Integrin \(\alpha_2\) and Extracellular Signal-regulated Kinase Are Functionally Linked in Highly Malignant Autocrine Transforming Growth Factor-\(\alpha\)-driven Colon Cancer Cells*

Recently, we have shown that autocrine transforming growth factor-\(\alpha\) (TGF-\(\alpha\)) controls the expression of integrin \(\alpha_2\), cell adhesion to collagen IV and motility in highly progressed HCT116 colon cancer cells (Sawhney, R. S., Zhou, G-H. K., Humphrey, L. E., Ghosh, P., Kreisberg, J. I., and Brattain, M. G. (2002) J. Biol. Chem. 277, 75–86). We now report that expression of basal integrin \(\alpha_2\) and its biological effects are controlled by constitutive activation of the extracellular signal-regulated/mitogen-activated protein kinase (ERK/MAPK) pathway. Treatment of cells with selective mitogen-activated protein kinase kinase (MEK) inhibitors PD098059 and U0126 showed that integrin \(\alpha_2\), expression, cell adhesion, and activation of ERK are inhibited in a parallel concentration-dependent fashion. Moreover, autocrine TGF-\(\alpha\)-mediated epidermal growth factor receptor activation was shown to control the constitutive activation of the ERK/MAPK pathway, since neutralizing antibody to the epidermal growth factor receptor was able to block basal ERK activity. TGF-\(\alpha\) antisense-transfected cells also showed attenuated activation of ERK. Using a real time electric cell impedance sensing technique, it was shown that ERK-dependent integrin \(\alpha_2\)-mediated cell micromotion signaling is controlled by autocrine TGF-\(\alpha\). Thus, this study implicates ERK/MAPK signaling activated by endogenous TGF-\(\alpha\) as one of the mechanistic features controlling metastatic spread.

Epidermal growth factor receptor (EGFr)\(^1\) activation is known to control cancer cell growth as a result of autocrine and/or paracrine stimulation. We have recently shown that constitutive endogenous activation of the EGFr by TGF-\(\alpha\) provides a critical growth and survival advantage to colon cancer cells (1, 2). Similarly, it is conceivable that human colon cancer cells involved in metastasis may derive an advantage in cell motility from a strong autocrine TGF-\(\alpha\) loop, because the initial number of cells contributing to metastatic behavior is small. Recently, we showed that in HCT116 cells, the DNA synthesis response is saturated by a relatively low EGFr occupation resulting from autocrine TGF-\(\alpha\) (3). The expression level of integrin \(\alpha_2\) and its functions, on the other hand, show a much wider window of response, increasing from low EGFr occupation by autocrine TGF-\(\alpha\) to saturation by complete receptor occupation with exogenous EGF. The initiation of this response at low level receptor occupation was shown by its attenuation with treatment by an EGFr blocking antibody that inhibits basal EGFr activation resulting from autocrine TGF-\(\alpha\) as well as by stable transfection with a TGF-\(\alpha\) antisense cDNA to inhibit basal EGFr activation in HCT116 cells. We also observed that the addition of exogenous EGF results in further EGFr activation, which is associated with higher expression of integrin \(\alpha_2\), enhanced cell adhesion, and micromotility. Thus, there is a difference in response windows based on the extent of EGFr activation. While the activation of EGFr was demonstrated to be critical to \(\alpha_2\), integrin-mediated adhesion and motility, the EGFr-mediated downstream mechanism(s) and the intracellular pathways that control autocrine TGF-\(\alpha\)-mediated cell adhesion and motility remain unclear.

In the present study, selective pharmacological inhibitors of signaling intermediates have been used to link cell signaling to integrin \(\alpha_2\) expression, cell adhesion, and motility in HCT116 colon cancer cells. This cell line has been shown to generate liver metastases when implanted orthotopically in athymic nude mice (4–6). The link between metastases and motility suggests that endogenous TGF-\(\alpha\) also has a role in the control of metastatic tumor formation.

The binding of a growth factor to its receptor may result in the activation of multiple signaling pathways, including the superfamily of mitogen-activated protein kinases (MAPKs)/extracellular signal-regulated kinases (ERK) (7, 8). Our work shows that in highly malignant growth factor-independent, metastatic HCT116 colon cancer cells, ERK plays an important role mediating endogenous cellular control of integrin \(\alpha_2\) expression, cell adhesion, and motility. This study implicates ERK activation by endogenous TGF-\(\alpha\) as one of the mechanistic features controlling metastatic spread.

**EXPERIMENTAL PROCEDURES**

*Materials—Collagen type IV (CN IV), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and bovine serum albumin (BSA) were purchased from Sigma. Polyclonal antibodies specific for integrin \(\alpha_2\) subunit (Ab 1936, integrin \(\alpha_2\) subunit (Ab 1934), and...*
functional monoclonal antibody specific for anti-human integrin α5 (clone P1E6) was procured from Chemicon International Inc. (Temecula, CA). The mouse IgG, isotype control was purchased from R&D Systems (Minneapolis, MN), and the secondary fluorescein isothiocyanate AffiniPure goat anti-mouse IgG (H + L) antibody was from Jackson ImmunoResearch (West Grove, PA). EGF, monoclonal blocking antibody, mAb 528, was obtained from Oncogene Science (Manhasset, NY), whereas J018, J023, and J024 inhibitors, PD098059 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), U0126 (1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadene) were purchased from Calbiochem and Promega, respectively. The phosphatidylinositol 3-kinase (PI3K) inhibitor LY 294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) was purchased from Calbiochem. Polyclonal anti-actin antibody was produced in rabbit (Sigma). Anti-ERK2 functional monoclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). McCoy’s 5A medium, transferrin, and insulin were obtained from Sigma, whereas EGF was purchased from R&D Systems. Arrays of gold film-coated electrodes for cell motility experiments were purchased from Applied Biophysics Inc. (Troy, NY). NHS-LC-Biotin was purchased from Pierce, whereas NHS-LC-Biotin was purchased from Genlantis, Inc. (Temecula, CA). EGFr monoclonal blocking antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).}

**Cell Culture and Adhesion Assay**—These experiments were performed as described previously (3). HCT 116 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 and 20 μg/ml insulin either in the absence or presence of EGF (10 ng/ml) depending upon experimental conditions.

For adhesion assays, 96-well tissue culture plates were coated overnight at room temperature with 5% fetal bovine serum (FBS) at concentrations of 0–0.25 μg/ml, blocked with 3% BSA in PBS for 3 h, and then rinsed once with PBS. Subsequently, the colorimetric MTT procedure was followed as described previously (3, 9).

After trypsinization, cells were incubated at 37 °C with inhibitors for 3 h to determine autocrine TGF-α or exogenous EGF-mediated cell adhesion functions. Cells were plated at 6 × 104 cells/well on CN IV-coated plates and incubated for 90 min in the absence or presence of MEK inhibitors PD098059 and U0126. Nonadherent cells were removed by washing three times with serum-free medium. The relative number of attached cells was determined by the MTT method. All inhibitors were dissolved in Me2SO as stock solutions and diluted at the third day. The cells were transfected with activated MEK1 DNA, a gift of Drs. Weber and Slack-Davis (10) at a 4:1 ratio of FuGENE to plasmid DNA. Forty-eight hours after transfection, the medium was replaced. Sixty-two hours after transfection, cells were harvested and cell lysates were prepared as described earlier. The cell lysates were analyzed for ERK activation and integrin α5 protein expression by Western blot.

**RNase Protection Assay**—Total cellular RNA was isolated from control or treated HCT116 cells by lysing the cells with the Trizol reagent following the supplier’s protocol (11). Equivalent amounts of RNA samples (40 μg) were used in RNase protection assays. The α5 subunit template was constructed by subcloning a 292-base pair EcoRI-Hinc II fragment of the human α5 subunit cDNA into plasmid PBSK(−). A high specific activity α5 subunit riboprobe was synthesized by T7 RNA polymerase, whereas actin antisense probe was prepared by Sphi-RNA polymerase in the presence of [32P]UTP (3000 Ci/mmol; Amersham Biosciences). Normalization of sample loading was assessed as previously described (3).

**Determination of Cell Surface Integrin α5 by Fluorescence-activated Cell Sorter Analysis**—HCT116 cells either were treated with MeSO, PD098059 (25 μM), or U0126 (10 μM) for 48 h. The cells were harvested with Joklik’s EDTA and washed once in culture medium. For each experimental condition, ~1 × 106 cells were pelleted and fixed in 2% formaldehyde at 4 °C overnight. The cells were washed twice with a cold 1% BSA in PBS solution. Cells were resuspended in 0.25 ml of cold 0.1% BSA in PBS and incubated with the primary antibody (IgG2a and P1E6; 1:50 dilution) for 45 min at 4 °C. Cells were washed three times with 1% BSA in PBS, resuspended in 0.1% BSA in PBS, and incubated with a secondary fluorescein isothiocyanate antibody (1:100 dilution) for 45 min on ice in the dark. The cells were washed twice with 1% BSA in PBS, resuspended in PBS, and maintained on ice. Cells were analyzed on a Beckton-Dickinson FACScan Analyzer using CellQuest and WinList software.

**Cell Motility Measurements by the Electrical Cell Impedance Sensor (ECIS) Technique**—These experiments were performed as described previously (3). Briefly, to determine cell motion, HCT116 cells were plated at 4×104 cells/well on small active gold electrodes (diameter, 250 μm) at the bottom of tissue culture wells (area, 0.5 cm²) (12–14). Four hundred microliters of medium, which served as an electrolyte, were used per well. Depending on the experimental design, arrays consisting of either five or eight individual small electrodes were used.

![Figure 1](http://www.jbc.org/)

**FIG. 1.** A, effect of EGF on activation of ERK. HCT116 cells were maintained in the absence of exogenous EGF. Lane 1, basal level of ERK activation at 4 °C; lane 2, increase in activation of ERK when cells were incubated for 20 min at 37 °C in the absence of exogenous EGF; lane 3, shows further enhancement in activation of ERK when cells were incubated for 20 min at 37 °C in the presence of exogenous EGF. Proteins were analyzed by Western blot analysis using mAb against the active phosphorylated ERK (upper panel). The lower panel shows total ERK B, densitometric quantitation in percentage activation of ERK by endogenous and exogenous ligands.
In these measurements, a 1-kHz AC signal from a constant current source was applied between the small electrode and a much larger counter electrode (0.15 cm²). This signal was too weak to disturb the cells or to change cell behavior (12). The voltage of the system was monitored by a lock-in amplifier, which can detect both magnitude and phase of the voltage appearing across the sample. The in-phase and out-of-phase voltages across the electrode were recorded by the lock-in amplifier once every second for measuring micromotion. The ECIS software ( Applied BioPhysics, Troy, NY) calculated the impedance (resistance and capacitance) values of the electrode over a designated period of time. The movement of the cells on the active electrode interfered with the flow of the current, resulting in fluctuations in the electrode impedance. These real time cellular movements were called micromotion (13) and were a measure of the motile ability of the cells under investigation. As the cells moved on the gold electrode, the sensitive nature of the lock-in amplifier detected the fluctuations in the resistance and capacitance values of impedance (14). These fluctuations were then statistically analyzed by the ECIS software, thus revealing the percentage variation in resistance, which in turn was a reflection of protein expression in TGF-α antisense cells. Upper panel, comparison of activation of ERK in control TGF-α antisense cells (lane 1) and activated MEK1-transfected cells (lane 2). Total ERK was used as a loading control. Lower panel, comparison of integrin α2 protein expression in control TGF-α antisense cells (lane 1) and activated MEK1-transfected cells (lane 2). Actin was used as a loading control. The TGF-α antisense cells were transfected with activated MEK1 DNA as described under “Experimental Procedures.”

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**Fig. 2.** A, inhibitory effect of mAb 528 on ERK activation. Cells maintained in the absence of EGF were treated with 10 μg/ml EGF-blocking mAb 528 or with mouse IgG for 48 h. Cells were lysed, and equal amounts of protein were analyzed by Western blot as described under “Experimental Procedures.” Total ERK was used as a loading control. B, inhibition of ERK in TGF-α antisense cells. HCT116 and HCT116 TGF-α antisense transfected cells were maintained in the absence of EGF. Lane 1, basal level of ERK activation in HCT116 cells; lane 2, basal level of ERK activation in TGF-α antisense cells; lane 3, increased activation of ERK when antisense cells were incubated for 20 min at 37 °C in the presence of exogenous EGF. Cells were lysed, and equal amounts of protein were analyzed by Western blot analysis as described under “Experimental Procedures,” using antibodies either against phosphorylated ERK (upper panel) or total ERK (lower panel). C, effect of MEK1 transfection on ERK activation and integrin α2 expression in TGF-α antisense cells. Upper panel, comparison of activation of ERK in control TGF-α antisense cells (lane 1) and activated MEK1-transfected cells (lane 2). Total ERK was used as a loading control. Lower panel, comparison of integrin α2 protein expression in control TGF-α antisense cells (lane 1) and activated MEK1-transfected cells (lane 2). Actin was used as a loading control. The TGF-α antisense cells were transfected with activated MEK1 DNA as described under “Experimental Procedures.”
FIG. 4. A (left panel), effect of MEK inhibitors on expression of integrin α2. HCT116 cells were treated with different concentrations of PD098059 and U0126 for 48 h. Cells were lysed, and equal amounts of protein were analyzed by Western blot analysis using specific antibody against integrin α2 as described under “Experimental Procedures.” Actin was used as a loading control.

A (right panel), effect of MEK inhibitors on expression of integrin α1. HCT116 cells were treated with PD098059 (25 μM) and U0126 (10 μM) for 48 h. Cells were lysed, and equal amounts of protein were analyzed by Western blot analysis using specific antibody against integrin α1 (Ab 1934) (upper panel) as described under “Experimental Procedures.” Actin was used as a loading control (lower panel).

B, effect of PD098059 and U0126 on the expression of integrin α2 mRNA. Total RNA (40 μg) isolated from control, PD098059 (10, 25, and 50 μM), and U0126 (5 and 10 μM)-treated cells was hybridized with 32P-labeled RNA probe.
Motility Responses of ERK/MAPK Signaling

RESULTS

ERK/MAPK Activity Is Induced by Endogenous and Exogenous EGFr Ligands—Since ERK/MAPK is a downstream effector of EGFr-mediated signaling, cells were sustained and then incubated at 37 °C for 20 min in the absence or presence of exogenous EGFr. Both ERK-1 (44 kDa) and ERK-2 (42 kDa) activities were enhanced by endogenous and exogenous ligands as determined by Western blot analyses using a mAb against the active phosphorylated ERK (p-ERK). In the upper panel (Fig. 1A), a Western blot of ERK using anti-phospho antibodies is shown, whereas the lower panel shows total ERK. Densitometric quantitation showed that endogenous ligand increased ERK activation about 80%, whereas exogenous EGFr enhanced ERK activation about 230%, as compared with the control (Fig. 1B).

Constitutive ERK Activation through Endogenous EGFr-mediated EGFr Activation—Previously, we showed that mAb 528 inhibited autocrine TGF-α-mediated EGFr activation and cell adhesion in HCT116 cells (3). To further define the role of activated ERK as a downstream event of EGFr activation, HCT116 cells were treated with EGFr-blocking mAb 528, and its effect on ERK levels was observed by Western blot analysis. The anti-EGFr antibody mAb 528 was effective in blocking EGFr activity in cells maintained in the absence of EGFr. Fig. 2A shows that mAb 528 inhibited phosphorylation of ERK as compared with control HCT116 cells. The antibody did not have any effect on total ERK. The results indicate that autocrine TGF-α contributes to ERK/MAPK signaling.

If TGF-α were acting in an autocrine manner to effect ERK activation, it would be expected that anti-TGF-α antisense-transfected cells would block activation of ERK relative to parental HCT116 cells. We have previously used well characterized HCT116 cells stably transfected with a full-length antisense TGF-α cDNA to show that the TGF-α antisense mRNA can be detected in these stably transfected cells (15, 16). The antisense mRNA forms duplexes with the sense TGF-α mRNA, which results in the reduced steady state of TGF-α mRNA but not of other genes (16). All of these effects as well as the biological effects (loss of tumorigenicity, gain of dependence on exogenous EGFr ligand for DNA synthesis, and cell proliferation) are reversed when the antisense mRNA is lost by revertant transfected cells or by the addition of exogenous ligand. Similarly, we have previously shown that exogenous EGFr ligand rescues the effects of TGF-α antisense transfection on basal steady state integrin α2 expression as well as on the subsequent steady state cell adhesion and micromotion properties (3).

The TGF-α antisense transfected cells were used to characterize autocrine TGF-α effects on ERK. Fig. 2B (lane 1) shows activation of ERK in parental HCT116 cells in the absence of exogenous EGFr, whereas lane 2 exhibits attenuation of ERK activation in HCT116 TGF-α antisense transfected cells showing that ERK activation is sensitive to TGF-α antisense expression. Furthermore, the decreased activation of ERK was rescued by treating TGF-α antisense cells with exogenous EGFr, showing that reactivation of EGFr rescues the antisense effect. However, levels of total ERK were not altered. These results are consistent with the effects of EGFr blocking mAb 528 on ERK phosphorylation in HCT116 cells.

To demonstrate that the decrease in integrin α2 expression in TGF-α antisense cells can be rescued by overexpressing ERK, we transfected TGF-α antisense cells with constitutively activated MEK1 (10). The activation of ERK and expression of integrin α2 were analyzed by immunoblotting. Fig. 2C shows that by constitutively overexpressing ERK in TGF-α antisense transfected cells, inhibition of integrin α2 is rescued. Fig. 2C shows activation of ERK in antisense cells (lane 1), whereas lane 2 shows activation of ERK in MEK1-transfected cells. Fig. 2C (lower panel) shows expression of integrin α2 in TGF-α antisense cells (lane 1), whereas lane 2 shows expression of integrin α2 in MEK1-transfected antisense cells.

PD098059 and U0126 Inhibit Cell Adhesion, Integrin α2 Expression, and ERK Activation in a Parallel Fashion—Because ERK can be activated by EGFr signaling, we determined whether autocrine TGF-α contributes to cell adhesion functions via ERK activation in HCT116 cells. We utilized highly selective MEK inhibitors to further define the role of the MAPK pathway in the control of integrin α2 and its functions (17, 18). Treatment with PD098059 or U0126 selectively inhibits MEK activity, which is responsible for phosphorylation and activation of ERK. PD098059 inhibits MEK1, whereas U0126 is an inhibitor of MEK1 and -2. Both inhibitors were effective for inhibition of cell adhesion (Fig. 3). The effect of PD098059 on CN IV-mediated adhesion in the absence of exogenous EGFr on cell adhesion was characterized. Inhibition of adhesion (35–67%) by different concentrations of the drug indicated that basal control of cell adhesion on CN IV is mediated by endogenous MAPK activation (Fig. 3A). HCT116 cells showed 33% inhibition of cell adhesion to CN IV (in the absence of exogenous EGFr) when incubated with MEK inhibitor U0126 relative to Me2SO-treated controls under identical conditions (Fig. 3B). The inhibitory effect of MEK inhibitors on cell adhesion was directly correlated with reduced expression of integrin α2, both at the protein and mRNA levels, as shown by Western blot analysis (Fig. 4A) and by an RNase protection assay (Fig. 4B).

To determine the effect of MEK inhibitors on functional cell surface integrin α2 expression, cells were treated with MEK inhibitors (U0126 and PD098059) or with the Me2SO vehicle alone. The effect of the inhibitors was analyzed by fluorescence-activated cell sorter analysis with integrin α2-specific (P1E6) functional antibodies. Fig. 4C shows that both MEK inhibitors induce a loss of cell surface integrin α2 expression. To confirm that MEK activation was directly related to ERK

The sizes of the protected fragments on urea-polyacrylamide gel electrophoresis are indicated by the arrows. Lane 1, a mixture of probes; lane 2, a negative control yeast tRNA; lanes 3–8, expression of integrin α2 mRNA after treating cells with either Me2SO (DMSO) or with the appropriate MEK inhibitor, as shown. Actin mRNA levels are shown for normalization of sample loading. C, fluorescence-activated cell sorter analysis of HCT116 cells treated with MEK inhibitors. a, control cells (Me2SO-treated) stained with anti-α2 integrin antibody (P1E6); b, cells treated with PD098059 (25 μM) for 48 h, stained with anti-α2 integrin antibody (P1E6); c, cells transfected with U0126 (10 μM) for 48 h, stained with anti-α2 integrin antibody (P1E6). As a point reference, the dotted lines indicate mean fluorescence intensity (MFI) of control cells. The mean fluorescence intensity and percentage of integrin α2-positive cells are indicated. D, effect of LY 294002 on the expression of integrin α2 protein. HCT116 cells were treated with different concentrations (0–40 μM) of LY 294002. Cells were biotinylated and lysed, and equal amounts of protein were analyzed by Western blot analysis. The upper panel shows the effect of LY 294002 on the expression of integrin α2, whereas the lower panel shows levels of actin as a loading control. E, dose-response effect of LY 294002 on adhesion of HCT116 cells. A comparison of the adhesion to CN IV of HCT116 parental and HCT116 cells treated with 20 and 40 μM concentrations of LY 294002. Substrates were prepared by coating tissue culture 96-well plates with CN IV at a concentration of 0.025 μg/ml overnight at room temperature. Cells were seeded at 6 × 104 cells/well onto coated plates and incubated for 90 min at 37 °C. The relative number of attached cells was determined by MTT assay as described under “Experimental Procedures.”

of the integrin α2 subunit (0.5 × 106 cpm) and actin (8500 cpm) simultaneously according to the details given under “Experimental Procedures.”

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activation of ERK by MEK inhibitors was also observed in the presence of exogenous EGF (Fig. 5B). Our results demonstrate that inhibition of cellular adhesion functions parallels the inhibition of activation of ERK by MEK inhibitors.

LY 294002 Does Not Inhibit Integrin α2 Protein Expression and Cell Adhesion—To determine the role of PI3K signaling in cell adhesion and integrin α2 expression, HCT116 cells were treated with various concentrations (0–40 μM) of LY 294002, a selective inhibitor of PI3K, and the expression of integrin α2 was analyzed by immunoblotting (Fig. 4D). We did not observe any decrease in the expression of integrin α2 protein by the PI3K inhibitor. Similarly, the adhesion assay, after treating cells with LY 294002, did not show any inhibition in cell adhesion (Fig. 4E). These results show that the EGFr-mediated expression of integrin α2 protein and its function are not under the control of the PI3K signaling pathway in HCT116 cells.

PD098059 Inhibits Basal Cell Micromotion in a Concentration-dependent Fashion—The real time ECIS technique was used to quantitate cell motility. Using this technique, cell motion may be measured at a nanometer level and is, therefore, called micromotion (13). Micromotion detected by the ECIS technique is directly related to conventional cell motility (19). Drugs that inhibit cell migration and cell motility in cultured cells, such as cytochalasin B, also inhibit micromotion (14, 20). Micromotion detected by the ECIS technique has been used to detect cell motility, cell morphology, and cell-ECM interactions in different systems (20, 21). More recently, ECIS has been used to establish the metastatic behavior of cells in culture (22). Cell motility is one of the salient features of invasive tumors, enabling tumor cells to migrate and metastasize into other tissues. Our results are consistent with recent findings that EGFr-mediated aberrant motility may lead to metastatic and invasive behavior in cancer cells (23).

In the ECIS technique, a small AC signal is applied across the gold electrode on which cells are plated, while the resistance and the capacitance of the electrode are measured over time (3). To determine the role of autocrine TGF-α in cell locomotion, cells (4 × 104) were grown on electrodes precoated with CN IV, in EGF-free medium. The subconfluent cultures were treated with 10 and 25 μM of PD098059 for 48 h, and the micromotion was recorded. Fig. 6A shows that in the control HCT116 cells, the percentage variation in resistance was found to be 2.421% (Me2SO; DMSO, upper panel). Treatment of the cells with PD098059 (10 μM; middle panel) decreased the fluctuations, indicating a decrease in cell motility, such that the percentage variation in resistance was now 1.580%. When cells were treated with higher concentrations of PD098059 (25 μM), we observed a further decrease in basal levels of cell micromotion (percentage variation in resistance was 0.743%), thus demonstrating that the inhibitory effect of PD098059 on cell micromotion was concentration-dependent. These results show that the basal levels of HCT116 cell micromotion are selectively under the control of endogenous ERK/MAPK signaling mediated by autocrine activation of TGF-α.

EGF Enhances CN IV-induced Cell Motility, whereas MEK Inhibitor PD098059 Abrogates EGF Effects—Following cell attachment and spreading, the micromotion of cells was studied as shown in Fig. 6B. In untreated HCT116 cells, the percentage variation in resistance was found to be 2.703 (Fig. 6B, upper panel). The addition of EGF (10 ng/ml) to the cell medium increased the fluctuations, indicating an increase in cell motility, such that the percentage variation in resistance was now 7.818 (Fig. 6B, middle panel). This indicated that the increase in cell micromotion may have been due to the activation of ERK by EGF. To determine whether this effect of EGF was indeed via MAPK signaling, we then treated these cells with PD098059 (25

![Fig. 5. A, effect of MEK inhibitors on ERK in the absence of EGF. Cells in the absence of EGF were treated with different concentrations of PD098059 (10, 25, and 50 μM; upper panel) and U0126 (5 and 10 μM; lower panel) for 48 h. Cells were lysed, and equal amounts of protein were analyzed by Western blot analysis as described under “Experimental Procedures,” using antibodies either against phosphorylated ERK or total ERK as shown. B, effect of MEK inhibitors on ERK in the presence of EGF. Cells in the presence of EGF were treated with different concentrations of U0126 (5 and 10 μM) for 48 h. Cells were lysed and equal amounts of protein were analyzed by Western blot analysis as described under “Experimental Procedures,” using antibodies either against phosphorylated ERK (upper panel) or total ERK (lower panel). DMSO, Me2SO.](https://www.jbc.org/doi/10.1074/jbc.M106095200)
Motility Responses of ERK/MAPK Signaling

We have previously reported that in HCT116 cells, the biological responses of EGFr-mediated functions including DNA synthesis, integrin α2 expression, cell adhesion, and cell micro-motion depend upon autocrine EGFr activation at the basal state as well as response to exogenous ligand (3). To further elucidate the mechanism(s) of EGFr-mediated integrin α2 expression and its biological functions in HCT116 cells, we have examined downstream signaling of basal and stimulated EGFr activation. Fig. 1 showed that ERK was endogenously activated in HCT116 cells, and incubation of cells in the presence of exogenous growth factor further activated ERK. These results are supported by our earlier observations that endogenous TGF-α does not fully activate EGFr and further incubation of HCT116 cells with exogenous EGF or TGF-α fully saturates unoccupied EGFr (3). We have now shown that the endogenous phosphorylation of ERK is due to autocrine TGF-α. Autocrine TGF-α was demonstrated to be responsible for ERK activation via EGFr signaling by two approaches. These included the use of mAb 528 and the use of TGF-α antisense-transfected cells. These results were further supported by our earlier observations that TGF-α antisense transfected cells showed reduced expression of integrin α2, cell adhesion, and micromotion (3). The decrease in ERK activation in TGF-α antisense cells as compared with parental HCT116 cells (Fig. 2B) may be critical in inhibiting cell adhesion and integrin α2 expression (3). Our MEK1 DNA transient transfection experiments (Fig. 2C) suggest that ERK/MAPK pathway is sufficient to mediate autocrine TGF-α-induced control of integrin α2. TGF-α autocrine loops have been reported in many cancer cells including colon carcinoma cells in which it has been demonstrated that malignancy is dependent upon TGF-α autocrine activity (1, 24–27).

In addition to the above experiments, selective inhibitors of MEK (PD098059 and U0126) also inhibited activation of ERK in HCT116 cells. Both inhibitors significantly inhibited the activation of ERK, in a dose-dependent fashion, both in the absence and presence of exogenous EGF. This showed that the activation of ERK is under MEK control. We further provide evidence that ERK activation in HCT116 cells is linked with cell adhesion. The inhibitory effect of MEK inhibitors on cell adhesion was concentration-dependent. To determine whether the inhibitory effect of MEK inhibitors on cell adhesion was mediated by expression of integrin α2, we treated cells with different concentrations of PD098059 and U0126. The inhibitors down-regulated the expression of integrin α2, both at protein and mRNA levels. To demonstrate the selectivity of inhibition of integrin α2 expression by ERK/MAPK signaling pathway, HCT116 cells were treated with selective MEK inhibitors (PD098059 and U0126), and expression of integrin α1 protein was determined by immunoblotting. The results show that HCT116 cells express the integrin α1 subunit; however, the levels of integrin α1 protein do not alter by either MEK

IV-coated gold electrodes were monitored for micromotion. B, EGF enhances cell micromotion, whereas PD098059 abrogates EGF effect. Electrode arrays after precoating with CN IV (5 μg/ml) were used in these experiments. HCT116 cells were cultured in the absence of EGF. Subconfluent (70–80%) cultures were either not treated (upper panel) or treated with EGF (10 ng/ml) (middle panel) and treated with EGF plus PD098059 (50 μM) (bottom panel). Cells growing on collagen-coated gold electrodes were monitored for micromotion.

DISCUSSION

Fig. 6. A, PD098059 inhibits basal levels of cell micromotion in a dose-response fashion in the absence of EGF. Electrode arrays after precoating with CN IV (5 μg/ml) were used. HCT116 cells (4 × 10⁴) were plated in medium devoid of EGF. Subconfluent cultures were either treated with Me₂SO (DMSO; upper panel), PD098059 10 μM (middle panel), or 25 μM (bottom panel) for 48 h. Cells growing on CN μM). The addition of PD098059 to the EGF-stimulated cells abrogated the increase in cell motility caused by EGF (percentage variation in resistance was 3.050; Fig. 6B, bottom panel). This demonstrated that, in addition to cell adhesion, cell micromotion is also mediated by ERK MAPK signaling.
inhibitor (Fig. 4A, right panel). These results show that MEK inhibitors selectively inhibit integrin \( \alpha_2 \) expression in HCT116 cells.

We observed the down-regulatory effect of MEK inhibitors on cell surface integrin \( \alpha_2 \) at two levels. First, the total number of cells expressing integrin \( \alpha_2 \) was reduced. In addition, inhibitors caused a marked decrease in overall surface density of integrin \( \alpha_2 \) at the individual cell level. Taken together, these results provide an explanation for the decrease in cell adhesion by MEK inhibitors. These data are consistent with the concept that a threshold level of cell surface integrin \( \alpha_2 \) is required to support optimal adhesion. The inhibitory effect of MEK inhibitors on cell adhesion and integrin \( \alpha_2 \) expression indicates that ERK activation is critical in "inside-out" signaling in HCT116 cells. Other studies have shown that ERK is critical mediator of "outside-in" signaling as well. For example, it has been shown that active ERK is present in focal adhesions formed by ECM-integrin interaction (28). The activation of ERK2/MPK by serum or growth factors has been shown to depend strongly on cell adhesion to ECM proteins (29). Integrin-mediated cell adhesion to the ECM allows efficient EGF-mediated activation of ERK. Lai et al. (30) have shown that ERK is important for osteoblast adhesion and integrin expression. ERK/MAPK signaling has previously been shown to be involved in expression of integrins (31, 32). Our experiments provide direct evidence linking endogenous ERK activation to integrin \( \alpha_2 \) expression and its biological functions.

In addition to mitogens, integrin ligation can also trigger ERK/MAPK signaling (33–36). Miyamoto et al. (36) have shown that integrin aggregation with or without ligand occupancy can initiate activation of the ERK signal transduction pathway. Aplin et al. (37) have shown that integrin \( \alpha_2 \)-mediated ERK activation was via focal adhesion kinase activation, whereas growth factors respond via Shc signaling, suggesting important differences in the activation of ERK/MAPK signaling by different ligands. Further, the adaptor protein Shc was not involved in integrin and growth factor collaborative signaling.

A second important target of EGF-mediated downstream signaling is via PI3K. This kinase is widely involved in a variety of signal transduction pathways including cell adhesion and cell survival (38). We have investigated the potential role of PI3K signaling in integrin \( \alpha_2 \) functions in HCT116 cells. Treatment of HCT116 cells with LY 294002, a selective inhibitor of PI3K signaling, did not significantly affect integrin \( \alpha_2 \) protein expression (Fig. 4D) or cell adhesion (Fig. 4E). Our results show that ERK/MAPK signaling and not PI3K signaling has a key role in the EGF-mediated control of integrin \( \alpha_2 \) function in autocrine TGF-\( \alpha \)-driven HCT116 cells. Recently, it was reported that the integrin \( \alpha_2 \beta_1 \) does not interact effectively with the PI3K/Akt pathway, whereas integrin \( \alpha_2 \beta_1 \) promptly activates this pathway in intestinal cells (39). However, the precise mechanism(s) underlying integrin-specific activation of the PI3K remains elusive.

To investigate whether EGF-mediated cell motility is linked with ERK/MAPK signaling, we examined the effect of PD098059 on cell motion. To determine cell motion, a real time quantitative and very sensitive technique, ECIS, was used (13). Further, to determine whether autocrine TGF-\( \alpha \) controls micromotion via ERK/MAPK signaling, experiments were performed in the absence of exogenous growth factor. Treatment of cells with PD098059 resulted in the inhibition of endogenous cell micromotion in a concentration-dependent fashion (Fig. 6A). These results demonstrate that autocrine TGF-\( \alpha \) not only controls cell adhesion and integrin \( \alpha_2 \) expression but also cell micromotion via ERK/MAPK signaling. Treatment of cells with exogenous EGF also dramatically enhanced cell locomotion (Fig. 6B, middle panel). The increase in percentage variation in resistance caused by EGF was abrogated by treating cells with the MEK inhibitor PD98059, thus showing that the EGFr-mediated cell micromotion, in addition to cell adhesion and integrin \( \alpha_2 \) expression, is also under the control of ERK/MAPK signaling. Autocrine growth factor-mediated signaling is believed to play an important role in cell biology. However, it is still not well understood whether autocrine effects on cell functions are different from the effects of ligand in the exogenous mode. Maheshwari et al. (40) have elegantly shown that in human mammary epithelial cells, autocrine EGF provides a directional motility signal involved in tissue organization that is not provided by exogenous ligand.

Cell micromotion may have a significant role to play in cancer metastasis, which is a complex process that includes changes in cell adhesion, allowing cancer cells to invade and migrate through the ECM. Some of these changes occur in focal adhesions that are formed due to ECM-integrin interaction when cells attach to the ECM. Cell motility is dependent on cell-substrate attachment at the leading edge of the cell coordinated with cell-substrate detachment at the rear of the cell. The attachment in the leading edge of the cell is associated with the formation of focal adhesion complexes, whereas detachment at the rear of the cell is associated with the disassembly of focal adhesion complexes and the proteolytic cleavage of the proteins that make up the focal adhesion complexes (23).

Since in HCT116 cells, growth factors EGF and TGF-\( \alpha \) enhance integrin \( \alpha_2 \) expression irrespective of mitogeneity, integrin \( \alpha_2 \) may play a role in metastatic behavior. This observation is consistent with reports indicating a relationship between integrin-mediated cell adhesion and motility on CNIV that is controlled by ECM density, integrin expression, and integrin affinity (41–44). Since integrin \( \alpha_2 \) is a receptor for CNIV, which is the major component of the basement membrane, this integrin may enhance metastasis and invasiveness. Thus, constitutive enhancement of motility by autocrine growth factors via ERK/MAPK signaling in malignant cells may be an important contributory factor to invasive and metastatic properties exhibited by HCT116 cells.

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Motility Responses of ERK/MAPK Signaling

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Integrin α2 and Extracellular Signal-regulated Kinase Are Functionally Linked in Highly Malignant Autocrine Transforming Growth Factor- α-driven Colon Cancer Cells

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