A Novel Mechanism for Integrin-Mediated Ras Activation in Breast Carcinoma Cells: The $\alpha_6\beta_4$ Integrin Regulates ErbB2 Translation and Transactivates Epidermal Growth Factor Receptor/ErbB2 Signaling

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Abstract

ErbB2 (HER2, Neu) and Ras play key roles in tumor invasion and metastasis. We identified a novel mechanism by which integrin $\alpha_6\beta_4$ regulates ErbB2 expression, Ras activation, and the invasion of breast carcinoma cells. Here we show that integrin $\alpha_6\beta_4$ regulates Ras activity especially in serum-depleted condition. Down-regulation of β_4 integrin by β_4 short hairpin RNA (shRNA) decreased Ras activity and carcinoma invasion whereas reexpression of this integrin restored Ras activity. ErbB2, a binding partner of epidermal growth factor receptor (EGFR), and EGFR modulated Ras activity, and integrin $\alpha_6\beta_4$ regulated phospho-EGFR level without affecting EGFR expression. We also found that integrin $\alpha_6\beta_4$ is involved in ErbB2 expression. Depletion of β_4 by shRNA reduced ErbB2 protein level without affecting ErbB2 mRNA level and reexpression of β_4 increased ErbB2 protein level. Reduction of eukaryotic initiation factor 4E, a rate-limiting factor for cap-dependent translation, decreased ErbB2 protein level, and β_4 shRNA cells exhibited a shift in ErbB2 mRNA to light polysomes compared with control cells. These results show that integrin $\alpha_6\beta_4$ regulates ErbB2 through translational control. In summary, we propose a novel mechanism for ErbB2 up-regulation and Ras activation in serum-depleted breast cancer cells; integrin $\alpha_6\beta_4$ regulates the expression of ErbB2 and the subsequent phosphorylation of EGFR and activation of Ras. These findings provide a mechanism that substantiates the reported role of $\alpha_6\beta_4$ in carcinoma invasion. (Cancer Res 2006; 66(5): 2732-9)

Introduction

Integrins regulate cell adhesion to the extracellular matrix and relay molecular cues about the cellular environment that influence cell shape, survival, proliferation, gene transcription, and migration (1). Integrins have short cytoplasmic domains but an exception is integrin $\alpha_6\beta_4$. Integrin β_4 has an atypical cytoplasmic domain; two pairs of fibronectin type III repeats separated by a connecting segment characterize this domain and it is distinct both in size ($\sim$1,000 amino acids) and structure from any other integrin subunit. This integrin is often up-regulated in carcinoma cells and there is compelling evidence that $\alpha_6\beta_4$ facilitates the formation of some carcinomas as well as the migration, invasion, and survival of carcinoma cells (2–4). Recently, $\alpha_6\beta_4$ integrin was found to promote the onset of the invasive phase of pathologic angiogenesis (5).

Sustained activation of the Ras GTPase is observed in a significant fraction of invasive breast carcinomas and it has been linked to the development and progression of this disease (6). Interestingly, however, mutations in the Ras gene are relatively infrequent in breast cancer (<5% of tumors; ref. 7). These data have led to the hypothesis that persistent activation of signaling pathways upstream of Ras accounts for its activation in invasive breast carcinomas. In this direction, signaling through EGF family receptors is known to activate Ras in many cellular contexts. For breast cancer, in particular, ErbB2 (HER2, Neu), which is persistently activated in $\sim$30% of breast cancers, has been implicated in Ras activation (8). What has emerged from these findings is that the ErbB2/Ras pathway is a critical signaling pathway in breast cancer and a prime target for therapeutic intervention (9).

Many growth factor receptors have been shown to interact with integrins to transduce stronger and more efficient signals to downstream molecules (10). ErbB2 can associate with integrin $\alpha_6\beta_4$ on the surface of breast carcinoma cell lines (11). Subsequent studies showed that both ErbB2 and integrin $\alpha_6\beta_4$ are required for phosphoinositide 3-kinase (PI3K) activation and the stimulation of cell invasion (12). Despite the fact that ErbB2 is a major regulator for Ras activity and ErbB2 interacts with integrin $\alpha_6\beta_4$, little is known about whether integrin $\alpha_6\beta_4$ can regulate Ras activity via ErbB2-dependent pathway and how these three molecules relate to each other. Given the important roles of integrin $\alpha_6\beta_4$, ErbB2, and Ras in breast carcinoma cells, we sought to investigate a more detailed relationship among $\alpha_6\beta_4$, Ras, and EGF receptor signaling in these cells, especially those cells that exhibit persistent Ras activation. More specifically, we focused on cells that can sustain Ras activation in the absence of exogenous growth factor stimulation. The results obtained indicate that Ras activation in such cells requires expression of $\alpha_6\beta_4$, which can phosphorylate the epidermal growth factor receptor (EGFR). Interestingly, further analysis revealed that $\alpha_6\beta_4$ regulates eukaryotic initiation factor 4E (eIF4E) activity and translation of ErbB2.

Materials and Methods

Cells and reagents. SUM-159 breast carcinoma cells were obtained from Dr. Steve Ethier (University of Michigan, Ann Arbor, MI). Cells were maintained in Ham's F-12 supplemented with 5% fetal bovine serum, 5 μg/mL insulin, 1 μg/mL hydrocortisone, 100 units/mL penicillin, and 100 μg/mL streptomycin. β_4 short hairpin RNA (shRNA)-pSuper.Retro and β_4 Scr-pSuper.Retro vectors were generated and these were stably expressed in SUM-159 cells as previously described (13). The sorting and subsequent surface labeling analysis of the β_4-deficient cell line were done with 3E1, a mouse anti-β_4 integrin antibody (Chemicon, Temecula, CA). The decreased level of β_4 expression in this cell line was further confirmed by Western blotting with a rabbit polyclonal anti-β_4 integrin antibody (505). The β_4 was reexpressed in the β_4 shRNA cells with a human β_4 retrovirus. The β_4 retrovirus was generated by cotransfecting 293T cells with β_4-pCLXSN

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(Alex Toker, Beth Israel Deaconess Medical Center, Boston, MA) and expression plasmids containing envelope and packaging proteins required for viral propagation using Lipofectamine as described by the manufacturer. Following infection, resistant cells were selected with 250 μg/mL G418 and β_4 shRNA cells that reexpressed β_4 were generated. Robust expression of β_4 is permitted in these cells because the enzymatic complex responsible for shRNA is saturable at high expression levels. For cells expressing 5R (a c-erbB2-specific single-chain antibody; Nancy E. Hynes, Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland), pBABE-5R and pBABE-control plasmids were introduced into the GP2-293 cells with vesicular stomatitis virus-G plasmids, and viral supernatants were used to infect SUM-159 cells. EGFR, phospho-EGFR, and phospho-tyrosine antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). ErbB2 and ErbB3 antibodies were purchased from NeoMarkers (Fremont, CA). A pan-ras antibody was purchased from Oncogene (Cambridge, MA). An eIF4E antibody was obtained from Transduction Laboratories (San Diego, CA). EGF was from Sigma (St. Louis, MO) and AG1478 was obtained from Calbiochem (San Diego, CA).

Apoptosis assays. Cells were washed once with serum-containing medium, once with PBS, once with Annexin V-FITC buffer [10 mmol/L HEPES-NaOH (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$], and then incubated for 15 minutes at room temperature with 5 μg/mL Annexin V-FITC (Biosource International, Camarillo, CA). After washing once with Annexin V buffer, the samples were resuspended in the same buffer. Immediately before analysis, 5 μg/mL propidium iodide (Biosource International) was added to distinguish apoptotic cells from necrotic cells, and the cells were analyzed using a flow cytometer.

Transfections. The eIF4E-sense and eIF4E-antisense constructs were a kind gift from Dr. Arigo De Benedetti (Louisiana State University, LA). The Dominant-negative H-Ras (N17 H-Ras) was kindly provided by Dr. Kun-Liang Guan (University of Michigan, Ann Arbor, MI). Cells were transfected with LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA) according to the protocol of the manufacturer. The transfection efficiency for SUM-159 cells was 55% to 60% as determined by green fluorescent protein plasmid transfection.

Invasion assays. The lower and upper parts of Transwells (Corning Costar) were coated with 10 μL of type I collagen (0.5 mg/mL) and 0.5 μg of cold water–diluted Matrigel (Collaborative Research, Bedford, MA), respectively. Subsequently, the coated Transwell membranes were incubated with Ham's F-12 media for 1 hour and 5×10^4 cells were plated in the upper chambers. The Ham's F-12 in the lower chambers contained 0.1 mg/mL bovine serum albumin (BSA). The inserts were incubated for 8 hours for cells that were grown in the presence of serum or for 8 hours for cells that were grown in the serum-depleted media for 28 hours. The cells that had invaded the lower surface of the membrane were fixed with methanol and stained with 0.2% crystal violet in 2% ethanol. The number of cells that had invaded was quantified by counting random fields using a light microscope equipped with a reticle.

Ras assay. Ras activity was measured utilizing a glutathione *S*-transferase (GST) fusion protein containing the Ras-binding domain (RBD) of Raf-1 as described (14). GST-Raf1-RBD construct was kindly provided by Dr. Johannes L. Bos (University Medical Centre Utrecht, Netherlands). The plasmid was transformed into *Escherichia coli* strain and protein production was initiated by adding isopropyl-D-thiogalactopyranoside to the cultures. Bacteria were resuspended in sonication buffer [20% sucrose, 10% glycerol, 50 mmol/L Tris-HCl (pH 8.0), 2 mmol/L DTT, 2 mmol/L $MgCl_2$, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, 2 μg/mL aprotinin] and lysed by sonication. The lysate was centrifuged at $12,000 \times g$ for 1 hour in the cold. Bacterially produced GST-Raf1-RBD was precoupled to glutathione-agarose beads and washed in cell lysis buffer [1% NP40, 10% glycerol, 50 mmol/L Tris (pH 7.4), 200 mmol/L NaCl, 2.5 mmol/L $MgCl_2$, 1 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L sodium orthovanadate, 1 μg/mL leupeptin, 2 μg/mL aprotinin, 10 μg/mL trypsin inhibitor, 10 μg/mL NaF]. The cells were lysed in cell lysis buffer and cleared lysates were split and used for the determination of Ras activity or for the analysis of protein expression. For Ras assay, cleared lysates were incubated with bead-GST mixture for 45 minutes. The beads mixtures were then washed four times with cell lysis buffer and bound proteins were eluted with SDS-PAGE sample buffer. Samples were separated by SDS-PAGE (12.5% polyacrylamide), blotted, and probed with anti-Ras antibody.

Analysis of protein expression. Cells were analyzed for their expression of specific proteins by either fluorescence-activated cell sorting (FACS) or immunoblotting. For FACS analysis, cells were washed twice with ice-cold PBS containing 0.2% BSA. Aliquots of cells were incubated for 1 hour at 4°C with antibodies in the PBS/BSA solution. The cells were washed thrice with PBS/BSA and then incubated with secondary antibodies coupled to R-phycoerythrin for 1 hour at 4°C. After washing thrice with PBS/BSA, the cells were resuspended in PBS and analyzed using a FACScan (Becton Dickinson, Franklin Lakes, NJ). Immunoblot analysis was done as previously described (15).

Immunoprecipitation. Cells were extracted with a buffer [20 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L EGTA, 10% glycerol, 1 mmol/L sodium orthovanadate, 1 mmol/L NaF, 2 mmol/L phenylmethylsulfonyl fluoride, 2 mg/mL aprotinin, 2 mg/mL leupeptin, and 1 mg/mL pepstatin]. After centrifugation, supernatants were collected and these were preabsorbed for 2 hours with protein A-Sepharose and protein G-Sepharose beads (Amersham Biosciences, Piscataway, NJ). After centrifugation at 2,500 rpm for 5 minutes, the supernatants were incubated overnight at 4°C with ErbB2 antibody. After precipitation with protein A-Sepharose and protein G-Sepharose, these were washed four times with a buffer and eluted in 2× reducing sample buffer.

Polysome isolation. Cells were maintained in medium without serum for 36 hours and then pretreated with 100 μg/mL cycloheximide for 15 minutes at 37°C before being harvested. After washing once with PBS containing 100 μg/mL cycloheximide, the cells were resuspended in 0.5 mL of a modified U+S buffer (16). This buffer was composed of 200 mmol/L Tris-HCl (pH 8.8), 25 mmol/L $MgCl_2$, 5 mmol/L EGTA (pH 8.0), 150 mmol/L KCl, 10 μg/mL heparin, 5 mmol/L DTT, 1% sodium deoxycholate, 2% polyoxyethylene 10-tridecy ester, 100 μg/mL cycloheximide, and 200 mmol/L sucrose. RNase inhibitor (Amersham Biosciences) was added to a final concentration of 0.5 units/μL. Cells were homogenized with 20 to 25 strokes in a Kontes tissue homogenizer, followed by centrifugation for 5 minutes at $14,000 \times g$. The supernatant was collected and frozen at −80°C until further use. Sucrose gradients (15-50%, w/w) were layered with 300 μL of cleared cell extract, which was then centrifuged at $160,000 \times g$ for 2 hours. Fractions (0.75-0.375 mL) were withdrawn from the top of the gradient and monitored for absorbency at 254 nm using an Isco syringe pump with UV-6 detector. Total RNA from the sucrose gradient fractions was extracted using Trizol LS (Life Technologies, Rockville, MD) according to the instructions of the manufacturer. Northern blot analysis was used to measure the mRNA level in each fraction.

Northern blot analysis. RNAs were electrophoresed on 1% agarose gels containing 6% formaldehyde and transferred to Hybond-N membrane (Amersham Biosciences) by capillary transfer. The membrane was fixed using an optimized UV cross-linking procedure. Prehybridization and hybridization were done at 68°C in ExpressHyb hybridization solution (Clontech, Palo Alto, CA). cDNA probes were labeled with [^{32}P]dCTP (3,000 Ci/mmol; Perkin-Elmer, Norwalk, CT) using a random primer kit (New England Biolabs, Ipswich, MA). The blot was washed twice with 20× SSC [300 mmol/L NaCl, 30 mmol/L sodium citrate (pH 7.0)] containing 0.05% SDS at 25°C and then with 0.1× SSC containing 0.1% SDS at 55°C and autoradiographed at −70°C.

Results

Constitutive activation of Ras in serum-depleted cells requires expression of the $\alpha_6\beta_4$ integrin. To examine the relationship between the $\alpha_6\beta_4$ integrin and constitutive Ras activation, we used a retrovirus containing a β_4 shRNA to deplete β_4 expression in SUM-159 cells, which were derived from an aggressive, anaplastic breast tumor (17). This approach, which we previously described (13), resulted in the generation of a β_4-RNAi cell line that exhibited a substantial reduction in total β_4 expression as well as cell-surface β_4 level compared with cells that expressed

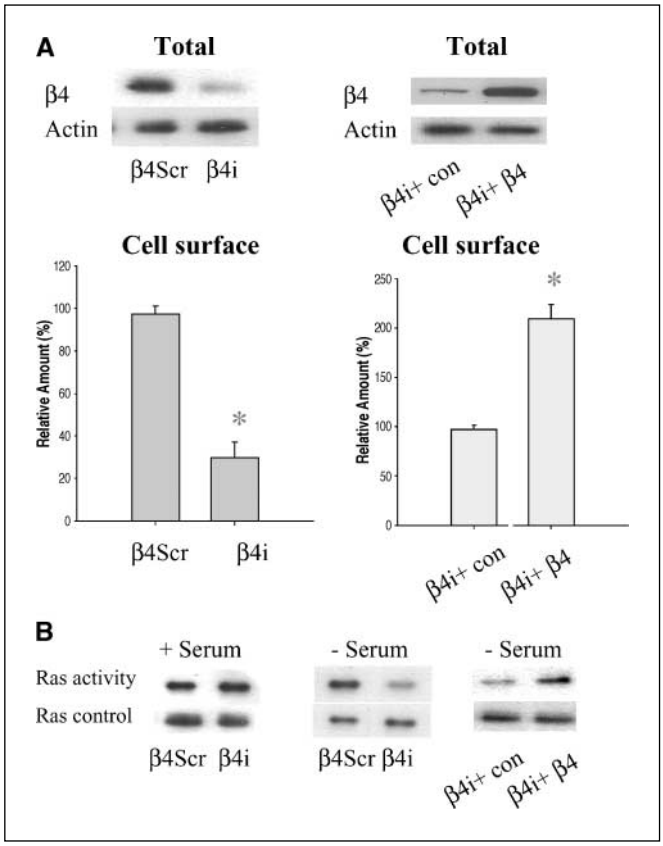

Figure 1. Integrin β_4 regulates Ras activity in the serum-depleted cells. *A,* cells that stably express β_4-Scr or β_4 shRNA were used for the determination of integrin β_4 levels. For reexpression of β_4, β_4-RNAi cells were infected with pCLXSN or pCLXSN-β_4 and selected as described in Materials and Methods. Total protein levels of integrin β_4 were detected using Western blot analysis. Protein loadings were normalized using the signal obtained with actin. Analysis of integrin surface expression was done by flow cytometry. Relative fluorescence intensity was calculated from mean channel fluorescence of 10,000 cells. *Columns,* mean of three independent experiments; *bars,* SD. *, $P < 0.01$ by Student's *t* test. *B,* β_4-Scr and β_4-RNAi cells were incubated in serum condition or in serum-free condition for 36 hours and Ras assay was done as described in Materials and Methods. For the Ras activity assay in β_4-RNAi-pCLXSN and β_4i-RNAi-pCLXSN-β_4 cells, these cells were incubated in serum-free condition for 36 hours and the assay was done.

a scrambled shRNA sequence (Fig. 1*A*). To control for the specificity of the β_4 shRNA in subsequent functional experiments, we reexpressed the human β_4 integrin in the β_4-RNAi cells to generate a β_4-RNAi + β_4 SUM-159 cell line that expresses the β_4 integrin at a relatively high level (Fig. 1*A*).

SUM-159 cells exhibit constitutive Ras activation that is not dependent on the presence of serum (Fig. 1*B*). Interestingly, however, the ability of these cells to sustain Ras activation in serum-deprived cells depends on expression of $\alpha_6\beta_4$ (Fig. 1*B*). Depletion of β_4 expression had no effect on Ras activity in cells maintained in the presence of growth factor–rich serum. The specificity of this effect is evidenced by the fact that enhanced expression of β_4 in the β_4 shRNA cells significantly increased Ras activity in comparison with control cells (Fig. 1*B*).

A functional consequence of the influence of integrin $\alpha_6\beta_4$ expression on Ras activity is invasion. As shown in Fig. 2*A*, depletion of β_4 integrin expression resulted in a significant decrease in the ability of serum-depleted SUM-159 cells to invade Matrigel (Fig. 2*A*), a process that was also impeded by expression of a dominant-interfering Ras construct (Fig. 2*B*). Of note, loss of β_4 integrin expression did not increase the apoptotic rate under these conditions (Fig. 2*C*).

Constitutive Ras activation depends on EGFR and ErbB2. Given the extensive literature on Ras activation by EGF family receptors and the importance of these receptors in breast cancer (18), we investigated the contribution of EGFR and ErbB2 to Ras activation in SUM-159 cells. These cells lack expression of ErbB3 (data not shown). Exposure of cells to AG1478, a specific inhibitor of the EGFR, resulted in a concentration-dependent reduction in Ras activity in serum-deprived cells (Fig. 3*A*). Similarly, infection of SUM-159 cells with a vector (5R) that expresses a c-erbB2-specific single-chain antibody, which has been shown to block ErbB2 function (19), diminished Ras activity in serum-deprived cells (Fig. 3*B*). Neither AG1478 treatment nor 5R expression had a significant effect on the apoptotic rate of SUM-159 cells (data not shown). To examine the effects of EGFR on invasion of β_4-Scr cells, AG1478 treatment followed by invasion assay was done. As shown in Fig. 3*C*, AG1478 decreased cell invasion by ~50%. Blocking of ErbB2 function by 5R expression also resulted in a significant decrease of β_4-Scr cell invasion (Fig. 3*D*).

The $\alpha_6\beta_4$ integrin "transactivates" EGFR phosphorylation. The finding that both EGFR/ErbB2 and $\alpha_6\beta_4$ contribute to Ras activation in serum-deprived cells suggested a relationship between growth factor receptor and integrin signaling in this process. To test this possibility, we treated β_4-Scr and β_4 shRNA cells with EGF and did Ras assay. As shown in Fig. 4*A*, Ras activity was not stimulated further by EGF in serum-deprived control cells. Exogenous EGF, however, increased Ras activity >2-fold in the β_4 shRNA cells. The finding that exogenous EGF did not increase Ras activation in cells that express $\alpha_6\beta_4$ supports the possibility that $\alpha_6\beta_4$ is "transactivating" EGFR signaling to some extent in serum-depleted condition. To test this hypothesis, we examined the tyrosine phosphorylation of the EGFR, which is indicative of receptor activation, as a function of $\alpha_6\beta_4$ expression. Indeed, as shown in Fig. 4*B*, depletion of β_4 expression resulted in an ~50% decrease in EGFR phosphorylation in comparison with control cells. As predicted from our previous data, this effect of $\alpha_6\beta_4$ on EGFR phosphorylation was only seen in serum-deprived cells (Fig. 4*B*). Of note, $\alpha_6\beta_4$ had no effect on the expression of the EGFR in SUM-159 cells (Fig. 4*B*). The specificity of the effect of $\alpha_6\beta_4$ expression on EGFR phosphorylation is evidenced by the finding that expression of β_4 in the β_4 shRNA cells increased EGFR phosphorylation ~2-fold (Fig. 4*B*).

Phosphorylation of the EGFR is also dependent on ErbB2 signaling in SUM-159 cells. Inhibition of ErbB2 function with the 5R antibody resulted in a marked decrease in EGFR phosphorylation (Fig. 5*A*).

The $\alpha_6\beta_4$ integrin can influence ErbB2 translation. We next examined the relationship between ErbB2 and EGFR in β_4-Scr and β_4 shRNA cells by coimmunoprecipitation. As shown in Fig. 5*B*, depletion of $\alpha_6\beta_4$ expression resulted in a loss of direct and indirect interaction of ErbB2 and EGFR. More specifically and surprisingly, a significant reduction in ErbB2 expression was evident in the β_4 shRNA cells relative to the control cells (Fig. 5*B*). This finding, which was confirmed in a separate immunoblotting experiment (Fig. 5*C*), prompted us to examine the relationship between $\alpha_6\beta_4$ and ErbB2 expression more rigorously. Reexpression of β_4 in the β_4 RNAi cells increased ErbB2 expression (Fig. 5*C*), a finding that supports the specificity of the shRNA results. We further studied the relationship between $\alpha_6\beta_4$ integrin and ErbB2 expression in another breast carcinoma cell line, MDA-MB-231. As shown in Fig. 5*D*, β_4

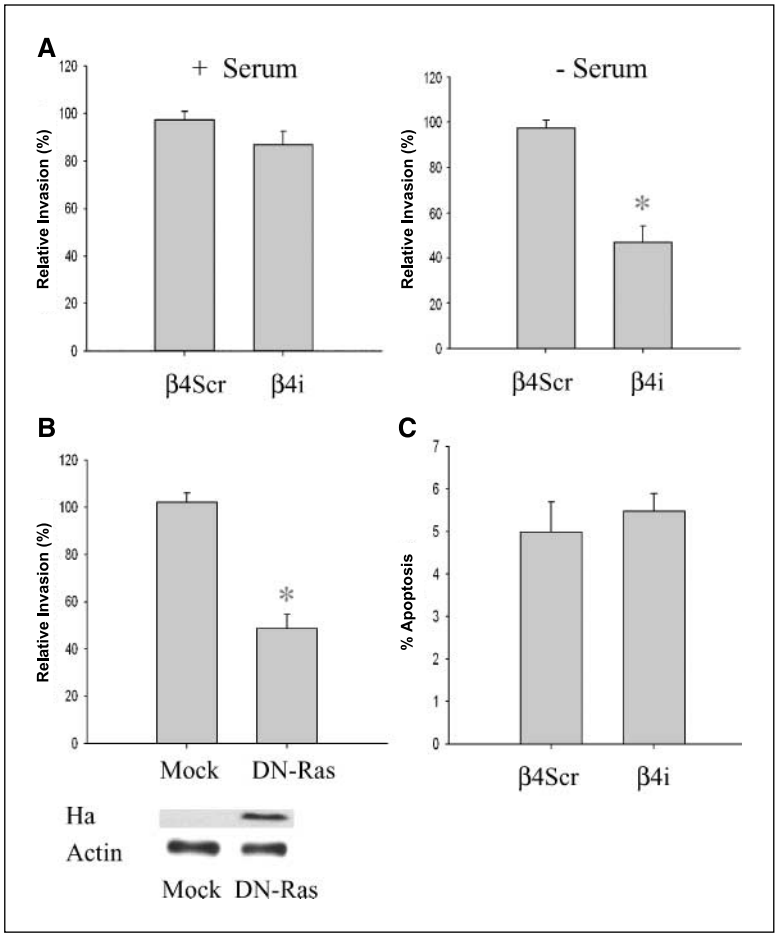

Figure 2. Integrin β_4 is involved in the invasion of serum-deprived SUM-159 cells. *A,* cells were incubated in serum condition or in serum-free condition for 28 hours and invasion assay was done for 8 hours. *Columns,* mean of three independent experiments; *bars,* SD. *, $P < 0.01$ by Student's *t* test. *B,* cells were transfected with either control vector or Ha-tagged dominant negative (*DN*) H-Ras (N17 H-Ras) vector. After 28 hours, the ability of cells to invade Matrigel was evaluated. Transfection was confirmed by anti-Ha immunoblot analysis. *Columns,* mean of three independent experiments; *bars,* SD. *C,* cells were incubated in serum-free condition for 36 hours and cell apoptosis was assessed using Annexin-FITC as described in Materials and Methods. *Columns,* mean of three independent experiments; *bars,* SD.

shRNA cells exhibited a significant reduction in β_4 integrin as well as ErbB2.

Although a decrease in ErbB2 protein expression was evident in the β_4 RNAi cells, no significant changes in erbB2 mRNA were apparent as assessed by Northern blot analysis (Fig. 6*A*). These findings suggested that $\alpha_6\beta_4$ may influence the translation of erbB2 in SUM-159 cells. Given that the concentration of eIF4E is rate-limiting for Cap-dependent translation, we reduced eIF4E expression in SUM-159 β_4 control and β_4 shRNA cells using an antisense oligonucleotide. This approach reduced eIF4E expression by ~60% compared with cells that expressed the sense oligonucleotide, and it resulted in a concomitant decrease in phospho-ErbB2, ErbB2,

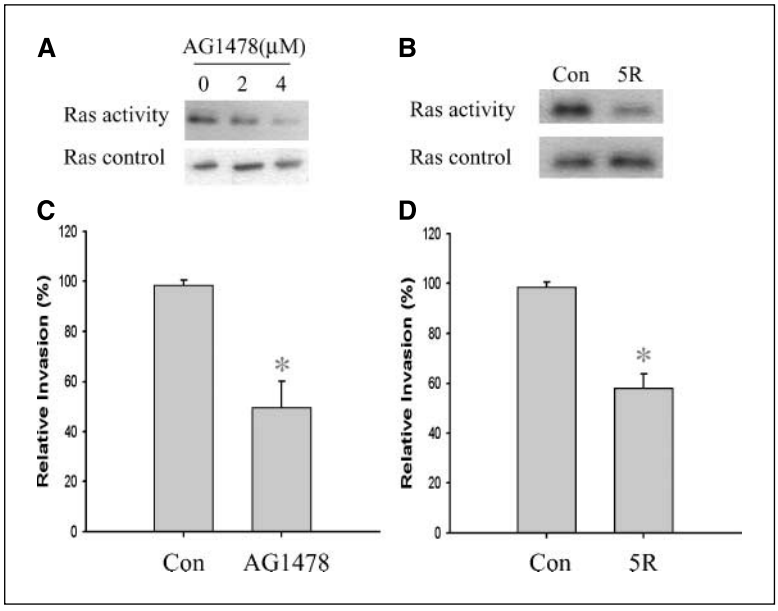

Figure 3. EGFRs pathways regulate Ras activity and invasion. *A,* cells were incubated in serum-free media for 36 hours and treated with AG1478 during the last 3 hours. Ras assay was done as described in Materials and Methods. *B,* for the generation of cells expressing 5R (a c-erbB2-specific single-chain antibody), SUM-159 cells were infected with pBABE-control or pBABE-5R viruses and stably infected cells were selected. These cells were incubated in serum-free condition for 36 hours and Ras assay was done. *C,* integrin β_4-Scr cells were incubated in serum-free condition for 28 hours. During the last 3 hours, AG1478 (4 μmol/L) treatment was done. Invasion assay was done for 8 hours in either the absence or presence of 4 μmol/L AG1478 in the upper chamber. *Columns,* mean of three independent experiments; *bars,* SD. *, $P < 0.01$ by Student's *t* test. *D,* pBABE-control and pBABE-5R cells were incubated in serum-free media for 36 hours and invasion assay was done. *Columns,* mean of three independent experiments; *bars,* SD. *, $P < 0.01$ by Student's *t* test.

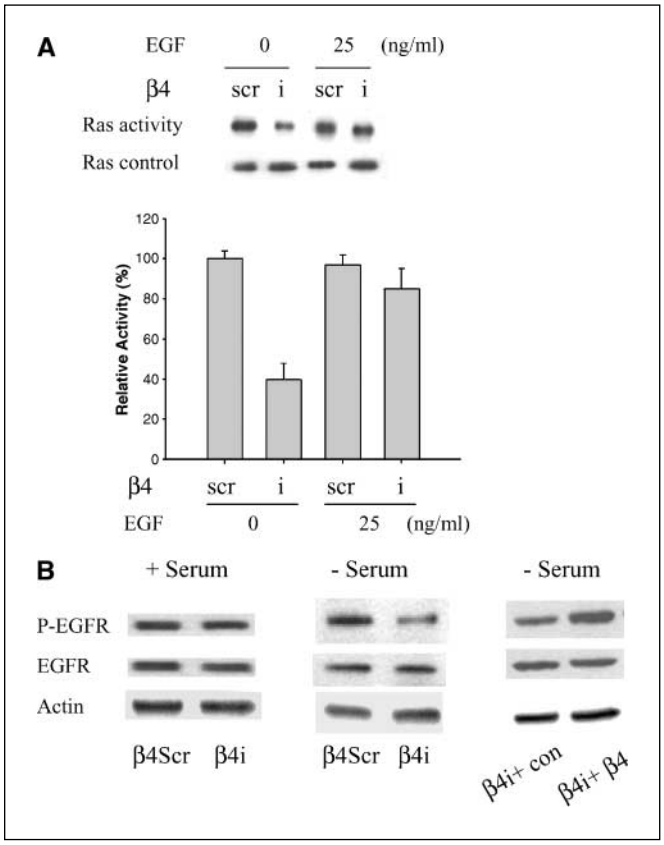

Figure 4. Integrin β_4 regulates EGFR phosphorylation. *A,* cells were incubated in serum-free condition for 36 hours and treated with 25 ng/mL EGF. After 30 minutes, Ras assay was done. *B,* β_4-Scr and β_4 shRNA cells were incubated in serum condition or in serum-free condition for 36 hours and protein levels of phospho-EGFR and EGFR were detected by immunoblot analysis. Detection of protein level in β_4-RNAi-pCLXSN and β_4i-RNAi-pCLXSN-β_4 cells was done by incubation in serum-free condition for 36 hours followed by immunoblot analysis.

and phospho-EGFR expressions in β_4 control cells (Fig. 6*B*). eIF4E reduction in β_4 shRNA cells, however, did not affect those protein levels (Fig. 6*B*), suggesting that β_4 shRNA cells may already have low eIF4E activity. To test this hypothesis, we examined phospho-eIF4E level in the β_4 shRNA and control cells. As shown in Fig. 6*C*, β_4 shRNA cells exhibited lower phospho-eIF4E level that was consistent with lower ErbB2 level in these cells. To obtain more definitive evidence that $\alpha_6\beta_4$ is regulating erbB2 translation, we did polysome analysis of the erbB2 message. mRNA isolated from the β_4 shRNA and control cells was fractionated on a sucrose gradient and the relative expression of erbB2 mRNA in each fraction was determined by Northern blotting. As shown in Fig. 6*D*, a striking difference in the distribution of erbB2 mRNA was evident in the two populations of cells. In the control cells, erbB2 mRNA fractionated in the heavy polysomal region. In contrast, in the β_4 shRNA cells, the majority of erbB2 mRNA showed a shift toward the light polysomal region. This result strongly supports the hypothesis that $\alpha_6\beta_4$ can regulate the cap-dependent translation of erbB2 in SUM-159 cells. We next examined the involvement of eIF4E in cell invasion. As shown in Fig. 6*E*, the reduction of eIF4E expression resulted in an $\sim$45% decrease in invasion.

Discussion

The involvement of integrin $\alpha_6\beta_4$ in tumor cell survival, migration, and invasion is well established. Clinical studies also support the positive correlation between integrin $\alpha_6\beta_4$ and tumor progression and poor clinical outcome (20, 21). An emerging consensus is that $\alpha_6\beta_4$ synergizes with specific molecules such as ErbB2, EGFR, Ron, Fyn, c-Met, protein kinase C (PKC), CD151, and CD9 to activate key signaling pathways for the invasion and migration of carcinoma cells (1–4, 22). The most studied signaling molecule activated by integrin $\alpha_6\beta_4$ is PI3K, an important molecule for carcinoma invasion. For instance, integrin $\alpha_6\beta_4$ functions together with ErbB2, c-Met, and insulin receptor substrates to activate PI3K (1–4, 23). This PI3K, in turn, affects clustering of integrin $\alpha_6\beta_4$ thereby increasing tumor invasion (24), suggesting a positive feedback loop between $\alpha_6\beta_4$ and PI3K. In addition to the stimulation of PI3K signaling, the $\alpha_6\beta_4$ integrin has also been shown to promote migration by the activation of other signaling molecules such as Ras and RhoA GTPase, Rac, mitogen-activated protein kinase pathways (1–4, 23). However, not much is known about the roles of this integrin on the expression of genes that are important for the carcinoma invasion and migration. It has been shown that $\alpha_6\beta_4$ integrin regulates the activities of nuclear factor of activated T cells and nuclear factor κB transcription factors (25, 26). The ability of the $\alpha_6\beta_4$ integrin to signal to transcriptional factors suggests that

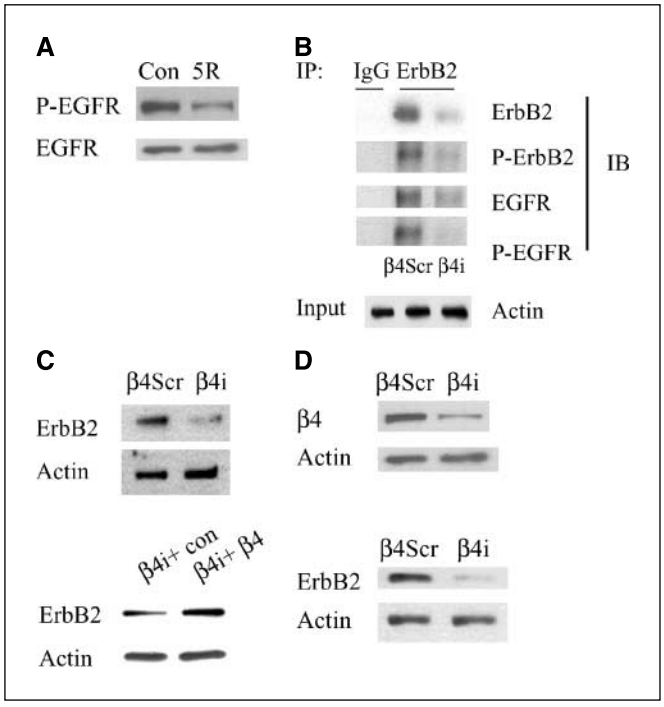

Figure 5. Integrin β_4 regulates ErbB2 protein level. *A,* cells were infected with either pBABE or pBABE-5R. The protein level of phospho-EGFR was detected using Western blot analysis. *B,* cells were lysed and the lysates were immunoprecipitated with either immunoglobulin G (*IgG*) or ErbB2 antibody as described in Materials and Methods. Protein levels of ErbB2, phospho-ErbB2, EGFR, and phospho-EGFR were detected by Western blot analysis. Actin was detected in total cell lysates before immunoprecipitation (input control). *C,* cells were incubated in serum-free condition for 36 hours and the protein level of ErbB2 was detected using immunoblot analysis. Protein loadings were normalized using the signal obtained with actin. Detection of ErbB2 protein level in β_4-RNAi-pCLXSN and β_4i-RNAi-pCLXSN-β_4 cells was done by incubation in serum-free condition for 36 hours followed by Western blot analysis. Protein loadings were normalized using the signal obtained with actin. *D,* for the generation of β_4-deficient cells, MDA-MB-231 cells were infected with integrin β_4-Scr or integrin β_4 shRNA viruses and stably infected cells were selected as described in Materials and Methods. Cells were incubated in serum-free condition for 36 hours and the expression level of integrin β_4 and ErbB2 was detected using immunoblot analysis. Protein loadings were normalized using the signal obtained with actin.

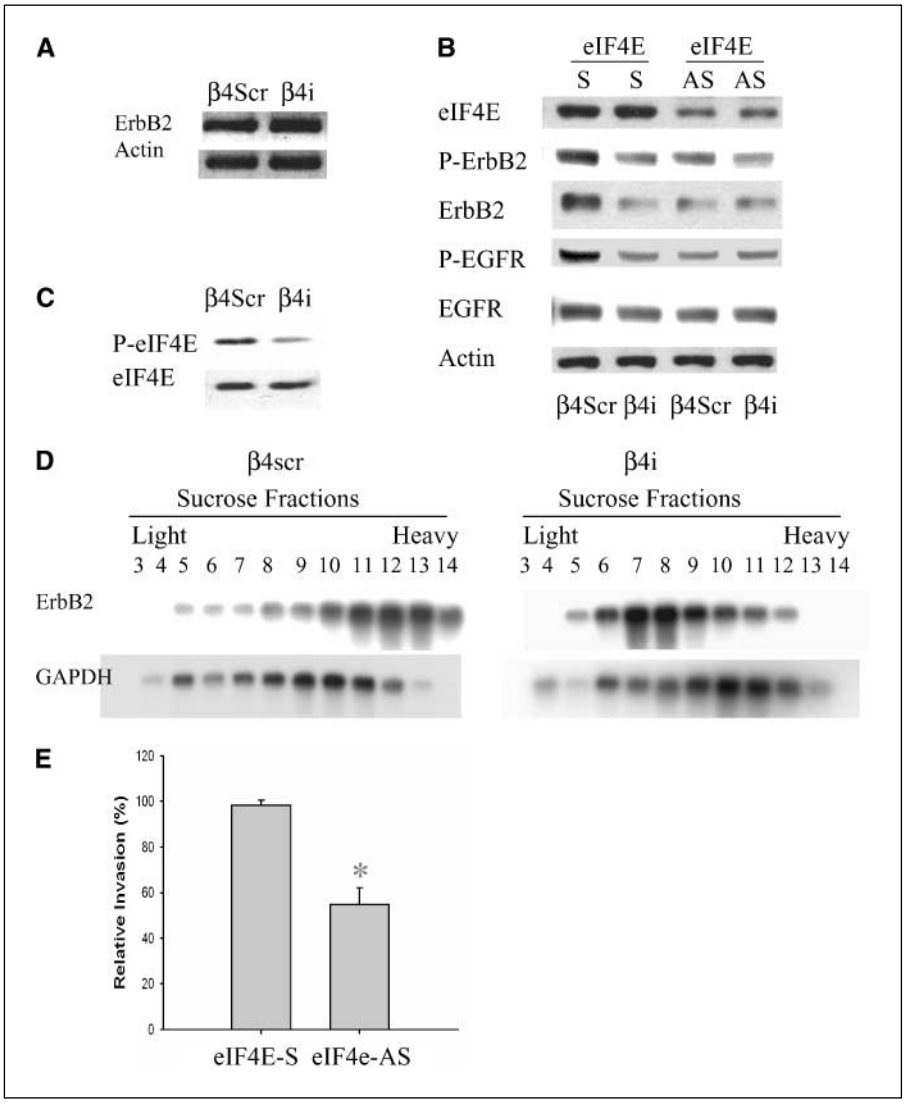

Figure 6. Integrin β_4 regulates erbB2 translationally. *A,* cells were incubated in serum-free condition for 30 hours and mRNA level of ErbB2 was detected by Northern blot analysis. *B,* β_4 control and shRNA cells were transfected with either eIF4E-sense oligonucleotide–expressing or eIF4E-antisense oligonucleotide–expressing constructs. After 36 hours, cells were lysed and protein levels of eIF4E, phospho-ErbB2, and phospho-EGFR were detected using immunoblot analysis. The blots were stripped and reprobed for the ErbB2 and EGFR proteins. Protein loadings were normalized using the signal obtained with actin. *C,* β_4 control and shRNA cells were incubated in serum-free condition for 36 hours and protein levels of phospho-eIF4E and eIF4E were measured using immunoblot analysis. *D,* β_4 control and shRNA cells were incubated in serum-free condition for 36 hours. After that, cells were lysed and RNA was fractionated on sucrose gradients. The erbB2 mRNA and glyceraldehyde-3-phosphate dehydrogenase mRNA levels were detected by Northern blot analysis. *E,* cells were transfected with either eIF4E-sense oligonucleotide–expressing or eIF4E-antisense oligonucleotide–expressing constructs. After 28 hours, invasion assay was done. *Columns,* mean of three independent experiments; *bars,* SD. *, $P < 0.01$ by Student's *t* test.

this integrin could regulate the transcription of genes. Recently, it has been shown that integrin $\alpha_6\beta_4$ promotes transcription of autotaxin, an important autocrine motility factor in breast carcinoma cells, via the nuclear factor of activated T cell–dependent pathway (27). Little is known about the roles of this integrin on translation. Only one report shows that integrin $\alpha_6\beta_4$ stimulates vascular endothelial growth factor (VEGF) translation via mammalian target of rapamycin (mTOR)–dependent inactivation of 4E-binding protein 1 (28). Here, we reveal that integrin $\alpha_6\beta_4$ regulates the activity of Ras, a key player in cell invasion, and this is achieved by regulating ErbB2 translation in serum-depleted cells. All these data indicate that integrin $\alpha_6\beta_4$ can interact and/or modulate specific molecules to synergistically activate signal molecules, which in turn regulate transcription and translation of molecules that are important for carcinoma invasion and migration.

Ras proteins control key signaling pathways that are involved in cell invasion and metastasis (8). Ras mutation can be found in 90% of pancreatic cancers, 50% of colon cancers, 50% of thyroid cancers, and 5% of breast cancers (7). Low percentages of *ras* mutations are found in breast cancers; however, Ras proteins are active in breast tumors by highly activated upstream signaling molecules, growth factor receptors (29). EGFR and ErbB2 overactivation is the most common reason for Ras pathway activation in many cancer cells (8). ErbB2 is a very potent oncoprotein (30). It is overexpressed in ~30% of invasive breast cancer cases and other cancers and is associated with enhanced tumorigenecity, metastatic potential, a poor prognosis, and resistance to chemotherapy (30). Recent reports show that ErbB2 up-regulates the chemokine receptor CXCR4 and also inhibits degradation of CXCR4 thereby increasing tumor metastasis (31). ErbB2 has been an important target in breast cancer therapy where treatment with the anti-ErbB2 monoclonal antibody trastuzumab (Herceptin) has proved effective (32). Therefore, an understanding of the mechanisms that regulate ErbB2 has clinical importance. Regulation of ErbB2 expression by integrin $\alpha_6\beta_4$ suggests a novel mechanism by which this integrin functions in the regulation of growth factor receptor activity. Previous studies on the relationship between growth factors and integrin $\alpha_6\beta_4$ have shown that growth factors regulate integrin $\alpha_6\beta_4$ functions (1). For example, epidermal growth factor (EGF) stimulates phosphorylation of the β_4 subunit on serine residues via PKCα thereby inducing hemidesmosomes disassembly (33). EGF also activates one of the Src family kinase, Fyn, which in turn phosphorylates tyrosine residues of integrin β_4 cytoplasmic

domain and induces cell migration (1). In addition, integrin $\alpha_6\beta_4$ is necessary for EGF-induced Rac activity and Rac mediates tumor survival (1–4). Hepatocyte growth factor also has been shown to induce tyrosine phosphorylation of the integrin β_4, which in turn recruits Src homology and collagen (protein) and PI3K, thereby increasing cell migration (1). These studies suggest that integrin $\alpha_6\beta_4$ plays its role as a mediator when it is activated by growth factors. In contrast, our data provide evidence that $\alpha_6\beta_4$ integrin regulates the expression of ErbB2 and the subsequent phosphorylation of EGFR and activation of Ras. Of interest, integrin $\alpha_6\beta_4$ had no effect on Ras activity, ErbB2 expression, and cell invasion in the presence of serum but has profound effects on these in the absence of exogenous growth factor stimulation. Given the facts that Ras and ErbB2 play key roles in tumor cell invasion and metastasis, and carcinomas express elevated levels of integrin $\alpha_6\beta_4$ (1, 4), our studies suggest the possibility of integrin $\alpha_6\beta_4$ for facilitating tumor invasion in growth factor–deprived circumstances.

The involvement of integrin $\alpha_6\beta_4$ on the control of ErbB2 expression through eIF4E suggests essential roles of translation regulation by this integrin in tumor invasion. Although transcriptional regulation is crucial, the flow of information from genes to proteins is too slow to accommodate rapid changes in the environment (34). Cells maintain a pool of mRNA and modulate the rates of key protein levels by a variety of sophisticated means such as translational controls. Especially, tumor cells have to escape the region that is devoid of nutrients and they encounter a variety of environments during metastasis. Therefore, tumor cells develop an efficient and powerful translational machinery by several mechanisms: aberrantly activated signal transduction pathways, changes of the expression or availability of translational machinery components, and/or variations in mRNA sequences (35). The key component for translation is eIF4E. This functions in the rate-limiting steps of translation initiation, and phosphorylation of eIF4E increases the rate of protein synthesis during tumor growth, invasion, and metastasis (34, 35). Most mRNAs are not affected by low eIF4E but some proteins, such as VEGF, c-myc, ornithine decarboxylase, and cyclin D1, are poorly translated when free eIF4E is limiting (35). The major regulators of eIF4E are Ras and Akt/mTOR (36, 37). Integrin $\alpha_6\beta_4$ regulates eIF4E, Ras, and Akt/mTOR; therefore, this integrin may function as a major regulator for translation in carcinomas. Of interest, the SUM-159 cells that we used expressed low levels of integrin β_4; however, these cells exhibited a great effect on ErbB2 translation and Ras activity. This suggests that even low levels of this integrin can function as a major translation regulator.

Because Ras and Akt/mTOR are activated by molecules such as integrin $\alpha_6\beta_4$ in many carcinomas, a good correlation is often observed among relative protein level, mRNA level, and gene copy number in tumors. However, many carcinomas have proteins that do not correlate with gene copy number and mRNA abundance. In the case of ErbB2, *erbB2* gene amplification or mRNA overexpression has been thought to cause the elevated expression of the ErbB2 protein (38). However, the level of erbB2 mRNA is not always proportional to the gene copy number, nor does the abundance of ErbB2 protein always reflect the mRNA levels. Therefore, some reports suggest the possibility of posttranscriptional regulation of erbB2 (39, 40) but no reports have identified this. Our finding that integrin $\alpha_6\beta_4$ regulates ErbB2 protein level through eIF4E suggests a novel mechanism for ErbB2 overexpression in tumor cells and new roles of this integrin for translational regulation. Taken together, it can be said that ErbB2 overexpression is regulated by complex and multiple levels: gene copy number, transcriptional, and translational control. Therefore, tumor cells that have an elevated level of erbB2 mRNA coupled with efficient translational machinery, which is strengthened by molecules such as integrin $\alpha_6\beta_4$, would benefit the most for ErbB2-dependent carcinoma invasion.

Here, we provided mechanistic understanding of integrin $\alpha_6\beta_4$-mediated breast carcinoma invasion. A potentially important consequence of our work and other reports is that integrin $\alpha_6\beta_4$ may be an attractive therapeutic target for breast cancer, especially in invading and metastasizing cells. It could be an ideal target in terms that it is a cell-surface receptor and exists with other growth factor receptors and cell-surface molecules as complexes that influence carcinoma invasion. In this regard, development of function-blocking anti-β_4 antibodies could offer a novel treatment for breast cancer patients.

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