Epidermal Growth Factor (EGF) Regulates $\alpha_5\beta_1$ Integrin Activation State in Human Cancer Cell Lines through the p90RSK-dependent Phosphorylation of Filamin A*

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Background: Regulation of integrin activation has important implications for tumor cell invasion and metastasis.

Results: EGF activates ERK/p90RSK and Rho/Rho kinase signaling in A431 and DiFi colon cancer cells, leading to phosphorylation of filamin A (FLNa) and inactivation of the $\alpha_5\beta_1$ integrin receptor.

Conclusion: EGF promotes $\alpha_5\beta_1$ inactivation through the p90RSK-dependent phosphorylation of FLNa.

Significance: We have identified a novel EGF-dependent mechanism controlling the $\alpha_5\beta_1$ integrin activation state.

Cell adhesion, motility, and invasion are regulated by the ligand-binding activity of integrin receptors, transmembrane proteins that bind to the extracellular matrix. Integrins whose conformation allows for ligand binding and appropriate functional activity are said to be in an active state. Integrin activation and subsequent ligand binding are dynamically regulated by the association of cytoplasmic proteins with integrin intracellular domains. In this study, we evaluated the role of EGF in the regulation of the activation state of the $\alpha_5\beta_1$ integrin receptor for fibronectin. The addition of EGF to either A431 squamous carcinoma cells or DiFi colon cancer cells resulted in loss of $\alpha_5\beta_1$-dependent adhesion to fibronectin but no loss of integrin from the cell surface. EGF activated the EGF receptor/ERK/p90RSK and Rho/Rho kinase signaling pathways. Blocking either pathway inhibited EGF-mediated loss of adhesion, suggesting that they work in parallel to regulate integrin function. EGF treatment also resulted in phosphorylation of filamin A (FLNa), which binds and inactivates $\beta_1$ integrins. EGF-mediated FLNa phosphorylation was completely blocked by an inhibitor of p90RSK and partially attenuated by an inhibitor of Rho kinase, suggesting that both pathways converge on FLNa to regulate integrin function. A431 clonal cell lines expressing non-phosphorylated dominant-negative FLNa were resistant to the inhibitory effects of EGF on integrin function, whereas clonal cell lines overexpressing wild-type FLNa were more sensitive to the inhibitory effect of EGF. These data suggest that EGF-dependent inactivation of $\alpha_5\beta_1$ integrin is regulated through FLNa phosphorylation and cellular contractility.

The extracellular matrix regulates basic biological processes by providing the cell with chemical, structural, and mechanical information about the tissue microenvironment. Changes in matrix properties are perceived by the cell through complex structural linkages between transmembrane receptors and the cytoskeleton. These structural linkages are dynamic and regulated by a system of scaffolding proteins, adaptor molecules, enzymes, and lipids (1). The interaction of cells with the extracellular environment is dependent on integrins, a family of heterodimeric transmembrane proteins that represent the primary connection between the extracellular matrix and the intracellular cytoskeleton. The binding of integrins to the extracellular matrix regulates bidirectional signaling cascades controlling the basic cellular functions of survival, growth, and migration (2). The ability of integrins to bind extracellular matrix proteins is regulated intracellularly by proteins that bind to the integrin cytoplasmic domain, resulting in structural alterations in the ectodomain that regulate ligand accessibility (3). Integrins whose conformation promotes ligand binding and appropriate functional activity are said to be activated. Integrin activation is under the control of “inside-out signaling,” a process by which intracellular signaling networks regulate the association of integrin cytoplasmic tails with binding proteins that impact integrin conformation. In adherent cells, integrin activation states are dynamic and carefully regulated to coordinate the requisite binding and release of matrix proteins required for cell migration, cytokinesis, and tissue remodeling (4).

Cytoplasmic actin-binding proteins, such as talins and filamins, bind directly to integrin tails and positively or negatively regulate integrin function. Talin is a cytoskeletal adaptor protein that binds simultaneously to actin and integrin, thereby providing a structural continuum between the extracellular matrix and filamentous actin. The binding of talin to the integrin cytoplasmic domain causes changes in the integrin ectodomain that result in the integrin being in a high-affinity state and able to bind ligand (5). Filamin A (FLNa)$^2$ is an actin-binding protein that cross-links actin into orthogonal networks. FLNa binds to the integrin cytoplasmic domain, but unlike talin, FLNa promotes an inactive non-ligand binding conformation (6). Several studies have provided evidence that FLNa is a negative regulator of integrin functions, such as cell adhesion and

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2 The abbreviations used are: FLNa, filamin A; EGFR, EGF receptor; MLC2, myosin light chain 2; RSK, ribosomal S6 kinase; FAK, focal adhesion kinase.
migration (7, 8). The FLNa monomer is a 240–280-kDa protein that consists of an N-terminal actin-binding domain and 24 Ig-like domains. FLNa and talin have overlapping binding sites on the β1 cytoplasmic tail, and FLNa can prevent the binding of talin to integrin, thereby keeping integrin in an inactive conformation (6). Integrin cytoplasmic domains bind directly to FLNa IgG domain 21, and this binding site is cryptic and appears to be under the regulation of cellular tension (9, 10).

Integrins can physically and functionally associate with growth factor receptor tyrosine kinases to promote reciprocal signaling mechanisms required for the regulation of proliferation, migration, and survival (11). Co-clustering of EGF receptor (EGFR) and integrins is seen during cell adhesion, where integrin ligation can initiate ligand-independent EGFR aggregation and signaling (12). Integrin-dependent adhesion also enhances growth factor-initiated signaling by increasing the strength and duration of the signal (13). However, the role that growth factor receptor tyrosine kinases, such as EGFR, play in the regulation of integrin activation through inside-out signaling is less well understood. Earlier studies have shown that EGF/EGFR can modulate cell adhesion in several cancer cell lines (14, 15); however, the mechanisms by which EGF regulates integrin function have been largely unexplored. In this study, we show that treatment of A431 squamous carcinoma cells or DiFi colon cancer cells with EGF prevents α5β1 integrin-dependent adhesion to fibronectin. The inhibitory effect of EGF on adhesion is due to the inactivation of the α5β1 integrin receptor. Integrin inactivation is dependent on the ERK/p90RSK and Rho/Rho kinase signaling pathways, which are activated in response to EGF and converge to regulate the phosphorylation of FLNa at Ser-2152. These data identify the p90RSK-dependent phosphorylation of FLNa as an essential step in the regulation of α5β1 integrin function by EGF.

**EXPERIMENTAL PROCEDURES**

*Reagents and Antibodies—Unless indicated otherwise, reagents were obtained from Sigma-Aldrich. Non-tissue culture-treated bacterial plates were from Greiner Bio-one North America (Monroe, NC). EGF was purchased from R&D Systems (Minneapolis, MN). The anti-α5 integrin (clone P1D6) and anti-β1 integrin (clone P5D2) blocking antibodies were from EMD Millipore Corp. (Billerica, MA), and control mouse IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA). Mouse monoclonal antibody to phosphorylated-ERK; rabbit antibodies to phosphoryl-Thr18/Ser19 myosin light chain 2 (MLC2), phospho-Ser–2152 FLNa, phospho-Ser–380 ribosomal S6 kinase (RSK), and total RSK; and the MEK inhibitor U0126 were purchased from Cell Signaling Technology (Danvers, MA). The MEK inhibitor PD184352 (also called CI-1040) and rabbit anti-ERK2 and anti-focal adhesion kinase (FAK) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Anti-FLNa monoclonal antibody was from AbD Serotec (Raleigh, NC). The EGFR inhibitor AG1478 and the p90RSK inhibitor BI-D1870 were from Enzo Life Sciences (Plymouth Meeting, PA). The vectors expressing wild-type or mutant FLNa were obtained from Addgene (Cambridge, MA) and were initially deposited by John Blenis (16). The cell-permeable form of C3 transferase was obtained from Cytoskeleton (Denver, CO). Human plasma fibronectin was purified to homogeneity from a fibronectin- and fibrinogen-rich byproduct of Factor VIII production by ion exchange chromatography on DEAE-cellulose (Amersham Biosciences) as described previously (17).

**Cell Culture—**Human epidermoid A431 carcinoma cells were grown in DMEM (Invitrogen) containing antibiotics (penicillin/streptomycin) and 10% FBS (HyClone Laboratories, Logan, UT). The DiFi colorectal carcinoma cells have been described previously (18) and were kindly provided by Dr. Zhen Fan (The University of Texas MD Anderson Cancer Center). DiFi cells were cultured in DMEM medium F-12 containing 10% FBS plus antibiotics.

**Isolation of Wild-type and Mutant FLNa-overexpressing A431 Cell Lines—**Three μg of the expression vector pcDNA3 (control) or the vector expressing wild-type (pcDNA3-WT-FLNa) or mutant (pcDNA3-FLNa(S2152A)) FLNa were transfected into A431 cells (2 × 10⁵ cells cultured overnight) using Lipofectamine 2000 (Invitrogen). Two days later, cells were trypsinized and placed in selection medium (500 μg/ml G418). Pools of G418-resistant A431 cells were obtained, and G418-resistant clones were isolated by limiting dilution. Two clones, each expressing wild-type FLNa, mutant FLNa(S2152A), or vector alone, were selected for expansion and used for experiments.

**Cell Adhesion Assay—**Polystyrene non-tissue culture-treated 48-well plates (Lab Planet) were coated with fibronectin (10 μg/ml, unless stated otherwise) in PBS overnight at 4°C. Wells were then blocked at room temperature in PBS plus 3% (w/v) BSA for 2 h. For adhesion assays, A431 cells in DMEM containing 0.1% BSA and 15 mM Hepes were seeded onto fibronectin-coated wells. Control experiments showed that none of the cell lines used in the studies were adherent in the absence of fibronectin. Pharmacological inhibitors were added to cells prior to plating. EGF was added at the time of seeding. Specific treatment times and EGF doses are provided in the figure legends. Adherent cells were washed once in PBS, fixed in PBS containing 3% formaldehyde for 40 min, and stained with 0.05% toluidine blue for 1 h. Wells were then washed three times in water. Toluidine blue was extracted with 10% acetic acid, and its absorbance was measured at 650 nm. Measurements were corrected for light scattering by subtracting the absorbance obtained at 405 nm.

**Immunoblotting—**Signaling intermediates activated in response to EGF were identified by immunoblotting. For these experiments, cells in DMEM containing 0.1% BSA and 15 mM Hepes were seeded onto non-tissue culture-treated plates (12-well), which were coated overnight at 4°C with poly-L-lysine (100 μg/ml). After 40 min at 37°C, cells were stimulated with EGF (3 nM) for 20 min. When pharmacological inhibitors were used, cells were pretreated with inhibitors for 1 h prior to EGF treatment. After EGF treatment, cells were placed immediately on ice, washed once in cold PBS, and lysed with 2 × hot sample buffer (0.12 M Tris (pH 6.8), 4% SDS, 20% glycerol, and 5% β-mercaptoethanol). In some experiments, nuclear free lysates were prepared. Cells on ice were lysed with cold lysis buffer (10 mM Tris-Cl (pH 7.4), 1% Triton X-100, 0.5% Nonidet P-40, 140 mM NaCl, 10 mM NaF, 1 mM Na₂O₄, and 10% FBS plus antibiotics.

**Isolation of Wild-type and Mutant FLNa-overexpressing A431 Cell Lines—**Three μg of the expression vector pcDNA3 (control) or the vector expressing wild-type (pcDNA3-WT-FLNa) or mutant (pcDNA3-FLNa(S2152A)) FLNa were transfected into A431 cells (2 × 10⁵ cells cultured overnight) using Lipofectamine 2000 (Invitrogen). Two days later, cells were trypsinized and placed in selection medium (500 μg/ml G418). Pools of G418-resistant A431 cells were obtained, and G418-resistant clones were isolated by limiting dilution. Two clones, each expressing wild-type FLNa, mutant FLNa(S2152A), or vector alone, were selected for expansion and used for experiments.

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tease inhibitors (Roche Applied Science)). Cell lysates were cleared by centrifugation (14,000 rpm for 15 min at 4 °C), and protein concentrations were determined with a bicinchoninic acid protein assay reagent kit (Thermo Scientific, Rockford, IL) using BSA as a standard.

Samples were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Schleicher & Schüll). Membranes were blocked in Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween 20 containing 5% (w/v) BSA and incubated overnight with primary antibodies at 4 °C. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies, and proteins were detected using an enhanced chemiluminescence reagent (GE Healthcare). For reprobing, membranes were incubated in 62.5 mM Tris-Cl (pH 6.8), 2% SDS, and 1% β-mercaptoethanol for 30 min at 60 °C to remove previously bound antibodies. ImageJ software was used to measure the intensity of Western blot bands.

RESULTS

EGF Inhibits Cell Adhesion—Earlier studies have shown that EGF can modulate adhesion in several cancer cell lines, suggesting that EGF signaling regulates integrin function (15). A431 is a metastatic squamous carcinoma cell line that expresses high levels of EGFR, the tyrosine kinase receptor for EGF (19). Experiments were performed to determine the role of EGFR in the regulation of the function of α5β1 integrin. Incubation of A431 cells with EGF inhibited the binding of cells to fibronectin. The effect of EGF on A431 cell adhesion was dose-dependent, as increasing amounts of EGF resulted in a progressive loss of cell adhesion to fibronectin (Fig. 1A). There was no adhesion of cells to control wells, which were not coated with fibronectin (data not shown). A431 cell adhesion to fibronectin was inhibited by a blocking antibody (clone P1D6) to the α5β1 integrin receptor (Fig. 1B). These data are consistent with EGF inhibiting the adhesive function of α5β1 integrin. The inhibitory effects of EGF on cell adhesion were prevented when cells were pretreated with inhibitors of EGFR kinase or ERK activation (Fig. 1C and D), indicating a role for EGFR signaling in mediating the inhibitory effects of EGF on cell adhesion. Although the addition of the EGFR kinase inhibitor AG1478 nearly completely reversed the inhibitory effect of EGF on adhesion, inhibition of MEK using PD184352 significantly attenuated but did not completely inhibit the effects of EGF on cell adhesion. This partial reversal was seen with another inhibitor of MEK activity, U0126 (Fig. 1D), suggesting that EGFR may also activate ERK-independent pathways that contribute to the inhibitory effects of EGF on cell adhesion. Similar results were seen using the colon carcinoma cell line DiFi (18). These cells also express high levels of EGFR and were inhibited from binding to fibronectin in the presence of EGF (Fig. 2A). The inhibitory effects of EGF were dose-dependent, and the effective range of inhibition was similar to that seen in A431 cells (compare Figs. 1A and 2A). EGF-dependent inhibition of cell adhesion in DiFi cells was reversed when cells were pretreated
EGF Regulates Integrin Activation State

FIGURE 2. EGF signaling inhibits α5β1 integrin-dependent adhesion of DiFi colon cancer cells to fibronectin. A, DiFi cells were seeded onto fibronectin (10 μg/ml)-coated wells for 75 min in the presence of the indicated concentrations of EGF. Cell adhesion was assayed as described in the legend to Fig. 1. B and C, DiFi cells were pretreated for 1 h with the EGFR kinase inhibitor AG1478 (B) or the MEK inhibitor PD184352 (C) at the indicated concentrations prior to assay with or without EGF (10 nM). Error bars indicate S.D. of triplicate samples for one of three representative experiments.

with AG1478, an inhibitor of EGFR kinase (Fig. 2B), or with PD184352, an inhibitor of MEK (Fig. 2C).

To determine the mechanism by which EGF inhibits cell adhesion to fibronectin, we considered whether EGF causes a loss of integrin from the cell surface or whether EGF induces a change in the activation state of the integrin. Flow cytometry studies showed that there was no loss of β1 integrin from the cell surface in either A431 or DiFi cells following the addition of EGF (data not shown). To determine whether EGF causes a decrease in the activation state of the integrin, cells were pre-treated with Mn2+, which stabilizes integrins in an active conformation. As shown in Fig. 3, pretreatment of cells with Mn2+ prevented the inhibitory effect of EGF on cell adhesion in both A431 and DiFi cells. The Mn2+-dependent adhesion of both cell types was completely inhibited by a blocking antibody to β1 integrin (clone P5D2), indicating that the effects of Mn2+ on adhesion are dependent on β1 integrin. The addition of a control IgG had no effect on Mn2+-induced adhesion. These data suggest that EGF inhibits cell adhesion through changes in integrin activation state and that stabilization of the high-affinity, ligand binding conformation of the integrin with Mn2+ prevents EGF-dependent inactivation. These data are consistent with a mechanism in which EGF inhibits cell adhesion by causing a decrease in the affinity of α5β1 integrin for fibronectin. Taken together, these data suggest that EGF regulates the interaction of cells with fibronectin by regulating intracellular signaling pathways that impact on the activation state of α5β1 integrin.

Effects of EGF on α5β1 Integrin Function Are Mediated through p90RSK and Rho Signaling—To understand how EGF might regulate the activation state of α5β1 integrin, a detailed analysis of the molecular events regulating integrin adhesive function was carried out in A431 cells. As ERK signaling was shown to modulate integrin function by EGF (Fig. 1), experiments were done to address whether p90RSK, a major target of ERK in the cytoplasm, is involved in the regulation of integrin function by EGF. As shown in Fig. 4A, phosphorylation of both ERK and p90RSK could be seen within 2–5 min after the addition of EGF to A431 cells. Quantitation of Western blots of EGF-treated A431 cell lysates showed a rapid parallel increase in the phosphorylation of both ERK and p90RSK within 5 min (Fig. 4, B and C). ERK activity was required for p90RSK activation, as inhibition of ERK activity with PD184352 completely blocked the phosphorylation of p90RSK in response to EGF (Fig. 4D). To determine whether p90RSK is required for the inhibitory effects of EGF on cell adhesion, A431 cells were pre-incubated with the p90RSK inhibitor BI-D1870 prior to incubation with EGF. As shown in Fig. 4E, BI-D1870 prevented the inhibitory effect of EGF on cell adhesion, thus confirming that p90RSK activation is required for the inhibitory effect of EGF on α5β1 integrin.

EGF is a known regulator of cytoskeletal dynamics that can also impact on integrin function. Therefore, experiments were done to determine whether Rho signaling might be involved in the regulation of integrin function by EGF. To evaluate this possibility, A431 cells were preincubated with inhibitors of the Rho signaling pathway prior to treatment with EGF. The addition of increasing amounts of the Rho inhibitor C3 transferase nearly completely prevented the loss of cell adhesion in response to EGF (Fig. 5A). Similar results were obtained when cells were pretreated with Y27632, an inhibitor of the Rho effector Rho kinase, which also attenuated the inhibitory effect of EGF on cell adhesion (Fig. 5B). Western blot analysis showed that EGF stimulated the phosphorylation of MLC (Fig. 5C), which could be completely inhibited by pretreatment of cells with Y27632 (data not shown). Quantitation of the Western blot data showed a 2.5-fold increase in MLC phosphorylation within 10 min of treatment with EGF (Fig. 5D). These data suggest that cellular contractility pathways contribute to the inactivation of integrin function by EGF. Interestingly, the effects of the Rho kinase inhibitor Y27632 and the MEK inhibitor PD184352 were additive when combined at suboptimal amounts (Fig. 5E), suggesting that contractility and EGFR signaling pathways work in parallel to regulate integrin function.

EGF Regulates α5β1 Integrin Activation State through Phosphorylation of FLNa—FLNa is a cytoskeleton-associated protein that binds integrin cytoplasmic domains and is known to regulate integrin activation states. FLNa is a direct target of p90RSK that phosphorylates FLNa at Ser-2152 (16). Western blot analysis of EGF-treated A431 cell lysates showed that phosphorylation of FLNa at Ser-2152 began 5–10 min following treatment of cells with EGF (Fig. 6A) and was increased by 3-fold within 30 min (Fig. 6B). FLNa phosphorylation in response to EGF was completely blocked by specific inhibitors of MEK (PD184352) and RSK (BI-D1870) (Fig. 6C), confirming that FLNa is a downstream target of an ERK/p90RSK signaling pathway in these cells. FLNa phosphorylation was also partially inhibited by the Rho kinase inhibitor Y27632 (Fig. 6D). Quantitation of the data from four separate experiments showed that...
inhibition of Rho kinase reduced the phosphorylation of FLNa by about half after EGF treatment (Fig. 6E). These data indicate that although the FLNa phosphorylation in response to EGF was dependent on p90RSK, the extent of phosphorylation could be modulated by Rho kinase. These findings suggest that the ERK/RSK and Rho/Rho kinase pathways converge on FLNa to effect changes in FLNa function. Parallel experiments done on DiFi colon cancer cells showed that EGF also activated the same pathways, leading to the phosphorylation of FLNa and the inhibition of FLNa function (data not shown).

To address the role of FLNa phosphorylation in the regulation of integrin activity, stable A431 cell lines that expressed either Myc-tagged WT-FLNa or the non-phosphorylatable mutant S2152A (Mut-FLNa) were constructed. Two clonal cell lines expressing either wild-type FLNa (WT-FLNa clones 1 and 2) or mutant FLNa (Mut-FLNa clones 1 and 2) were selected for further analysis. In the absence of EGF, all cell lines attached to fibronectin (Fig. 7, A and C, black bars). Following the addition of EGF, cells expressing WT-FLNa were significantly less adherent to fibronectin than controls (Fig. 7A, white bars), whereas cells expressing FLNa(S2152A) were more adherent than control cells (Fig. 7C, white bars). As shown in Fig. 7B, cells transfected with WT-FLNa expressed 2–3-fold more FLNa than control cells, suggesting that the increased amount of FLNa had sensitized the cells to the inhibitory effects of EGF. Fig. 7D shows that cells expressed mutant FLNa in amounts equal to or slightly greater than endogenous levels, suggesting that the FLNa mutant functions as a dominant-negative to prevent EGF-dependent effects on integrin function. These findings suggest that in response to EGF, A431 cells regulate the
functional activity of $\alpha_5\beta_1$ integrin through the phosphorylation of FLNa.

To characterize the effect of FLNa phosphorylation on integrin affinity, cell lines expressing wild-type or mutant FLNa were compared for their ability to adhere to substrates coated with increasing amounts of fibronectin. As shown in Fig. 8A, all cell lines adhered equally to all doses of fibronectin, indicating that ectopic expression of wild-type or mutant FLNa does not affect the baseline affinity of $\alpha_5\beta_1$ integrin for fibronectin. In the presence of EGF, however, the cell lines demonstrated differential adhesion to fibronectin (Fig. 8B). The cell line expressing WT-FLNa was significantly less adherent than either the A431 parental or vector control cells. In contrast, the cell line expressing the non-phosphorylatable S2152A mutant exhibited significantly greater adhesion to fibronectin than control cells. These data indicate that in the presence of EGF, FLNa modulates the efficiency of cell adhesion to fibronectin, consistent with a role for FLNa in regulating the affinity of $\alpha_5\beta_1$ integrin for fibronectin. To evaluate the amount of phosphorylated FLNa in each cell line, cell extracts were analyzed by Western blotting. As shown in Fig. 8C, EGF treatment resulted in an increase in phosphorylation of FLNa in control cells (A431 and vector) and in cells expressing WT-FLNa. However, EGF treatment did not result in an increase in phosphorylation in cells expressing mutant FLNa, suggesting that the non-phosphorylatable FLNa mutant was acting as a dominant-negative to block the phosphorylation of endogenous FLNa, thereby preventing the EGF-dependent inactivation of $\alpha_5\beta_1$ integrin. The addition of 0.5 mM Mn$^{2+}$ to cells expressing WT-FLNa rescued adhesion of cells to fibronectin (Fig. 8D), consistent with a role for FLNa in regulating integrin function by changing its activation state. Taken together, our data are consistent with a model in which EGF signaling activates the Rho/Rho kinase and ERK/p90RSK signaling pathways, which converge on FLNa to regulate inactivation of $\alpha_5\beta_1$ integrin. The partnering of FLNa with the $\beta_1$ integrin cytoplasmic domain is regulated by both contractile forces and phosphorylation. An earlier analysis of the crystal structure of a three-domain FLNa fragment containing the primary integrin-binding site (IgFLNa19–21) showed

DISCUSSION

In this study, we defined a molecular pathway by which EGF controls the activation state of integrin receptors. Our data show that in colon and squamous cancer cells, EGF activates EGFR signaling, which leads to loss of $\alpha_5\beta_1$ integrin function. Further investigation of the molecular mechanisms underlying this phenomenon indicated that in A431 as well as DiFi cells, EGF activates both the ERK/p90RSK and Rho/Rho kinase signaling pathways, which converge on FLNa to promote FLNa binding to and inactivation of $\alpha_5\beta_1$ integrin.
that the binding of integrin cytoplasmic domains to FLNa is regulated by an autoinhibition mechanism whereby the primary integrin-binding site on IgFLNa21 is sterically blocked by the partially unfolded IgFLNa20 domain (9). Molecular dynamics simulations based on this structure have predicted that mechanical forces can unfold FLNa to expose the integrin-binding site and that phosphorylation of Ser-2152 within IgFLNa20 will facilitate this unfolding by lowering the force threshold required (10, 20). By preventing the association of activating molecules, such as talin, with the integrin cytoplasmic tail, FLNa helps to maintain the integrin in an inactive state (6). Our data showing that inactivation of \(\alpha_5\beta_1\) integrin in response to EGF is dependent on both FLNa phosphorylation and cellular contractility provide the first in vivo evidence in support of this model.

Dysregulation of signaling through EGFR and other ErbB family members is seen in many cancers. The molecular basis for this dysregulation varies considerably among different tumor types, and the impact of these altered signaling pathways on tumor progression, treatment, and subsequent relapse is not well understood but remains a highly important question in the design of EGFR-based treatment protocols (21, 22). Earlier studies have shown that adhesion of various tumor cell lines to the extracellular matrix can be modified either positively or negatively by EGF (14, 15). These studies provided suggestive evidence that in some tumors, EGFR signaling could regulate the activation state of the integrin. More recent studies have now demonstrated that EGF signaling can either positively or negatively control the activation state and function of several integrin receptors. Studies indicate that EGF can regulate integrin function by several mechanisms, including changing the amount of integrins on the cell surface, activating inside-out signaling pathways to affect ligand binding, and regulating outside-in signaling to selectively control integrin functions. In keratinocytes and squamous carcinoma, EGF signaling promotes \(\alpha_6\beta_4\) integrin inactivation and hemidesmosome disassembly (23, 24). In contrast, EGF positively regulates \(\alpha_5\beta_1\) and \(\alpha_5\beta_5\) integrin activation in ovarian and pancreatic cancer cells to promote invasion and metastasis (25, 26). These data suggest that EGF regulation of integrin function is likely to be both integrin- and cell type-specific.

EGF-mediated inactivation of \(\alpha_5\beta_1\) in A431 cells is dependent on phosphorylation of FLNa by p90RSK. p90RSK is a member of the RSK family of Ser/Thr kinases and is a substrate for
ERK1/2. In response to several different stimuli, including EGF, p90RSK is activated at the plasma membrane and controls a number of cellular processes, including cell growth, motility, and survival. These same cellular processes are also regulated by integrins, suggesting cross-talk between RSK signaling and integrin function. However, data that specifically address the details of this cross-talk are lacking. In response to mitogen stimulation, the canonical pathway to p90RSK activation proceeds through sequential ERK-dependent and PDK1-dependent phosphorylation events that are required for complete activation (27). Our data indicate that inhibition of p90RSK catalytic activity completely restores integrin function in EGF-treated cells. However, inhibition of ERK signaling only partially restores integrin activity in A431 cells. The inability of ERK inhibitors to completely restore integrin function suggests that ERK1/2-independent pathways to p90RSK activation may function in these cells. Such alternative mechanisms to p90RSK activation include membrane translocation and phosphorylation by p38 or ERK5 (28–30). Our findings indicate that FLNa is phosphorylated by p90RSK in A431 and DiFi cells and that, in these cancer cell lines, p90RSK-dependent phosphorylation of FLNa at Ser-2152 promotes the inactivation of α5β1 integrin in response to EGF.

The tumor microenvironment, particularly contact with the extracellular matrix, is now recognized as an essential regulator of tumor dormancy, a state of prolonged latency in which disseminated tumor cells remain in secondary sites, such as bone marrow and lymph nodes, for years with no clinical manifesta-
tion of malignancy (reviewed in Ref. 31). Loss of cell adhesion is thought to induce a state of dormancy in which the tumor cell is non-proliferative but capable of activating survival pathways, allowing it to avoid apoptosis and remain dormant. Recent studies have provided evidence that activation of β1 integrin plays a critical role in the release of metastatic tumor cells from dormancy. Three-dimensional culture systems used to model conditions of tumor dormancy have shown that ligation of β1 integrins and subsequent organization of the actin cytoskeleton are required for the conversion of quiescent tumor cells into proliferating cells (32–34). Identifying the mechanisms by which the integrin activation state regulates metastatic cell growth represents an important avenue of investigation. Understanding the complex interactions between the microenvironment and the tumor cell, particularly those that regulate the switch between proliferation and dormancy, may provide important clues toward developing strategies designed to control metastatic disease by maintaining the dormant state. Our studies define a molecular pathway by which metastatic cells may remain dormant under conditions in which prevailing levels of EGF are sufficient to activate EGFR kinase.

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