The Intracellular Functions of \( \alpha_6\beta_4 \) Integrin Are Regulated by EGF

Fabrizio Mainiero, Angela Pepe, Mitchell Yeon, Yunling Ren, and Filippo G. Giancotti
Department of Pathology, Kaplan Cancer Center, New York University School of Medicine, New York 10016

Abstract. Upon ligand binding, the \( \alpha_6\beta_4 \) integrin becomes phosphorylated on tyrosine residues and combines sequentially with the adaptor molecules Shc and Grb2, linking to the ras pathway, and with cytoskeletal elements of hemidesmosomes. Since \( \alpha_6\beta_4 \) is expressed in a variety of tissues regulated by the EGF receptor (EGFR), we have examined the effects of EGF on the cytoskeletal and signaling functions of \( \alpha_6\beta_4 \). Experiments of immunoblotting with anti-phosphotyrosine antibodies and immunoprecipitation followed by phosphoamino acid analysis and phosphopeptide mapping showed that activation of the EGFR causes phosphorylation of the \( \beta_4 \) subunit at multiple tyrosine residues, and this event requires ligation of the integrin by laminins or specific antibodies. Immunoprecipitation experiments indicated that stimulation with EGF does not result in association of \( \alpha_6\beta_4 \) with Shc. In contrast, EGF can partially suppress the recruitment of Shc to ligated \( \alpha_6\beta_4 \). Immunochemical analysis revealed that EGF treatment does not induce increased assembly of hemidesmosomes, but instead causes a deterioration of these adhesive structures. Finally, Boyden chamber assays indicated that exposure to EGF results in upregulation of \( \alpha_6\beta_4 \)-mediated cell migration toward laminins. We conclude that EGF-dependent signals suppress the association of activated \( \alpha_6\beta_4 \) with both signaling and cytoskeletal molecules, but upregulate \( \alpha_6\beta_4 \)-dependent cell migration. The changes in \( \alpha_6\beta_4 \) function induced by EGF may play a role during wound healing and tumorigenesis.

To fully understand embryonic development, tissue repair, and tumor invasion, it is important to elucidate the mechanisms by which growth factor- and integrin-dependent signals are integrated inside cells. It is known that integrins transmit positional cues from the extracellular matrix to the cell interior, and the mechanisms by which these signals affect cellular responses to growth and differentiation factors are being actively investigated (Juliano and Haskill, 1993; Giancotti and Mainiero, 1994; Schwartz et al., 1995). Conversely, growth factors and cytokines can modulate a number of integrin-dependent functions, including cell adhesion (Serve et al., 1995; Kishnani et al., 1995), cell migration (Chen et al., 1993; Mathai et al., 1993; Klemke et al., 1994), and cytoskeletal organization (Ridley and Hall, 1992; Ridley et al., 1992), but the mechanisms underlying these phenomena are less clear.

The interaction between growth factor receptors and integrins has been largely examined in fibroblasts and platelets. Most of the studies have focused on the focal adhesion kinase p125FAK (Schaller et al., 1992). In addition to being activated and undergoing autophosphorylation in response to ligation of \( \beta_1 \) and \( \beta_3 \) integrins (Guan and Shalloway, 1992; Hanks et al., 1992; Lipfert et al., 1992), p125FAK is the target of signals originating from a number of growth factors and mitogenic neuropeptides (Zachary and Rozengurt, 1992). The activation of p125FAK has been linked to changes potentially important for the regulation of actin cytoskeleton, such as the phosphorylation of paxillin and tensin (Burridge et al., 1992; Bockholt and Burridge, 1993) and the activation of Rho (McNamee et al., 1992; Chong et al., 1994) and PI-3 kinase (Chen and Guan, 1994). In addition, activated p125FAK can combine with the Grb2/mSOS complex potentially leading to stimulation of the ras-MAP (mitogen-activated protein) kinase pathway (Schlaepfer et al., 1994), and insulin stimulation promotes association of the \( \alpha_6\beta_3 \) integrin with the Insulin Receptor Substrate 1 and the Grb2/mSOS complex (Vuori and Ruoslahti, 1994). These observations suggest that integrin- and growth factor–dependent signals may converge on p125FAK and Insulin Receptor Substrate 1 to regulate gene expression and the actin cytoskeleton.

Much less is known about the integration of growth factor- and integrin-dependent signals in epithelial and other cells that are in contact with the basement membrane. The \( \alpha_6\beta_4 \) integrin is expressed in epithelial, endothelial, and Schwann cells and binds to various isoforms of the basement membrane component laminin (Lee et al., 1992; Niessen et al., 1994; Spinardi et al., 1995). Our previous studies have focused on the mechanisms by which this integrin interacts with the cytoskeleton and with signaling molecules. In contrast to other integrins that localize to fo-
cal adhesions or otherwise interact with the actin filament system, αβ6 is found in hemidesmosomes in close proximity to molecules linking to the keratin filament system (Carter et al., 1990; Stepp et al., 1990). There is evidence indicating that the association of αβ6 with the hemidesmosomal cytoskeleton requires the uniquely large cytoplasmic domain of β6 and specifically a ~300-amino acid region, which includes the first two type III fibronectin-like modules and the connecting segment (Spinardi et al., 1993). The ability of a tail-less mutant β6 subunit to produce a dominant negative effect on the assembly of hemidesmosomes without suppressing cell adhesion to laminins indicates that αβ6 plays an essential role in organizing the hemidesmosomal cytoskeleton (Spinardi et al., 1995). Taken together, these observations suggest that laminin binding to αβ6 promotes the nucleation of hemidesmosomal cytoskeleton, and this activity is mediated by the β6 cytoplasmic domain.

Recent studies have indicated that ligation of the extracellular portion of αβ6 causes tyrosine phosphorylation of the β6 subunit, and this event is mediated by protein kinase(s) physically associated with the integrin. Coimmunoprecipitation experiments have shown that, upon ligation of the extracellular portion of αβ6, the adaptor protein Shc forms a complex with the tyrosine-phosphorylated β6 subunit. Shc is then phosphorylated on tyrosine residues and recruits the adaptor protein Grb2, thereby potentially linking αβ6 to the ras pathway. The β6 subunit is phosphorylated on multiple tyrosine residues in vivo, including a tyrosine-based activation motif (TAM) resembling those found in the T cell and B cell receptors. Since phenylalanine substitutions at the β6 TAM disrupt the association of αβ6 with hemidesmosomes, but do not interfere with tyrosine phosphorylation of Shc and recruitment of Grb2, distinct sites in αβ6 mediate assembly of the hemidesmosomal cytoskeleton and linkage to the ras pathway (Mainiero et al., 1995).

The αβ6 integrin is expressed in a variety of epithelial tissues that are regulated by the EGF (Sonnenberg et al., 1990). In this study, we have examined the effects of EGF on the cytoskeletal and signaling functions of αβ6. Our results indicate that activation of the EGF receptor (EGFR) causes tyrosine phosphorylation of the β6 subunit, but this event is not followed by association of the integrin with Shc or by increased assembly of hemidesmosomes. In contrast, EGF-dependent signals interfere with the ability of activated αβ6 to associate with both signaling and cytoskeletal molecules. Exposure to EGF causes deterioration of hemidesmosomes and leads to increased αβ6-mediated cell migration toward laminins.

Materials and Methods

Cell Lines, Transfections, Antibodies, and Extracellular Matrix Molecules

Human epidermoid carcinoma A431 cells were cultured in DME with 10% FCS or bovine calf serum, respectively. Human primary keratinocytes were cultured in keratinocyte growth medium ( Gibco BRL, Gaithersburg, MD). The 804G cells were cotransfected with the expression vector pcDNA3, encoding a full-length human EGF (Ullrich et al., 1984), and the hygroycin resistance plasmid pHBD by the calcium coprecipitation method (Giancotti et al., 1994). Stable cell lines expressing moderate levels of recombinant EGF (25-35 times lower than the endogenous EGF in A431 cells) were selected by fluorescence-activated cell sorting analysis and cultured with medium supplemented with 200 μg/ml hygromycin (Calbiochem-Novabiochem Corp., La Jolla, CA). NIH-3T3 cells overexpressing a recombinant human EGF (clone HER 14) (Honegger et al., 1987) were cultured in DME supplemented with 10% bovine calf serum and gentamicin (Gibco BRL).

The mAb 3E1 reacting with the extracellular portion of human β6 and the rabbit polyclonal antiserum to the COOH-terminal peptide of β6 were described previously (Giancotti et al., 1992). The mAbs BV7 and TS2/16 bind to the extracellular portion of the human β6 subunit (Martin-Padura et al., 1994; Arroyo et al., 1992). The rabbit antiserum to the cytoplasmic domain of α6 was previously described (Vogel et al., 1993). The anti-major histocompatibility complex (MHC) mAb W5.32 reacts with human and cultured rat cells (Kahn-Perles et al., 1987). The rabbit polyclonal anti-P-Tyr antibody #72 was produced according to published procedures (Kamps and Selton, 1988). The monoclonal anti-P-Tyr antibody 4G10 was obtained from UBI (Lake Placid, NY). The monoclonal anti-P-Tyr antibody PY20 and the monoclonal anti-Shc antibody were from Transduction Laboratories (Lexington, KY). The polyclonal anti-Shc serum #554 was obtained by immunizing a rabbit with a glutathione-S-transferase fusion protein comprising the SH2 domain of the protein. The bullous pemphigoid antigen 2 (BPAG 2)-specific rabbit polyclonal antiserum was raised by immunization with a glutathione-S-transferase fusion protein comprising the major antigenic determinant of the mouse protein in the laboratory of Jouni Uitto (Thomas Jefferson University, Philadelphia, PA). The antisera against the α6β4 integrin purified from human placenta was generated in the laboratory of Erkki Ruoslahti (La Jolla Cancer Research Foundation, La Jolla, CA) as previously described (Argaves et al., 1986). This antisera cross-reacts with rodent β6 integrins and blocks their function. The agaro-coupled 1G2 mAb was purchased from Oncogene Science (Uniondale, NY).

Human plasma fibronectin and human placental laminin 4 were purchased from Gibco BRL. Laminin 5 matrices were prepared as described previously (Sonnenberg et al., 1993; Spinardi et al., 1995).

Biochemical Methods

To test the effect of EGF on αβ6, subconfluent A431 cells were serum-starved and then treated with human recombinant EGF (Intergen Co., Purchase, NY). When indicated, the cells were detached by 10 mM EDTA and either kept in suspension or plated on dishes coated with extracellular matrix proteins before EGF stimulation. To examine the effect of selective ligation of αβ6, the cells were plated on fibronectin-coated dishes, and then incubated with sulfate polystyrene latex beads coated with the 3E1 or control mAbs TS2/16 and W5.32. Stimulation of suspended cells with antibody-coated beads was performed as previously described (Mainiero et al., 1995). At the end of incubation, the cells were extracted for 30 min at 0°C with RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) or lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1% Triton X-100) containing 1 mM sodium orthovanadate, 50 mM sodium pyrophosphate, 100 mM sodium fluoride, 0.01% aprotinin, 4 μg/ml pepstatin A, 10 μg/ml leupeptin, 1 mM PMSF, 1 mM EDTA, and 1 mM EGTA (all from Sigma Chemical Co., St. Louis, MO).

Immunoprecipitation and immunoblotting were performed as previously described (Giancotti and Ruoslahti, 1990; Giancotti et al., 1992). Non-competitive antibodies were detected by chemiluminescence with ECL (Amersham Life Sciences, Little Chalfont, UK).

Phosphopeptide mapping was performed essentially as described by Boyle et al. (1991). Serum-starved cells were labeled metabolically with [32P]orthophosphate (5 mCi/ml, ICN Biochemicals, Inc., Irvine, CA) for 3 h and then either treated with 250 ng/ml EGF for 5 min at 37°C or with 500 μM sodium orthovanadate and 3 mM H2O2 for 10 min at 37°C. After immunoprecipitation with the 3E1 antibody, the samples were transferred to nitrocellulose. The nitrocellulose fragments containing β6 were soaked in 0.5% polyvinylpyrrolidone (PVP-360; Sigma Chemical Co.), 100 mM acetic acid at 37°C for 30 min. Complete digestion was achieved by incubating the bands in 200 μl of 50 mM phosphatase buffer, pH 7.8, containing 25 μg

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2; EGFR, EGF receptor; MHC, major histocompatibility complex; TAM, tyrosine-based activation motif.
of Staphylococcus aureus V8 protease (Worthington Biochemical Corp., Freehold, NJ) for 48 h at 37°C. The samples were separated by two-dimensional TLC. Separation in the first dimension was achieved by electrophoresis in pH 1.9 buffer (2.5% formic acid, 7.8% acetic acid) (1.5 kV, 50 min) and in the second, by ascending chromatography in phosphochromatography buffer (37.5% n-butanol, 25% pyridin, 7.5% acetic acid).

Phosphoamino acid analysis was performed as described by Boyle et al. (1991). 32P-labeled ββ was eluted from fixed polyacrylamide gels and precipitated with 10% TCA. 32P-labeled peptides were scraped off TLC plates, eluted in 20% acetonitrile and 0.08% trifluoroacetic acid, and lyophilized. Both types of sample were subjected to acid hydrolysis in 6 N HCl at 110°C for 1 h. Phosphoamino acids were separated by two-dimensional TLC: electrophoresis in pH 1.9 buffer for the first dimension (1.5 kV, 40 min) and in pH 3.5 buffer (5% acetic acid, 0.5% pyridine) for the second dimension (1.5 kV, 30 min). Nonradioactive standards were detected by ninhydrin staining.

Adhesion and Migration Assays

Adhesion assays were performed essentially as previously described (Giancotti et al., 1985). Before the assay, the cells were serum starved and either treated with 100 ng/ml EGF for 5 min or left untreated. After detachment by incubation in 10 mM EDTA, they were washed and plated on extracellular matrix-coated plates in the presence of anti-ββ serum at 1:50. The results were quantitated as previously described (Giancotti et al., 1986).

Cell migration assays were performed by using modified Boyden chambers containing porous (8-µm) polycarbonate membranes (Nunc, Roskilde, Denmark). To measure migration toward fibronectin and laminin 4, the lower aspect of the membrane was coated with 10 µg/ml of each extracellular matrix protein. To measure migration toward laminin 5, RAC-11P/SD cells were cultured on the lower aspect of the filter, and their migration was quantitated by immunofluorescence staining.

Immunofluorescence

The 804G transfectants and primary human keratinocytes were cultured on glass coverslips, starved for ~24 h and then treated with EGF, PDGF, or left untreated. After extraction with PBS containing 0.2% Triton X-100 for 5 min on ice, the cells were fixed with methanol and stained for 45 min with the various antibodies. The anti-ββ cytoplasmic peptide rabbit serum was diluted 1:200. The anti-BPAG 2 IgGs were used at 25 µg/ml, and the anti-P-Tyr antibodies showed that exposure to EGF does not induce tyrosine phosphorylation of ββ in the membrane fraction or in the cytoplasmic fraction (Fig. 1 A). This result suggests that the effect of EGF on ββ phosphorylation is selective. Experiments of [32p]orthophosphate labeling and phosphoamino acid analysis were performed to confirm the ability of EGF to induce tyrosine phosphorylation of ββ. The results indicated that the ββ subunit is constitutively phosphorylated on serine, and it becomes phosphorylated on tyrosine residues in response to EGF treatment (Fig. 1 B). Taken together, these results demonstrate that the ββ subunit is phosphorylated on tyrosine residues in cells exposed to EGF.

Immunoblotting with anti-phosphotyrosine antibodies indicated that the phosphorylation of ββ induced by EGF is dose dependent. In A431 cells, we detected a significant level of ββ phosphorylation in response to as little as 10 ng/ml EGF, and maximal phosphorylation in response to 250 ng/ml EGF (Fig. 2 A). The results of time course experiments indicated that the phosphorylation of ββ induced by EGF in A431 cells follows a biphasic kinetics characterized by a first rapid peak occurring at 2 min and a second one at ~120 min from the initial challenge (Fig. 2 B). The decline in ββ phosphorylation observed at 4 and 8 min after the initial stimulus may be related to the internalization of EGF receptor, a phenomenon that occurs rapidly after ligand binding (Beguinot et al., 1984). This interpretation is supported by the observation that the second peak of ββ phosphorylation induced by EGF occurs at a time when the downregulation of the EGF receptor has already subsided (Teslenko et al., 1987). The stoichiometry of EGF-induced ββ phosphorylation was estimated in A431 cells treated for 20 min with 50 ng/ml EGF. After extraction, the tyrosine-phosphorylated integrin was separated from the nonphosphorylated one by affinity chromatography on the anti-P-Tyr mAb 1G2, and both fractions were subjected to immunoblotting with anti-ββ antibodies (not shown).

Results

EGF-mediated Tyrosine Phosphorylation of the ββ Subunit

To test the hypothesis of a potential link between the intracellular responses elicited by EGF and the function of αββ integrin, we examined if treatment with EGF could induce tyrosine phosphorylation of αββ in cultured epithelial cells. The human epidermoid carcinoma A431 cells, which express high levels of the EGF, were serum starved and either left untreated or exposed to EGF. Immunoprecipitation with the anti-ββ mAb 3E1 followed by immunoblotting with anti-phosphotyrosine (anti-P-Tyr) antibodies indicated that treatment with EGF causes significant tyrosine phosphorylation of the ββ subunit, suggesting that αββ is a direct or indirect target of the EGFR (Fig. 1 A). To explore the selectivity of the effect of EGF on ββ phosphorylation, we asked if exposure to the growth factor also caused tyrosine phosphorylation of β1 or αα integrins. We reasoned that this experiment would have provided for a good control, as the cytoplasmic domains of β1, β3, β4, and β6 contain a conserved sequence motif resembling a major tyrosine autophosphorylation site in the EGFR (Hynes, 1992). As shown in Fig. 1 A, immunoprecipitation with the anti-ββ mAb 7B7 or an anti-αα cytoplasmic domain serum followed by immunoblotting with anti-P-Tyr antibodies showed that exposure to EGF does not induce tyrosine phosphorylation of β1 or αα containing integrins (Fig. 1 A). This result suggests that the effect of EGF on ββ phosphorylation is selective. Experiments of [32p]orthophosphate labeling and phosphoamino acid analysis were performed to confirm the ability of EGF to induce tyrosine phosphorylation of ββ. The results indicated that the ββ subunit is constitutively phosphorylated on serine, and it becomes phosphorylated on tyrosine residues in response to EGF treatment (Fig. 1 B). Taken together, these results demonstrate that the ββ subunit is phosphorylated on tyrosine residues in cells exposed to EGF. Immunoblotting with anti-phosphotyrosine antibodies indicated that the phosphorylation of ββ induced by EGF is dose dependent. In A431 cells, we detected a significant level of ββ phosphorylation in response to as little as 10 ng/ml EGF, and maximal phosphorylation in response to 250 ng/ml EGF (Fig. 2 A). The results of time course experiments indicated that the phosphorylation of ββ induced by EGF in A431 cells follows a biphasic kinetics characterized by a first rapid peak occurring at 2 min and a second one at ~120 min from the initial challenge (Fig. 2 B). The decline in ββ phosphorylation observed at 4 and 8 min after the initial stimulus may be related to the internalization of EGF receptor, a phenomenon that occurs rapidly after ligand binding (Beguinot et al., 1984). This interpretation is supported by the observation that the second peak of ββ phosphorylation induced by EGF occurs at a time when the downregulation of the EGF receptor has already subsided (Teslenko et al., 1987). The stoichiometry of EGF-induced ββ phosphorylation was estimated in A431 cells treated for 20 min with 50 ng/ml EGF. After extraction, the tyrosine-phosphorylated integrin was separated from the nonphosphorylated one by affinity chromatography on the anti-P-Tyr mAb 1G2, and both fractions were subjected to immunoblotting with anti-ββ antibodies (not shown). Densitometric analysis of the results indicated that 83% of the total ββ subunit bound to the anti-phosphotyrosine affinity column. From these experiments, we concluded that the tyrosine phosphorylation of ββ induced by EGF in A431 cells is rapid, dose dependent, and characterized by a high stoichiometry.

We next wondered if treatment with EGF caused tyrosine phosphorylation of ββ also in normal epithelial cells, which express lower levels of the EGFR than A431 cells. As shown in Fig. 2 C (left), treatment of primary human keratinocytes with 10 ng/ml EGF caused significant tyrosine phosphorylation of the ββ subunit. A similar result was obtained with rat epithelial 804G cells expressing moderate levels of recombinant human EGFR (35 times lower than the endogenous EGFR in A431 cells) (Fig. 2 C,
Figure 1. EGF-mediated tyrosine phosphorylation of the β₄ subunit. (A) A431 cells were serum starved and either left untreated or stimulated with 250 ng/ml EGF for 20 min. The samples were immunoprecipitated with control rabbit anti-mouse IgGs, anti-human β₄ mAb 3E1, anti-human β₁ mAb BV7, or anti-α, cytoplasmic domain serum, and then probed by immunoblotting with polyclonal anti-P-Tyr antibodies. (B) A431 cells were serum starved, metabolically labeled with [³²P]orthophosphate, and then either left untreated or stimulated with 250 ng/ml EGF for 20 min. The samples were immunoprecipitated with control rabbit anti-mouse IgGs or anti-human β₄ mAb 3E1. The radioactive bands corresponding to β₄ were subjected to phosphoamino acid analysis.

right). These results indicate that EGF induces tyrosine phosphorylation of β₄ in cells that express moderate levels of EGFR, including primary epithelial cells.

To explore the mechanism by which the EGFR induces tyrosine phosphorylation of the β₄ subunit, we examined the ability of immunopurified EGFR to phosphorylate in vitro the α₅β₄ integrin or fusion proteins reproducing the β₄ cytoplasmic domain. Despite undergoing significant autophosphorylation, the EGFR only weakly phosphorylated these potential substrates in in vitro assay (unpublished results). We also wondered if the EGFR and α₅β₄ stably interacted in A431 cells, but coimmunoprecipitation experiments performed under mild detergent conditions failed to demonstrate a specific association of the two molecules (unpublished results). Although not conclusive, the results of these experiments are consistent with the hypothesis that in vivo the EGFR does not directly phosphorylate β₄, but rather activates a signaling pathway that causes its phosphorylation.

EGF-mediated Tyrosine Phosphorylation of β₄ Requires Ligation of the Integrin by Extracellular Matrix Ligands or Antibodies

To examine if the ability of EGF to induce tyrosine phosphorylation of β₄ is influenced by cell adhesion, A431 cells were detached from the culture substratum, either kept in suspension or plated onto uncoated culture dishes for various times, and then treated with EGF. As shown in Fig. 3 A, in the absence of EGF treatment, no tyrosine phosphorylation of β₄ was detected in both suspended and stably adherent cells. Treatment with EGF did not result in tyrosine phosphorylation of β₄ in suspended cells. In contrast, the growth factor induced significant phosphorylation of β₄ in cells that had been plated on the culture dish for ≥4 h and were stably adherent. The ability of EGF to induce tyrosine phosphorylation of β₄ did not depend on cell-to-cell contact, as sparse and confluent cells were equally susceptible to the effect of the growth factor. Immunofluorescence experiments indicated that by 4 h of plating the α₅β₄ integrin had already redistributed to the basal cell surface, presumably in response to extracellular matrix ligands deposited onto the culture substratum during adhesion (data not shown). These observations suggest a correlation between the recruitment of α₅β₄ to the basal cell surface during adhesion and its susceptibility to EGF-mediated tyrosine phosphorylation.

To examine the hypothesis that the tyrosine phosphorylation of β₄ induced by EGF requires ligation of the integrin by extracellular matrix ligand, A431 cells were plated on dishes coated with the α₅β₄ ligands laminin 5 and laminin 4 or the control ligand fibronectin, and then treated with EGF. Coating concentrations were adjusted so as to obtain the same extent of cell adhesion and spreading at 30 min. As shown in Fig. 3 B, plating of the cells on laminin 5 and 4 rendered the β₄ subunit fully susceptible to EGF-mediated phosphorylation. In contrast, plating on fibronectin had a more modest effect. The effect of EGF on β₄ phosphorylation was maximal during initial adhesion to laminins and then declined. In accordance with the observation that A431 cells adhere with a faster kinetics to laminin 5 than to laminin 4, the peak of β₄ phosphorylation occurred earlier on laminin 5 than on laminin 4. Plating A431 cells onto laminin 5 in the absence of EGF also induced tyrosine phosphorylation of β₄, but this phosphorylation was of lower level and occurred with a slower kinet-
Dose dependence and kinetics of EGF-mediated tyrosine phosphorylation of $\beta_4$. Serum-starved A431 cells were treated for 20 min with the indicated concentrations of EGF (A), or treated with 50 ng/ml EGF for the indicated times (B). After immunoprecipitation with the 3El mAb, the samples were probed by immunoblotting with polyclonal anti-P-Tyr or anti-$\beta_4$ cytoplasmic domain antibodies. Growth factor-starved primary human keratinocytes (C, left) and 804G cells expressing a recombinant EGFR (C, right) were treated with the indicated concentrations of EGF for 15 min. Immunoprecipitation was with anti-$\beta_4$ cytoplasmic domain antibodies and immunoblotting with polyclonal anti-P-Tyr antibodies.

Control experiments revealed that the ability of EGFR to undergo autophosphorylation, as well as to induce tyrosine phosphorylation of several cellular substrates, was similar in cells freshly plated on each one of the extracellular matrix proteins tested, including fibronectin (Fig. 3 C). These results are consistent with the notion that ligand binding to $\alpha_6\beta_4$ is required for optimal tyrosine phosphorylation of $\beta_4$ in response to EGF stimulation.

Since the A431 cells express at least another integrin, $\alpha_3\beta_1$, capable of binding to laminin 5 and possibly to lamin 4, we wished to obtain direct evidence that ligation of $\alpha_6\beta_4$ at the cell surface is required for optimal phosphorylation of $\beta_4$. A431 cells were plated for 60 min on the control ligand fibronectin, and then incubated for different times with polystyrene beads coated with the anti-$\beta_4$ mAb 3E1, the anti-$\beta_4$ mAb TS2/16, or the control anti-MHC mAb W6.32. As shown in Fig. 4, treatment with EGF caused significant tyrosine phosphorylation of $\beta_4$ in cells exposed for 10 min to the anti-$\beta_4$ beads, but not in cells treated with anti-$\beta_1$ or anti-MHC beads. Incubation with soluble 3E1 mAb produced a very modest effect. Control experiments indicated that treatment of A431 cells with anti-$\beta_4$ beads in the absence of EGF induces, as expected, a significant tyrosine phosphorylation of p125FAK (data not shown). These results suggest that $\alpha_6\beta_4$ must be oligomerized at the cell surface to be susceptible to EGF-mediated phosphorylation.

Interestingly, while EGF could consistently induce significant tyrosine phosphorylation of $\beta_4$ in cells that had been plated onto a plastic culture substratum for 4 h or more (Fig. 3 A), maximal phosphorylation of $\beta_4$ occurred only transiently in cells plated on laminin 5 and 4 (Fig. 3 B) or incubated with anti-$\beta_4$ beads (Fig. 4). The transient nature of the effect induced by initial ligation of $\alpha_6\beta_4$ on tyrosine phosphorylation of $\beta_4$ may be explained by the pre-
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EGF-induced Phosphorylation of Multiple Tyrosine Residues

Phosphopeptide mapping experiments were performed to analyze the β4 sites phosphorylated in response to EGF treatment. Since cross-linking of αβ4 by antibodies or plating on laminin 5 did not induce a level of tyrosine phosphorylation of β4 sufficient for high resolution mapping, the sites phosphorylated in response to EGF were compared to those phosphorylated in response to pervanadate. Previous results have shown that treatment with pervanadate results in phosphorylation of multiple β4 residues, including the β4 TAM and presumably also the Shc binding sites, since pervanadate can induce association of αβ4 with Shc (Mainiero et al., 1995). A431 cells were metabolically labeled with \[^{32}P\]orthophosphate, and then either left untreated or stimulated with EGF or pervanadate. After immunoprecipitation, the β4 subunit was digested with Staphylococcus V8 protease, and the resulting peptides were separated by bidimensional TLC. As shown in Fig. 5A, the β4 subunit from unstimulated cells was resolved in a number of phosphopeptides (S1–S10). In accordance with the observation that β4 is phosphorylated constitutively on serine residues (Fig. 5B), phosphoamino acid analysis indicated that these peptides contained only phosphoserine. Treatment with EGF resulted in the appearance of a number of additional phosphopeptides (Y1–Y8) (Fig. 5B), and phosphoamino acid analysis of several of them (Y1–Y6) confirmed that they contained exclusively phosphotyrosine. These results indicate that exposure to EGF results in phosphorylation of multiple tyrosine residues in the β4 cytoplasmic domain. The phosphopeptide map of β4 from pervanadate-treated cells was similar, but not identical, to that of β4 from EGF-stimulated cells. It contained the peptides S1–S10 and Y1–Y8, but also an additional phosphotyrosine-containing peptide, Y9. Furthermore, the intensity of the spot corresponding to phosphopeptide Y1 was much larger in pervanadate than in EGF-treated cells, and conversely, phosphopeptide Y6 was more intensely labeled in EGF than in pervanadate-treated cells (Fig. 5C). Previous experiments of site-directed mutagenesis and phosphopeptide mapping of β4 from pervanadate-treated cells have indicated that peptide Y5 contains tyrosine 1440, the COOH-terminal element of the TAM, and have provided circumstantial evidence that peptide Y2 contains tyrosine 1422, the NH2-terminal element of the TAM (Mainiero et al., 1995). Since exposure to EGF resulted in the appearance of phosphopeptides Y5 and Y2, we concluded that EGF induces the phosphorylation of multiple tyrosine residues in β4 and that these include the COOH-terminal, and possibly the NH2-terminal, element of the TAM.

EGF-mediated Tyrosine Phosphorylation of β4 Does Not Result in Recruitment of the Adaptor Proteins Shc and Grb2

To examine if EGF-mediated tyrosine phosphorylation of β4 results in association of the adaptor protein Shc to αβ4, A431 cells were either incubated with anti-β4 beads in suspension or treated with EGF while adherent. The resulting extracts were immunoprecipitated with anti-β4 antibodies and probed by immunoblotting with anti-β4 and anti-Shc antibodies. As shown in Fig. 6A, ligation of αβ4 led to recruitment of Shc. In contrast, treatment with EGF did not result in association of this adaptor molecule to αβ4. Immunoprecipitation with anti-Shc antibodies followed by immunoblotting with anti-β4 antibodies confirmed that EGF stimulation does not result in recruitment of Shc to αβ4 (Fig. 6B). Control experiments indicated that a certain amount of the adaptor molecule remained available in the cytoplasm of EGF-treated cells (not shown; see also Fig. 6C). The results of these experiments suggest that EGF does not induce phosphorylation of the Shc binding sites in β4.

The inability of EGF to induce association of Shc with the αβ4 integrin raises the possibility that the EGFR and αβ4 may, when simultaneously ligated, compete for this adaptor molecule in vivo. To explore this possibility, we examined the effect of EGF on the recruitment of Shc to activated αβ4. As shown in Fig. 6C, the amount of Shc coimmunoprecipitated with αβ4 was lower in cells stimulated with anti-β4 beads and EGF than in cells treated only with anti-β4 beads. The inhibitory effect of EGF was especially evident in cells that had been incubated with anti-β4 beads for 5 or 10 min, irrespective of whether the EGF was...
applied together with the beads or before the beads. In addition to indicating that a certain amount of Shc remains available for binding to \( \alpha_\beta_4 \) in EGF-treated cells, these results indicate that the EGFR and \( \alpha_\beta_4 \) integrin compete for this adaptor molecule in cultured cells, raising the possibility that the ability of \( \alpha_\beta_4 \) to link to the ras pathway may be suppressed by EGF-dependent signals in vivo.

**Disruption of Hemidesmosomes by EGF**

To examine the effect of EGF on the ability of \( \alpha_\beta_4 \) to associate with the hemidesmosomal cytoskeleton, we elected to use the rat 804G bladder epithelial cells, which form hemidesmosomes in vitro. Since these cells express very low levels of the EGFR, we used cell lines expressing moderate levels of human EGFR from cDNA. Immunoblotting analysis of total proteins with anti-P-Tyr antibodies indicated that exposure of the EGFR-transfected cells to EGF resulted in tyrosine phosphorylation of the recombinant EGFR and of several of its cellular substrates, including the \( \beta_4 \) subunit (data not shown and Fig. 2 C, right).

The EGFR-transfected 804G cells were starved, and then either left untreated or treated for various times with 100 ng/ml EGF. Immunofluorescent analysis revealed that while in control cells, \( \alpha_\beta_4 \) and the BPAG 2 were concentrated at the basal cell surface within Triton X-100-resistant, "Swiss cheese"-like structures corresponding to hemidesmosomes (Fig. 7, a and b); in cells treated with EGF, these molecules had undergone a profound redistribution and were no longer detected in association with these structures (Fig. 7, d and e). To confirm the physiological significance of these observations, we examined the effect of EGF on the hemidesmosome-like structures formed by normal human primary keratinocytes in culture. As shown in Fig. 7 (c and f), treatment with EGF resulted in loss of hemidesmosomal staining also in these cells, suggesting that disassembly of hemidesmosomes may be one of the physiological consequences of activation of the EGFR in primary epithelial cells. Immunofluorescent analysis of EGF transfected 804G cells treated for various times with EGF indicated that the effect of the growth factor on hemidesmosomes was already significant after 1 h and
Disruption of hemidesmosomes by EGF. EGFR-transfected 804G cells (a, b, d, and e) and primary human keratinocytes (c and f) were cultured on glass coverslips for 48 h, serum starved, and either left untreated (a–c) or treated with 100 ng/ml EGF for 12 h (d–f). After extraction with 0.2% Triton X-100, the cells were fixed and stained with anti-BPAG 2 antibodies (a and d), anti-J34 cytoplasmic peptide serum (b and e), or the anti-J34 mAb 3E1 (c and f) followed by FITC-labeled affinity-purified secondary antibodies.

The ability of EGF to induce hemidesmosome disassembly was unexpected because phosphopeptide mapping had indicated that the β4 TAM, which mediates a signaling event required for the association of α6β4 with hemidesmosomes (Mainiero et al., 1995), is phosphorylated in response to EGF. We wondered if the effect of EGF on hemidesmosomes was caused by its ability to downregulate ligand binding to α6β4 by a mechanism of inside-to-outside signaling. The effect of EGF treatment on the adhesion of EGFR-transfected 804G cells to laminin 4 and 5 was therefore examined. To block β1-dependent adhesion, the cells were plated on the two extracellular matrix proteins in the presence of inhibitory anti-β1 antibodies. As shown in Fig. 9, the extent to which the EGFR-transfected 804G cells adhered to laminin 4 and 5 was not significantly changed after treatment with EGF. A similar result was obtained with A431 cells (not shown). These results indicate that exposure to EGF does not cause a significant change in ligand binding to α6β4, thus suggesting that the deterioration of hemidesmosomes observed in EGF-treated cells is not caused by a downregulation of ligand binding. Together with the observation that EGF induces phosphorylation of the β4 TAM, these data suggest the hypothesis that EGF-dependent signals suppress the association of α6β4 with the hemidesmosomal cytoskeleton by interfering with the functioning of signaling and cytoskeletal molecules downstream of the β4 TAM.

Increased α6β4-dependent Cell Migration in Response to EGF

To determine if the apparent disruption of hemidesmosomes caused by EGF correlates with a change in α6β4-
dependent cell migration, we measured the ability of control and EGFR-transfected 804G cells to migrate toward various extracellular matrix components by using a Boyden chamber system. As shown in Fig. 10, treatment with EGF resulted in increased migration of the EGFR-transfected 804G cells toward the two α6β4 ligands laminin 4 and 5, but not the control ligand fibronectin, suggesting that EGF-dependent signals can increase cell migration toward α6β4 ligands. In addition, the basal migration of EGFR-transfected 804G cells toward laminin 4 and 5 was greater than that of control 804G cells. This result suggests that the recombinant EGFR may be partially active in the absence of exogenous ligand in 804G cells, perhaps because these cells secrete EGF or TGF-α. In accordance with this hypothesis, we found that the medium conditioned by 804G cells is capable of stimulating the auto-phosphorylation of recombinant EGFR expressed in transfected 804G cells (data not shown). Inhibitory anti-β1 antibodies were able to suppress the migration of unstimulated cells toward laminin 5 by 91 ± 6%, but only inhibited the migration of EGF-treated cells by 11 ± 3%, indicating that the EGF-stimulated migration toward laminin 5 was largely dependent on α6β4 function. This conclusion was also supported by the observation that EGFR-transfected NIH-3T3 fibroblasts, which do not express α6β4, did not respond to EGF with increased migration toward laminin 4 (Fig. 10, top). Finally, the effect of EGF was specific, since it was not observed in response to PDGF or with the control 804G cells in response to EGF. Taken together, these results indicate that EGF specifically upregulates α6β4-dependent migration toward laminins.

**Discussion**

Several observations suggest that α6β4- and growth factor-dependent signals may cooperate to control epidermal cell proliferation and migration. In stratified epithelia, such as the epidermis, α6β4 mediates the interaction of basal keratinocytes with the basement membrane (Kajiji et al., 1989), and there is evidence indicating that these cells have to remain in contact with this extracellular matrix to maintain their proliferative potential (Green, 1977; Hall and...
Figure 9. EGF does not affect α6β4-dependent cell adhesion. EGFR-transfected 804G cells were starved and either left untreated or stimulated with 100 ng/ml EGF. The cells were plated in the presence of inhibitory anti-β4 antibodies on dishes coated with the indicated amounts of laminin 4 for 60 min (top) or on laminin 5 matrix-coated dishes for the indicated times (bottom).

Watt, 1989). Furthermore, the coincident expression of α6β4 and laminins by keratinocytes migrating into corneal wounds suggests a role for α6β4-mediated migration during the reepithelialization of wounds (Kurpakus et al., 1991). Prompted by the prominent role of EGF and transforming growth factor α in controlling keratinocyte growth and migration (Rheinwald and Green, 1977; Barrandon and Green, 1987), and by the coincident expression of α6β4 and EGFR in basal keratinocytes in vivo (Green et al., 1987; Kajiji et al., 1989), we have examined the effect of EGFR activation on the intracellular functions of α6β4. Our results indicate that EGF-dependent signals have a complex effect on α6β4 function: they cause tyrosine phosphorylation of β4 without promoting the association of Shc, induce disassembly of hemidesmosomes, and upregulate cell migration on laminins.

In this study, we provide direct evidence that activation of the EGFR causes tyrosine phosphorylation of the β4 subunit. This phosphorylation is characterized by a rapid kinetics and, at least in A431 cells, by a high stoichiometry. Since we have been unable to obtain evidence that the EGFR efficiently phosphorylate β4 in vitro, it is our hypothesis that the EGFR does not directly phosphorylate β4 in vivo, but rather it activates a signaling pathway that results in its phosphorylation. The observation that EGF-mediated phosphorylation of β4 requires ligation of the integrin by extracellular ligand or antibodies suggests that this phosphorylation event is mediated by an integrin-associated kinase acting in trans. Future studies will be required to determine if α6β4 is indeed an indirect target of
the EGFR and if it is associated with two distinct tyrosine kinases, one activated by EGF and the other by extracellular matrix binding, or with a single tyrosine kinase activated by both stimuli.

The results of phosphopeptide mapping indicate that EGF causes phosphorylation of several distinct β4 tyrosine residues. Although the majority of the tyrosine phosphorylation sites in β4 remain to be identified and their function assessed, the complexity of the tyrosine phosphorylation pattern induced by EGF suggests that many αβ4 functions may be regulated by the growth factor. One major intracellular function of αβ4 that is negatively regulated by EGF is the recruitment of the adaptor molecule Shc. Treatment with EGF does not result in the association of αβ4 with Shc and presumably Grb2. In fact, exposure to EGF partially suppresses the recruitment of Shc to the ligated integrin. Although it is possible that EGF causes a conformational change or another posttranslational modification of αβ4 that prevents it from binding to Shc, the most likely explanation of these results is that the growth factor does not induce phosphorylation of the Shc binding motifs in β4. The observation that the EGFR can compete with αβ4 for the recruitment of Shc is in accordance with the recognized ability of activated EGFR to associate with this adaptor molecule (Pellicci et al., 1992) and suggests that a significant activation of the EGFR may interfere with the ability of ligand-occupied αβ4 to activate signaling in vivo. In contrast, when suboptimally ligated, the EGFR and αβ4 are likely to cooperate with each other to activate the ras pathway. This latter prediction may be relevant to understanding anchorage-dependent cell growth in epithelial cells.

The results of our immunofluorescent analysis indicate that treatment with EGF causes disruption of hemidesmosomes in both EGFR-transfected 804G cells and primary human keratinocytes. What is the mechanism by which EGF interferes with the assembly of hemidesmosomes? Our previous studies suggest that the nucleation of hemidesmosomes requires a signal mediated by the β4 TAM (Mainiero et al., 1995). It is, however, unlikely that the phosphorylation of the TAM is the only αβ4 function necessary for the assembly of hemidesmosomes. Deletion mutagenesis experiments have indicated that the association of αβ4 with the hemidesmosomal cytoskeleton not only requires the connecting segment, which includes the TAM, but also sequences within the two type III fibronectin-like modules upstream of the connecting segment (Spinardi, L., and F.G. Giancotti, unpublished results). This observation is consistent with the hypothesis that a TAM-dependent signal renders one or more cytoskeletal elements of hemidesmosomes competent for binding to sequences within the first two type III fibronectin-like modules of β4. Further assembly of hemidesmosomes may then be driven by the cooperative binding of additional cytoskeletal elements. Based on this model, EGF-dependent signals may interfere with the assembly of hemidesmosomes at one or more of several steps. Since EGF does not affect αβ4-mediated adhesion to laminins and does not suppress phosphorylation of the β4 TAM, the growth factor may interfere with the functioning of one or more signaling or cytoskeletal molecules located downstream of the TAM in the pathway that controls the association of αβ4 with the cytoskeleton. Furthermore, it is possible that EGF induces the phosphorylation of tyrosine residues located within the first two type III fibronectin-like modules of the β4 tail, thus directly interfering with the association of cytoskeletal molecules. Finally, as the process of hemidesmosome formation is likely to be complex and to require the function of many components in addition to αβ4 and the molecules to which it binds, EGF may disrupt hemidesmosomes by acting on one or more of these additional components.

Most of the previous studies on the regulation of the cytoskeleton by growth factors have focused on the effects of EGF and PDGF on the actin filament system. It has been known for long that these growth factors can induce profound changes in the architecture of the actin cytoskeleton (Bockus and Stiles, 1984; Herman and Pledger, 1985). Recent studies have indicated that they can induce the sequential formation of filopodia, lamellipodia, and focal adhesions, and that these cytoskeletal changes are mediated by a GTPase cascade involving Cdc 42, Rac, and Rho (Nores and Hall, 1995). Our current observations clearly indicate that EGF can also profoundly affect the keratin filament system, thereby providing evidence for a novel mechanism of cytoskeletal regulation by EGF.

The changes in the association of αβ4 with the cytoskeleton induced by activated EGFR are likely to be significant in both physiological and pathological situations. Several lines of evidence support the notion that hemidesmosomes mediate stable adhesion to the basement membrane (Uitto and Christiano, 1992; Guo et al., 1995; Spinardi et al., 1995). Their disruption may therefore result in a more dynamic interaction with the extracellular matrix. In accordance with this hypothesis, we have observed that the disassembly of hemidesmosomes caused by EGF correlates with an increase in αβ4-dependent cell migration. This observation suggests that the ability of αβ4 to mediate cell migration on laminins can be upregulated by factors that interfere with its association with the hemidesmosomal cytoskeleton. It is well known that EGF and TGF-α can promote the reepithelialization of wounds (Schultz et al., 1991), and it has recently been observed that keratinocytes lose their hemidesmosomes as they migrate into corneal wounds (Gipson et al., 1993). Thus, the ability of activated EGFR to coordinate disassembly of hemidesmosomes and increase cell migration on laminins is likely to be important during wound healing. In addition, there is evidence indicating that keratinocytes of patients affected by the skin disease psoriasis overproduce TGF-α (Elder et al., 1989) and that squamous carcinoma cells overexpress the EGFR (Yamamoto et al., 1986; Ozanne et al., 1986). In both pathological situations, the expression of αβ4 is no longer restricted to the basal surface of those cells that abut the basement membrane, but extends suprabasally (Kimmel and Carey, 1986; Pellegrini et al., 1992). Our current results suggest that the loss of αβ4 polarity observed in these diseases may result from the ability of activated EGFR to disrupt the association of the integrin to the hemidesmosomal cytoskeleton. They further suggest that the ability of EGFR to affect the association of αβ4 with the cytoskeleton may contribute to the invasive ability of squamous carcinoma cells.

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