarily binds to activated tyrosine 1068 and with low affinity to tyrosine 1086, whereas Shc primarily binds to tyrosine 1173 and it binds to tyrosine 992 in a less sensitive fashion. However, the functional significance of secondary sites in the intracellular domain of EGFr has yet to be elucidated. One hypothesis is that in HCT 116 cells, autocrine TGF-α saturates primary high affinity docking sites by adaptor proteins, whereas the less sensitive function of EGFr may be due to the occupancy of secondary tyrosine autophosphorylation site(s) by exogenous EGF. Thus showing the functional regulatory role of minor autophosphorylated EGFr sites via an auxiliary mechanism.

It has been reported that in human A431 epidermoid carcinoma cells, EGFr activation leads to inhibition of cell growth through induction of p21cip1/WAF1 at high levels of receptor occupation (41, 42). In contrast, p21cip1/WAF1 is not induced at low levels of EGFr activation. This phenomenon, however, is somewhat different than the situation we observe in HCT116 cells. In the case of A431 cells it is likely a matter of the high vs. low affinity receptors typically seen in cells with amplified EGFr (43). In contrast to the delay of induction of p21cip1/WAF1 until
a relatively high receptor occupancy is obtained in A431 cells, our work deals with a situation in which the DNA synthesis response is saturated by the relatively low EGFr occupation level resulting from autocrine TGFα. HCT116 cells have approximately 6.8 X10^4 EGF cell surface receptors with an apparent dissociation constant (Kd) of approximately 10 nM and the B\text{max} was 110 fmol/10^6 cells. Unlike A431 cells, there is only one class of receptors, expressed by HCT116 cells based on Scatchard analysis. It is difficult to say what proportion of EGFr is endogenously bound. Acid treatment to remove receptor occupation prior to Scatchard experiments does not indicate large amounts of endogenous occupation relative to the 68,000 receptors seen on the cell surface. We believe this is due to intracellular activation of these receptors as based on the inability of exogenous EGFr and TGFα blocking antibodies to inhibit cell growth and inhibition of DNA synthesis by these cells (5,7). Alternatively, it may be due to occupation of a proportion of the cell surface receptors by transmembrane bound TGFα precursor which is not labile to the acid as we have described previously (44).

The expression level of integrin α2 shows a wide window of response, relative to mitogenesis, ranging from low EGFr occupation by autocrine TGFα to saturation by exogenous EGF or TGFα. It is initiated at low level receptor occupation as evidenced by its diminution by treatment with EGFr blocking antibody which inhibits basal EGFr activation resulting from autocrine TGFα. Moreover, stable transfection with a full-length TGFα antisense cDNA inhibits TGFα expression (5,7) and basal EGFr activation in these cells (data not shown). Addition of exogenous EGF or TGFα results in further EGFr activation (data not shown) which is associated with higher expression of integrin α2 (Fig 4). These changes in turn lead to alteration in cell adhesion (Fig. 1) and cell micromotion (Fig. 10). The reduction in the low
levels of basal integrin $\alpha_2$ by EGFr antibody (Fig. 6 C) and TGF$\alpha$ antisense on cell adhesion (Fig. 7A) and cell micromotion (Fig. 11) shows that this is an expanded window of response relative to mitogenesis. Thus, there is a difference in the response windows based on extent of EGFr activation. They may not be strictly hierarchical in the sense of cellular priorities or due to different subsets of receptors, but rather in the sense of degree of saturation of response at a given level of EGFr activation.

Results from this study are consistent with such a role for autocrine TGF-$\alpha$ as the EGFr blocking antibody was able to inhibit cell adhesion in HCT116 cells devoid of EGF in the medium. These results were further supported by adhesion assays in which the adhesion of HCT116 cells was directly compared to adhesion of TGF-$\alpha$ antisense transfected cells. The cell adhesion was markedly reduced in TGF-$\alpha$ antisense transfected cells as compared to the control HCT116 neo clones (Fig. 7A). Thus, showing that autocrine TGF-$\alpha$ contributes to basal levels of integrin $\alpha_2$ expression and cell adhesion. The presence of a strong TGF-$\alpha$ loop in HCT116 cells is one of the salient features contributing to the highly malignant properties to this cell line and anti-sense TGF-$\alpha$ transfectants show loss of basal EGFr activation as well as a requirement for exogenous EGF for optimal mitogenesis (5). It has been proposed that the major growth advantage of autocrine TGF-$\alpha$ in malignant cells may be due to the increased ability of cells to re-enter the cell cycle (6). Similarly, it is conceivable that cancer cells involved in metastasis will derive an advantage from a strong autocrine TGF-$\alpha$ loop in both growth and motility since initially the number of cells contributing to metastatic behavior is very small.

Cell locomotion may have significance in colon cancer metastasis (45). We used a cell-substrate electrical cell impedance sensor (ECIS) technique to determine the effects of EGF,
EGFr inhibitor AG1478, and integrin α2 blocking antibody on HCT116 cell motility. Using this technique, cell motion may be quantitatively measured at the nanometer level (micromotion) (25). In any dynamic cell system the cell-cell and cell-substrate interactions are constantly changing due to regular metabolic processes (26). As such, the physical spaces between two cells or between the cell and the surface on which it is growing, changes as well. This results in small cellular movements termed micromotion, which occur at the nanometer scale and cannot be detected in a regular microscope. As the gap between the cells or between the cell and its substrate fluctuates, so does the current flowing across the cell layer (25, 26). The sensitive nature of the lock-in amplifier detects the changes in this current and voltage and translates them into resistive and capacitive units as presented here. Micromotion detected by ECIS technique is directly related to conventional cell motility (46). Drugs which inhibit cell migration and motility, such as cytochalasin D, also inhibit micromotion (47). Micromotion detected by the ECIS technique has been successfully used to detect cell migration and morphological changes in a variety of systems (47, 48). In this study, we have shown that the basement membrane CN IV-mediated adhesion and exogenous EGF significantly enhance micromotion independent of cell growth in human colon cancer cells. The cell locomotion was not observed on BSA-coated electrodes (Fig. 8). Therefore, these cells must have an appropriate ECM for growth factor to affect cell motility suggesting that the signaling cascades for integrins and growth factors are linked. The cell motility on CN IV coated electrodes was mediated by integrin α2 as demonstrated by using blocking monoclonal antibody (PIE6) which markedly reduced cell micromotion (Fig. 9). The increase in amplitude of fluctuations (resistance) on CN IV caused by EGF (Fig. 10B), as compared to control (Fig. 10A), was reduced by AG1478 (Fig. 10C). These
locomotion fluctuations are typical of a cell phenotype and may be considered the cell signature of a particular cell phenotype (26,27). On the evidence that AG1478 abrogates induction of integrin α2 by EGF (data not shown), the reduction in micromotion may be attributed to the lower expression of integrin caused by tyrphostin AG1478. This indicates that EGFr is a transducing element in the control of cell locomotion. **Cell populations with higher expression levels of integrins (in the presence of EGF) exhibit increased cell micromotility (Fig. 10B) as compared to control cells (Fig. 10A).** These observations are consistent with evidence of a cause and effect relationship between integrin-mediated adhesion and motility on extracellular matrix that is tightly controlled by ligand density, integrin expression levels, and integrin affinity or avidity (34-36). Optimal levels of adhesion propel migration through a process in which adhesion molecules at the leading edge of the cell form complexes with matrix while molecules at the trailing edge release the substrate, allowing cells movement (35). Thus, higher integrin concentrations may lead to higher rates for this process at cellular interfaces involved in movement, especially in response to EGF receptor activation and associated changes in cytoskeletal arrangements. The role of autocrine TGFα in basal steady state cell micromotion is demonstrated by using TGFα antisense transfected cells (Fig. 11). The stimulation of HCT116 cell micromotion, cell adhesion and higher expression of integrin α2 via the EGFr signaling pathway may be one mechanism by which these cells become metastatic. The enhancement of integrin expression by EGFr activation in HCT116 cells may also contribute towards cell survival.
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The abbreviations used are: TGF-α, transforming growth factor-α; EGFr, epidermal growth factor receptor; EGF, epidermal growth factor; CN IV, collagen type IV; FN, fibronectin; LN, laminin; ECM, extracellular matrix protein; ECIS, electrical cell impedance sensor; BSA, bovine serum albumin; T, transferrin; I, insulin; E, EGF; TTBS, Triton Tris buffered saline; ECL, enhanced chemiluminescence; MTT, methylthiazole tetrazolium.
Figure Legends

Fig. 1. The effect of ECM protein concentration on adhesion of HCT116 cells continuously maintained in the absence or presence (10 ng/ml) of exogenous EGF or TGFα. 96-well tissue culture plates were coated with collagen IV at 0, 0.025, 0.1, 0.25, 1.0, 2.5 µg/ml or with FN and LN at 0, 0.1, 0.5, 1.0, 5.0, and 10.0 µg/ml for overnight at room temperature, non-specific sites were blocked with BSA (3%) for 3 hours and subsequently wells were washed once with PBS. Subconfluent cell cultures were trypsinized and seeded at 6x10^4 cells/well onto ECM and BSA-coated plates and incubated for 90 min at 37°C. Adhesion assays were carried out as described in the “Experimental Procedures”. The relative number of attached cells were expressed as percentage increased over BSA. (A) Cells maintained for 5 days in the EGF deficient medium were changed to the same fresh medium 48h prior to assay (open circle); cells maintained for 5 days in EGF-deficient medium changed to medium supplemented with EGF 48h prior to assay (closed circle). (B) Cells maintained for 5 days in EGF supplemented medium changed to the same fresh medium 48h prior to assay (open box); cells maintained for 5 days in EGF supplemented medium changed to fresh medium without EGF 48h prior to assay (closed box). HCT116 cells maintained in the absence (C) or presence (D) of EGF on FN (closed circles) or LN (open circles). (E) Cells were maintained for 5 days in the absence of exogenous growth factor. TGFα was added for 48h prior to adhesion assay; HCT116 cells in the absence (open squares) or presence (closed squares) of TGFα. Error bars represent the standard error of four experiments performed in triplicate.
Fig. 2. Inhibition of HCT116 cells adhesion to CN IV by antibodies to integrin receptors in the absence or presence of EGF. 96-well tissue culture plates were coated with (A)CN IV (5 μg/ml), (B) LN (10 μg/ml), and (C) FN (10 μg/ml). Monoclonal antibodies (Ab) to anti-integrin α2, α3 and α5 subunits were added at different dilutions as indicated. Adhesion assays were performed as detailed in “Experimental Procedures”. The α3 and α5 monoclonal antibodies were used as negative controls at the highest concentrations (1:50). Cells were maintained in the absence or presence of 10 ng/ml EGF. (A) Lane 1, without Ab; lanes 2-4, α2 Ab; lane 5, α3 Ab; lane 6, α5 Ab. (B) Lane 1, without Ab; Lanes 2-4, α2 Ab; lanes 5-7, α3 Ab; lane 8, α5 Ab. (C) Lane 1, without Ab; lane 2, α2 Ab; lane 3, α3 Ab; lanes 4-6, α5 Ab. Each binding value represents the mean of two individual experiments performed in triplicate.

Fig. 3. Kinetics of effects of addition (A) or withdrawal (B) of EGF on HCT116 cell adhesion to CN IV. Cells maintained in the absence or presence of 10 ng/ml EGF were plated into 6-well tissue culture plates (5x10⁴ cells/well) and allowed to grow for 3-5 days. At this point EGF containing medium was replaced with medium devoid of EGF while medium lacking EGF was replaced with EGF containing medium for various periods of time (2, 6, 12, 24, 36 and 48 hours). Control cells received the same fresh medium on which they were originally grown at the same times. Cells were harvested and plated at 6x10⁴ cells per well into 96-well tissue culture plates coated with BSA (open circle) or CN IV 5 μg/ml (closed triangle), incubated for 90 min at 37°C and assayed for attachment by the standard procedure as described in the “Experimental Procedures”. Data are reported as the percentage of either EGF-added or EGF-
withdrawn adherent cells relative to the attached cells maintained in the original medium. Error bars represent the standard error of the average of two experiments performed in triplicate.

Fig. 4A. Kinetics of EGF modulation of cell surface integrin \( \alpha_2 \) subunit protein expression in HCT116 cells. Cells were plated as described in Figure 3. At 3-5 days, cells maintained in the absence or presence of EGF (10 ng/ml) were changed to medium containing or lacking EGF for 0, 6, 12, 24, and 48 hours. All cells received a change of fresh medium at the same time and were then harvested and iodinated at the indicated times after medium changed as described in “Experimental Procedures”. Detergent extracts of surface labeled cells from adherent cells were treated with monoclonal antibodies against human integrin \( \alpha_2 \) subunit followed by complexing with rabbit anti-mouse IgG. Immune complexes were precipitated by protein A agarose beads and analyzed by electrophoresis on 7.5% polyacrylamide gel under reducing conditions. Actin was used as a control. Right panels show densitometry quantitation of integrin \( \alpha_2 \).

Fig. 4B. Kinetics of EGF modulation of integrin \( \alpha_1 \) protein expression in HCT116 cells.
Cells were maintained in the absence of EGF for 5 days, EGF (10 ng/ml) was added to the medium for 0, 12, 24, and 48 hours. The cells were harvested and biotinylated as described in “Experimental Procedures). Cell lysates were analyzed by Western blot using polyclonal Ab1934 (Chemicon) against integrin \( \alpha_1 \). Actin was used as a control.

Fig. 5A. Temporal expression of integrin \( \alpha_2 \) subunit mRNA levels detected by RNase protection assay in HCT116 cells. Total RNA (40 \( \mu \)g) isolated from the EGF (10 ng/ml)
treated cells for 0, 6, 12, 24, and 48 hours was hybridized with $^{32}$P-labeled RNA probes of the integrin $\alpha_2$ subunit (0.5x10$^6$ cpm) and actin (8,000 cpm) simultaneously according to the details given in “Experimental Procedures”. The sizes of the protected fragments on urea-polyacrylamide gel electrophoresis are indicated by the arrows. Actin mRNA levels are shown for normalization of sample loading. Yeast tRNA was used as a negative control. Fig 5A (lower panel) shows densitometry quantitation of integrin $\alpha_2$ mRNA.

**Figs. 5B-5D. Effect of EGF on expression of integrin $\alpha_3$, $\alpha_5$, and $\beta_1$ subunit mRNA levels detected by RNase protection assay in HCT116 cells.** Total RNA (40 $\mu$g) isolated from the untreated or EGF (10 ng/ml) treated cells for 48 hours was hybridized with $^{32}$P-labeled RNA probes of the integrin $\alpha_3$ subunit (left panel) (0.5x10$^6$ cpm), $\alpha_5$ subunit (middle panel), $\beta_1$ subunit (right panel) and actin (8,000 cpm) simultaneously according to the details given in “Experimental Procedures”. The sizes of the protected fragments on urea-polyacrylamide gel electrophoresis are indicated by the arrows. Actin mRNA levels are shown for normalization of the sample loading. Yeast tRNA was used as a negative control.

**Fig. 6. EGF receptor antibody blocks HCT116 cell adhesion to CN IV, integrin $\alpha_2$ expression and EGFr activation.** Cells maintained either in the absence or presence of EGF were treated with 10 $\mu$g/ml EGF receptor blocking monoclonal antibody (mAb 528) for 48 hours. Cells were then trypsinized and inoculated at 6x10$^4$ cells per well into BSA and CN IV coated plate and incubated at 37$^\circ$C for 90 min in the absence or presence of monoclonal
antibody as indicated. (A) Experiment was performed in the absence of EGF whereas (B) experiment was performed in the presence of EGF. Non-adherent cells were washed off and adherent cells were determined by MTT assay as described in “Experimental Procedures”. Error bars represent the standard error of two experiments performed in triplicate. (C) Cells cultured in a 6 well plate were either treated with 15 μg/ml EGFr blocking mAb528 (lane 1) or with mouse IgG (lane 2) for 48 h. Cells were biotinylated for integrin α2, lysed and equal amounts of protein were analyzed by western blot as described in the “Experimental Procedures”. Actin was used as a loading control.

Fig. 7A. Comparison of adhesion of HCT116 neo control (open bars) and HCT116 TGF-α antisense transfected (HCT116-A.S.33) cells (dark bars) to CN IV. Substrates were prepared by coating tissue culture 96-well plates with CN IV at concentrations of 0, 0.25, and 0.50 μg/ml overnight at room temperature. Cells were seeded at 6x10⁴ cells/well onto coated plates and incubated for 90 min at 37°C. The relative numbers of attached cells were determined by MTT assay as described in “Experimental Procedures”. Optical density values at 595 nm on BSA-coated wells were subtracted.

Fig. 7B. Comparison of integrin α2 expression of HCT116 and HCT116 TGFα antisense transfected cells. Cells were maintained in the absence of EGF for 5 days. EGF was added to the medium 48h prior to harvesting. Cells were biotinylated, lysed and equal amounts of protein were analyzed by Western blotting as given in the “Experimental Procedures”. Lanes 1 and 3 show basal levels of integrin α2 in HCT116 neo and antisense cells respectively; lanes 2 and 4
show stimulation of integrin α2 by EGF in HCT116 neo and antisense cells respectively. Actin was used as a loading control.

**Fig. 8.** Attachment of HCT116 cells to electrodes precoated with either BSA or CN IV as a function of time. Each electrode well was inoculated with 2.0 x 10^4 cells /400μl medium as given in “Experimental Procedures.” Electrodes E1 and E2 were coated with BSA (3%), whereas electrodes E3 through E5 were coated with CN IV (5μg/ml). The cell attachment was recorded for 23h.

**Fig. 9.** Functional blocking monoclonal antibody to integrin α2 decreases cell micromotion in a concentration dependent fashion. HCT116 cells (6x10^4 cells /condition) in serum-free medium were incubated with either mouse IgG (1:50 dilution; top panel) or with integrin α2 blocking mAb (clone PIE6) at dilutions 1:50 (middle panel) or 1:150 (bottom panel) for 30 min at 37°C. Subsequently, cells were transferred to electrode wells precoated with CN IV. After 3 hours of cell attachment to electrodes, cell micromotion was recorded for 70 min.

**Fig. 10.** EGF enhances cell motility whereas tyrphostin AG1478 abrogates EGF effect. Electrode arrays after precoating with CN IV (5 μg/ml) were used in these experiments. HCT116 cells were plated in serum-free medium at a density of 2x10^4 cells/electrode/well. At 24 hours, the serum-free medium was replaced by TI medium. Subconfluent (70-80%) cultures were either (A) not treated or (B) treated with EGF (10 ng/ml) and (C) treated with EGF + AG1478 (10 μM). Cells growing on collagen coated gold electrodes were monitored for cell
attachment for 20h followed by micromotion (resistance) over a period of 70 min. The effects of EGF and AG1478 on cell motility representing three experiments are shown in Fig. 13D.

**Fig. 11. Comparison of micromotion of HCT116 and HCT116α antisense transfected cells.**

Precoated electrode arrays with CN IV (5 μg/ml) were used in these experiments. HCT116 and HCT116α antisense cells were plated in serum-free medium at a density of 2x10⁴ cells/electrode/well. At 24 hours, the serum-free medium was replaced by TI medium. (A) Subconfluent (70-80%) HCT116 cultures were either not treated (upper left panel) or treated (upper right panel) with EGF (10 ng/ml) and (B) subconfluent (70-80%) HCT116α antisense cells were either not treated (lower left panel) or treated (lower right panel) with EGF (10 ng/ml). Cells growing on collagen coated gold electrodes were monitored for cell attachment for 20h followed by micromotion (resistance) over a period of 70 min.
M1:03268 Figure 1

A. EGF addition

B. EGF withdrawal

C.

D.

E.
M1:03268 Figure 5

**A.**

Integrin α2 → (292 bp)

Integrin α3 → (326 bp)

Actin → (135 bp)

**B.**

EGF

Integrin α3 → (405 bp)

**C.**

EGF

Integrin α5 → (214 bp)

**D.**

Probes 1 2

- +

Probes 1 2

- +

**Graph:**

- **Y-axis:** Densitometry Units
- **X-axis:** Time (hours)

- **Bars:**
  - 0
  - 6
  - 12
  - 24
  - 48

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Figure 7A

- Bar graph showing the concentration of Collagen IV (μg/ml) with two conditions:
  - HCT 116 Neo
  - HCT 116 a-as33

Figure 7B

- Western blot images showing:
  - Integrin α2
  - EGF
  - Actin

Conditions:
- 1: -
- 2: +
- 3: -
- 4: +
M1:03268 Figure 10

A. CN IV Control  Resistance 4.13%

B. CN IV +EGF  Resistance 7.103%

C. CN IV +EGF +AG1478  Resistance 3.119%

D. Resistance (Arbitrary Units)
M1:03268 Figure 11

(A) HCT116 cells

(B) HCT116 αAntisense cells
Differences in sensitivity of biological functions mediated by epidermal growth factor receptor activation with respect to endogenous and exogenous ligands

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