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Protein Kinase C-dependent Mobilization of the α6β4 Integrin from Hemidesmosomes and Its Association with Actin-rich Cell Protrusions Drive the Chemotactic Migration of Carcinoma Cells

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Abstract. We explored the hypothesis that the chemotactic migration of carcinoma cells that assemble hemidesmosomes involves the activation of a signaling pathway that releases the α6β4 integrin from these stable adhesion complexes and promotes its association with F-actin in cell protrusions enabling it to function in migration. Squamous carcinoma-derived A431 cells were used because they express α6β4 and migrate in response to EGF stimulation. Using function-blocking antibodies, we show that the α6β4 integrin participates in EGF-stimulated chemotaxis and is required for lamellae formation on laminin-1. At concentrations of EGF that stimulate A431 chemotaxis (~1 ng/ml), the α6β4 integrin is mobilized from hemidesmosomes as evidenced by indirect immunofluorescence microscopy using mAbs specific for this integrin and hemidesmosomal components and its loss from a cytokeratin fraction obtained by detergent extraction. EGF stimulation also increased the formation of lamellipodia and membrane ruffles that contained α6β4 in association with F-actin. Importantly, we demonstrate that this mobilization of α6β4 from hemidesmosomes and its redistribution to cell protrusions occurs by a mechanism that involves activation of protein kinase C-α and that it is associated with the phosphorylation of the β4 integrin subunit on serine residues. Thus, the chemotactic migration of A431 cells on laminin-1 requires not only the formation of F-actin-rich cell protrusions that mediate α6β4-dependent cell protrusions but also the disruption of α6β4-containing cell movement but also the disruption of α6β4-containing hemidesmosomes by protein kinase C.

Key words: integrins • cell movement • PKC • hemidesmosomes • cytoskeleton

Chemotactic migration is essential for embryonic development, tissue homeostasis, and the immune response (29, 36, 55). It is also a major factor in the pathogenesis of many human diseases (21, 30, 54). This complex and poorly understood process involves dynamic and coordinated interactions among integrins, chemottractant receptors and the actin cytoskeleton. The net result of these interactions is localized actin polymerization in the direction of the chemotactant gradient coupled with the establishment of traction forces necessary for migration (14, 36, 52). The chemotactic migration of epithelial-derived cells is of particular significance to the mechanism of wound healing and carcinoma invasion (21, 30, 54). However, the mechanisms involved in epithelial migration are not well understood. For example, an explanation needs to be provided for how integrin function is altered in response to stimuli that induce epithelial migration. At the very least, the hypothesis can be formulated that integrin function differs in stably adherent epithelial cells in comparison to migrating epithelial cells such as those cells at a wound edge or in an invasive carcinoma.

The α6β4 integrin is ideal for studying differences in integrin function between stably adherent and migrating epithelial cells. This integrin, which is a receptor for the laminins (reviewed in 34), is essential for the organization and maintenance of epithelial architecture. In many epithelia, α6β4 mediates the formation of stable adhesive structures termed hemidesmosomes that link the intermediate filament cytoskeleton with the extracellular matrix (4, 23). In addition to α6β4, the classical hemidesmosome contains at least three other known proteins: BPAG, bullous pemphigoid antigen; EGF, EGF receptor; PKC, protein kinase C; PLC, phospholipase C; P13K, phosphoinositide 3-OH kinase.

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1. Abbreviations used in this paper: BPAG, bullous pemphigoid antigen; EGF, EGF receptor; PKC, protein kinase C; PLC, phospholipase C; P13K, phosphoinositide 3-OH kinase.
linked to intermediate filaments through HD 1/plectin, and that this interaction is critical for hemidesmosomal formation (37, 45, 47). BPA G-1 has also been shown to link the hemidesmosome to intermediate filaments (24). The importance of the α6β4 integrin in hemidesmosome function and epithelial architecture has been reinforced by the generation of β4-nullizygous mice. The most obvious defect in these mice is a loss of hemidesmosomes and detachment of the epidermis (16, 58). In striking contrast to its function in normal epithelia, α6β4 can stimulate carcinoma migration and invasion through its ability to interact with the actin cytoskeleton and mediate the formation and stabilization of lamellae (44). This dynamic function of α6β4 in enhancing the migration of invasive carcinoma cells is quite distinct from its role in maintaining stable adhesive contacts in normal epithelia by associating with intermediate filament. In fact, we have established that the ability of α6β4 to stimulate carcinoma migration and invasion depends upon its activation of distinct signaling pathways including P13-K (51) and α CaMP-specific phosphodiesterase (39). In essence, our studies have defined an integrin-mediated mechanism of carcinoma invasion that involves the stimulation of chemotactic migration by the association of α6β4 with F-actin and the activation of a specific signaling pathway by this integrin.

The above observations raise the important issue of whether migratory stimuli influence the localization and cytoskeletal interactions of α6β4. Such changes could provide a mechanism to account for the dichotomy of α6β4 function in stably adherent and migrating cells. To address this issue, we used squamous carcinoma-derived A431 cells for several reasons. A431 cells express α6β4, as well as the EGF receptor that is known to stimulate their chemotactic migration and in vitro invasion (32). In addition, a previous report indicated that activation of the EGF receptor can disassemble hemidesmosomes in 804G bladder carcinoma cells (31). The results obtained in our study indicate that EGF, at concentrations that stimulate A431 chemotaxis, mobilizes the α6β4 integrin from hemidesmosomes and increases the formation of α6β4-containing lamellipodia and membrane ruffles. Importantly, we also demonstrate that this mobilization of α6β4 occurs by a protein kinase C (PKC)-dependent mechanism, and that it involves the phosphorylation of the β4 integrin subunit on serine residues.

Materials and Methods

Cells and Reagents

The A431 squamous carcinoma cell line was obtained from the American Type Culture Collection and maintained in DMEM with 10% fetal calf serum, at 37°C in a humidified atmosphere containing 5% CO2.

The following antibodies were used in this study: mouse mAb 2B7 (integrin α6-specific) was prepared in our laboratory (50); rat GoH3 mAb (integrin β4-specific; reference 18) was provided by Dr. Rita Falcioni (Regina Elena Cancer Institute, Rome, Italy). A peptide-specific antiserum elicited against the last 20 amino acids of the carboxy terminus of the β4 subunit was prepared commercially. A rabbit polyclonal antibody specific for the EGF receptor was purchased from Santa Cruz Biotechnology. The phosphotyrosine-specific antibodies PY20 and 4G10 were purchased from Transduction Labs and Upstate Biotechnology Incorporated, respectively. Mouse monoclonal antibodies specific for BPA G-1 (R3B5), BPA G-2 (1D1), and HD 1/plectin (121) (26, 40) were a gift of Dr. O. Waribe (Nagoya University, Japan). A nitrilotriacetic polyclonal antibody, pan-cytoketatin mAb, rat IgG, and mouse IgG were purchased from Sigma Chemical. Lamulin-1, prepared from the EHS sarcoma was provided by Dr. Hynda K. leinman (NIDR, Bethesda, MD). Collagen type I was purchased from Collagen Corp. Human recombinant EGF was purchased from Sigma Chemical. The PKC inhibitor GÖ6976 was obtained from Alexis Corp. PMA was obtained from Calbiochem-Novabiochem.

Chemotaxis Assays

Chemotaxis was analyzed using 6.5-mm Transwell™ chambers, 8-μm pore size (Costar). The separating membrane was coated with laminin-1 (20 μg/ml) on both sides for 2 h at room temperature and then blocked with 1% albumin in PBS for 30 min. A431 cells (3 × 104) were resuspended in DMEM containing 0.1% albumin. Some experiments, antibodies (10 μg/ml of 2B7 or mouse IgG control) were added to the cells. The cells were added to the top wells of the Transwell™ chambers and allowed to settle on the filters for 1 h at 37°C. Before EGF (1 ng/ml) was added to the lower chamber. In some cases, GÖ6976 (1 μM) or vehicle alone (DM SO) was added 30 min before EGF stimulation. A filter a 2-h incubation, cells that had not migrated from the upper surface of the membrane were removed using cotton swabs and the remaining cells on the lower side of the membrane were fixed in methanol, dried, and stained with a 0.2% solution of crystal violet in 2% ethanol. Migration was quantified by digital analysis as described below.

Analysis of Lamellar Area

A431 cells were plated on laminin-1 for 1 h and either not stimulated or stimulated with EGF (1 ng/ml) for 15 min in the presence or absence of antibody 2B7 or IgG (10 μg/ml). The antibodies were added 30 min before EGF stimulation. The lamellar area, defined as a characteristic flat and thin protrusion of the cell containing no vesicles, was measured using a Nikon Diaphot 300 inverted microscope with phase contrast optics. This microscope was connected to a CCD camera (Dage-MTI), a frame-grabber (Scion), and a 7600 Power Macintosh computer to capture the images. Images were collected and analyzed with IPLab Spectrum image analysis software. Lamellar area was determined by tracing the lamellae contour and quantifying the area digitally. For each individual experiment 50-80 cells were analyzed.

Indirect Immunofluorescence Microscopy

Bacteriological dishes were coated with 20 μg of laminin-1 or collagen type I for 2 h at room temperature and the dishes were then blocked with PBS containing 1% bovine serum albumin (BSA) for 30 min. A431 cells were resuspended in serum-free RPMI 1640 medium containing 10 mM Hepes and 0.1% BSA. The cells were plated at low density (2 × 104 cells/cm2) on the matrix-coated dishes and allowed to adhere for 1-2 h in a humidified atmosphere with 5% CO2 at 37°C. When indicated, GÖ6976 (0.5-1 μM) in DM SO or vehicle alone was added 30 min before stimulation. The cells were then stimulated with either EGF (0.5-100 ng/ml) for 15 min or PMA (25-50 ng/ml) for 30 min.

Cells were fixed with a buffer containing 2% paraformaldehyde, 100 mM KCl, 200 mM sucrose, 1 mM EGTA, 1 mM MgCl2, 1 mM PM SO, and 10 mM Pipes at pH 6.8 for 15 min. In some cases, the cells were extracted before fixation with a buffer containing 0.2% Triton X-100, 100 mM KCl, 200 mM sucrose, 10 mM EGTA, 2 mM MgCl2, 100 mM sodium vanadate, 1 mM PM SO, and 10 mM Pipes at pH 6.8 for 15 min. After fixation, the cells were rinsed with PBS and incubated with a blocking solution that contained 1% albumin and 5% goat serum in PBS for 30 min. Cells that were to be stained for HD 1/plectin, BPA G-1 or BPA G-2, were fixed with acetone/methanol 1:1 (vol/vol) instead of paraformaldehyde. Either primary antibodies or FITC phalloidin (20 μg/ml) in blocking solution were added to the fixed cells separately or in combination for 30 min. The cells were then rinsed three times and either a fluorescein-conjugated donkey anti-rabbit or a rhodamine-conjugated donkey anti-rat IgG (minimal cross-reaction inter-species; Jackson Immunoresearch Laboratories) in blocking buffer (1:150) were used separately or in combination to stain the cells for 30 min. Cells were rinsed with PBS and mounted in a mixture (8:2) of glycerol and PBS (pH 8.5) containing 1% propylgallate. The dishes were cut into slides and examined by confocal microscopy.
Detergent Extractions

To obtain a fraction enriched in cytokeratins (9, 19, 35), A 431 cells (2 × 10⁶) were incubated on laminin-1–coated dishes as described above for 1–2 h. In some cases, G66976 (1 μM) or vehicle alone was added 30 min before stimulation. EGF (0.5–2 ng/ml) was added and the cells incubated at 37°C for an additional 15 min. The cells were initially extracted with a buffer containing 0.2% Triton X-100, 100 mM KCl, 200 mM sucrose, 10 mM EGTA, 2 mM MgCl₂, 200 μM sodium vanadate, 1 mM PM SF, and 10 mM Pipes at pH 6.8 for 1 min (membrane/soluble fraction). The cells were rinsed several times before adding a second buffer containing 1% Tween-40, 0.5% deoxycholate, 10 mM NaCl, 2 mM MgCl₂, 20 mM Tris-HCl, pH 7.5, for 10 min (cytoskeletal fraction). The cells were rinsed and the residual fraction was extracted with a third buffer containing SDS 0.4%, 10 mM NaCl, 20 mM Tris-HCl, pH 7.5, sonicated and then boiled before adding Triton X-100 to a final concentration of 1% (vol/vol; cytokeratin fraction). The samples were immuno precipitated with the α4-specific polyclonal antibody, resolved by SDS-PAGE (6 or 8%) and immunoblotted with the same polyclonal antibody. Immune complexes were detected using a secondary antibody conjugated to horseradish peroxidase and visualized by enhanced chemiluminescence (A mersham, Inc.).

Analysis of Protein Phosphorylation

To examine tyrosine phosphorylation using phosphotyrosine-specific antibodies, A 431 cells were plated on laminin-1–coated dishes for 1 h at 37°C as described above. Cells were then stimulated with EGF (1–100 ng/ml) for 15 min and extracted with RIPA buffer containing 0.1% SDS, 1% Triton X-100, 0.5% deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 50 mM sodium pyrophosphate, 100 mM sodium fluoride, 1 mM sodium vanadate, 1 mM PM SF, 10 μg/ml each of leupeptin, pepstatin A, and aprotinin, and 50 mM Tris-HCl, pH 7.5. The samples were immunoprecipitated using the 439-9B antibody, resolved by SDS-PAGE and immunoblotted using a combination of both the PKY and 40/10 polypeptide-specific antibodies. Immune complexes were detected using a secondary antibody conjugated to horseradish peroxidase and visualized by enhanced chemiluminescence (A mersham, Inc.). Subsequently, the membranes were stripped and immunoblotted with the α4-specific polyclonal antibody.

For metabolic radiolabeling with 32P, A 431 cells (2 × 10⁶) were plated on laminin-1–coated dishes for 30 min as described above. Subsequently, the medium was removed and replaced with a phosphate-deficient medium (GIBCO). A 1 h incubation in this medium, 32P (0.5–2.0 μCi/ml (NEN)) was added and the cells were incubated for an additional 2 h. When indicated, G66976 (0.5–1 μM) in DM SO or vehicle alone was added 30 min before stimulation. The cells were then stimulated with either EGF (0.5–100 ng/ml) for 15 min or PMA (25–50 ng/ml) for 30 min. The cells were extracted with RIPA buffer as described above and the extracts were immunoprecipitated with 439-9B antibody, resolved by SDS-PAGE (6% gels) and transferred to PVDF membranes (Immobilon-P; Millipore). The membranes were exposed to x-ray film, developed, and then immunoblotted with the α4-specific polyclonal antibody to control for equivalent amounts of protein in the samples. A quantitative analysis of the relative intensities of the radioactive bands was made using a 2D image analyzer (Instant Imager; Packard, Meriden, CT). For phosphoamino-acid analysis, the area of the membrane that contained the α4 subunit was excised with a razor, acid hydrolyzed, and the hydrolysate was separated using 20-thin layer chromatography following standard techniques (49) and exposed to x-ray film.

Results

EGF Stimulation of A431 Cells Redistributes the Localization of the α6β4 Integrin from Hemidesmosomes to Lamellipodia and Membrane Ruffles

Indirect immunofluorescence microscopy revealed that the α6β4 integrin on the ventral surface of A431 cells plated on either laminin-1 (Fig. 1, A–D) or collagen (not shown) is localized primarily in discrete structures and plaques in areas that exclude stress fibers. This pattern of staining for α6β4 is characteristic of its localization in hemidesmosomes (11, 46). Moreover, a distinct colocalization of α6β4 with the hemidesmosomal components βPA G-1 (Fig. 1 A), βPA G-2 (Fig. 1 B), and H D 1 plectin (Fig. 1 C) was evident in these structures. These data establish that α6β4 is localized in structures that are characteristic of hemidesmosomes on the ventral surface of adherent A 431 cells.

Our previous data established that α6β4 participates in carcinoma migration through its ability to interact with the actin cytoskeleton (44). For this reason, we examined the involvement of this integrin in the chemotactic migration of A 431 cells towards EGF because these cells express high levels of the EGF receptor and this growth factor is known to stimulate their migration (32, 53). As shown in Fig. 2 A, relatively low concentrations of EGF (1 ng/ml) stimulated a robust migration response in A 431 cells. However, EGF did not stimulate significant migration when used at higher concentrations (>10 ng/ml) (Fig. 2 A). The effect of EGF on cell migration was mostly chemotactic in nature because the chemokinetic element represented ≈20% of the total migration (measured as the migration occurring in the presence of EGF in both chambers to disrupt gradients at the optimal dose of 1 ng/ml; data not shown). The fact that A 431 cells express α6β4 and no α6β1 integrin (1, 17, and data not shown) enabled us to use function-blocking, α6-subunit specific antibodies to examine the contribution of α6β4 to A 431 chemotaxis. Treatment of A 431 cells with the 287 bA b inhibited chemotaxis toward EGF on laminin-1 by 60% (Fig. 2 B). This mAb did not inhibit the attachment of the cells to laminin-1 (data not shown), indicating a distinct function for α6β4 in the chemotactic migration of A 431 cells. Lamellipodial protrusions are thought to be critical in cell migration and are the basis for generating lamellae, which are larger protrusions that are associated with the direction the cell migrates (36, 52). Indeed, A 431 cells displayed...
a striking increase in lamellipodia and ruffle formation for sustained periods of time in response to treatment with EGF at a concentration that stimulate optimal chemotaxis (1 ng/ml) (Fig. 1 E). High concentrations of EGF (>5 ng/ml), which were inefficient in stimulating chemotaxis, caused the cells to round up quickly after a short period of protrusive activity, leaving behind numerous retraction fibers (data not shown). For this reason, we used low concentrations of EGF to stimulate A431 cells in subsequent experiments (0.5-2 ng/ml). The importance of α6β4 in the formation of such protrusions is supported by the fact that the lamellar area of A431 cells was substantially reduced by pretreatment with the 2B7 mAb before plating on laminin-1 (Fig. 2 C).

The findings that α6β4 is localized in hemidesmosomes in adherent A431 cells and that EGF stimulated their α6β4-dependent migration raised the possibility that EGF also altered the localization and cytoskeletal interactions
of this integrin. Under these conditions of EGF stimulation, a striking change in the localization of \( \alpha 6 \beta 4 \) was apparent. Specifically, this integrin was substantially reduced in hemidesmosomes on the ventral surface (Fig. 1, D and E, and Fig. 7, left panels), but it was readily apparent in the lamellipodia and ruffles that are formed in response to EGF stimulation (Fig. 1 E). We observed also that EGF stimulation results in a reduction of HDL/plectin staining in hemidesmosomes, indicating a disassembly of hemidesmosome structure (Fig. 1 F).

Our observation that \( \alpha 6 \beta 4 \) is redistributed from hemidesmosomes to lamellipodia and membrane ruffles in response to EGF stimulation prompted us to examine its association with cytokeratins and F-actin in more detail using an in situ extraction scheme that solubilizes proteins to an extent that correlates with their cytosome associations (9, 19). Specifically, membrane, actin, and cytokeratin fractions were obtained from A431 cells that had been either left untreated or stimulated with EGF using sequentially a Triton X-100 buffer (fraction 1, membrane), a two-detergent buffer (1.0% Tween-40/0.5% deoxycholate, fraction 2, actin), and a third buffer containing SDS that solubilizes cytokeratins and associated proteins (fraction 3, cytokeratin; references 9, 19). The relative amount of \( \alpha 6 \beta 4 \) present in each fraction was detected by immunoprecipitation and subsequent immunoblotting with \( \beta 4 \)-specific antibodies. The relative distribution of actin and cytokeratin among the three fractions was also determined to assess the efficiency of the fractionation (Fig. 3). As expected, the cytokeratin and actin were present largely in fraction 3 and actin was distributed between fractions 1 and 2, which represent the G-actin and F-actin pools, respectively. Importantly, EGF stimulation did not alter this relative distribution of cytosomeal proteins among the three fractions. However, as shown in Fig. 3, EGF stimulation resulted in a substantial reduction in the amount of \( \alpha 6 \beta 4 \) in fraction 3 (cytokeratin) and an increase in the amount of \( \alpha 6 \beta 4 \) in the actin fraction in comparison to unstimulated cells. Densitometric analysis of the \( \beta 4 \)-specific bands in this figure revealed an approximate 63% reduction of \( \alpha 6 \beta 4 \) in the cytokeratin fraction and a 48% increase in the actin fraction. These observations provide evidence that the mobilization of \( \alpha 6 \beta 4 \) from hemidesmosomes that we detected by indirect immunofluorescence microscopy is associated with a disruption in its association with cytokeratins and an increase in its association with F-actin.

The localization of \( \alpha 6 \beta 4 \) in membrane ruffles and lamellipodia that form in response to EGF stimulation prompted us to explore the possibility of its association with F-actin in these structures because we had previously observed such an association in colon carcinoma cells (44). We found that the colocalization of \( \alpha 6 \beta 4 \) with F-actin in cell protrusions detected by immunofluorescence was retained in a significant number of protrusions after extraction of EGF-stimulated cells with a Triton X-100 buffer that preserves the actin cytoskeleton (Fig. 4, A and B). However, extraction of these EGF-stimulated cells with the Tween-40/DOC buffer described above eliminated both the F-actin and \( \alpha 6 \beta 4 \) staining (data not shown). Taken together, these findings indicate that EGF stimulation elicits a dissociation of \( \alpha 6 \beta 4 \) from cytokeratin-associated hemidesmosomes, as well as the formation of lamellipodia and ruffles that contain \( \alpha 6 \beta 4 \) in association with F-actin.

**The \( \alpha 6 \beta 4 \) Integrin Colocalizes with the EGF Receptor and Phosphotyrosine in Membrane Ruffles and Lamellipodia**

Our findings that EGF stimulation mobilizes \( \alpha 6 \beta 4 \) from hemidesmosomes and promotes \( \alpha 6 \beta 4 \)-dependent chemotaxis suggested a possible association between \( \alpha 6 \beta 4 \) and the EGF receptor. To address this possibility, EGF-stimulated A431 cells were stained for both \( \alpha 6 \beta 4 \) and the EGF receptor. As shown in Fig. 5 A, a striking colocalization of these two receptors was evident in membrane ruffles and lamellipodia. The specificity of this colocalization is evidenced by the finding that another surface protein, the HLA antigen, was not present in these F-actin–rich structures (data not shown). We were unable, however, to detect a specific, physical association between \( \alpha 6 \beta 4 \) and the EGF receptor by coimmunoprecipitation (data not shown).

Given the fact that the EGF receptor is a tyrosine kinase and the report that EGF stimulation of A431 cells results in a substantial increase in the tyrosine phosphorylation of the \( \beta 4 \) integrin subunit (31), we explored the distribution of phosphotyrosine in both EGF-stimulated, as well as unstimulated, A431 cells using indirect immunofluorescence. In unstimulated cells, most of the phosphotyrosine staining on the ventral surface is seen at the cell periphery in radial arrangements similar to focal adhesions (Fig. 5 B). A consistent colocalization of either \( \alpha 6 \beta 4 \) (Fig. 5 B) or EGF-R (data not shown) with phosphotyrosine in these structures was not evident. Moreover, significant phosphotyrosine staining was not evident in hemidesmosomes (Fig. 5 B). EGF stimulation, however, resulted in the colocalization of phosphotyrosine and \( \alpha 6 \beta 4 \) in lamellipodia and ruf-
fles (Fig. 5 C). From these results, we can conclude that α6β4 is associated with more phosphotyrosine-containing proteins in cell protrusions than in hemidesmosomes.

**EGF Stimulation Induces the Phosphorylation of the β4 Integrin Subunit on Serine Residues**

The colocalization of α6β4 with phosphotyrosine in the lamellipodia and ruffles of EGF-stimulated A431 cells prompted us to examine the phosphorylation of α6β4 induced by EGF. For this purpose, α6β4 was immunoprecipitated from stimulated A431 cells with the 439-9B mAb and the immunoprecipitates were blotted with two phosphotyrosine-specific Abs (PY20 and 4G10). In these experiments, the cells were extracted with RIPA buffer because we observed a nonspecific interaction between α6β4 and the EGFR using a Triton X-100 buffer (data not shown). Under these conditions, we detected no phosphotyrosine in the β4 subunit in response to the concentration of EGF (1 ng/ml) that induced α6β4 redistribution to lamellipodia and ruffles, and that stimulated optimal A431 chemotaxis (Fig. 6 A). Moreover, even high concentrations of EGF (100 ng/ml) that induced a rapid rounding-up of adherent A431 cells and did not stimulate chemotaxis (Fig. 2 A) induced only a marginal increase, at best, in the phosphotyrosine content of the β4 subunit as detected by these antibodies (Fig. 6 A). Similar results were obtained with other α6 and β4-specific mAbs including GoH3, 2B7, A9, as well as a β4-specific polyclonal antibody (data not shown). These findings are in contrast to the report that EGF stimulation induces a substantial increase in the tyrosine phosphorylation of the β4 subunit in A431 cells (31).

To exclude the possibility that the phosphotyrosine-specific Abs we used were unable to detect significant tyrosine phosphorylation of the β4 subunit after EGF stimulation, A431 cells were labeled metabolically with 32P-orthophosphate and then stimulated with EGF. As shown in Fig. 6 B, EGF stimulation did increase the phosphorylation of the β4 subunit substantially with half-maximal phosphorylation observed at ~1 ng/ml of EGF. The discrepancy between the phosphotyrosine-specific Ab results and the metabolic labeling results prompted us to do a phosphoamino acid analysis of the radiolabeled β4 subunit. Surprisingly, we found that the β4 subunit is phosphorylated almost exclusively on serine (Fig. 6 C). Indeed, both the basal and EGF-induced increases in β4 phosphorylation that we detected in Fig. 6 B result from serine phosphorylation (Fig. 6 C). This phosphoamino-acid analysis in conjunction with the phosphotyrosine antibody data provide convincing evidence that EGF stimulation induces significant phosphorylation of the β4 subunit on serine but not tyrosine residues.

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**Figure 4.** The α6β4 integrin associates with F-actin in response to EGF. A431 cells were plated on laminin-1 for 1 h before stimulation with EGF (1 ng/ml) for 15 min. The cells were extracted with a Triton X-100–containing buffer and then fixed as described in Materials and Methods. The fixed cells were double-stained for indirect immunofluorescence analysis using (A and C) GoH3 mAb and (B and D) FITC-phalloidin. Arrows indicate points of colocalization. Bars, 10 μm.

**Figure 5.** The α6β4 integrin colocalizes with the EGF receptor and phosphotyrosine in ruffles and lamellipodia but not in hemidesmosomes. A431 cells were plated on laminin-1 for 1 h and then either stimulated with EGF (1 ng/ml) for 15 min (A and C) or left untreated (B). All cells were processed for double-staining with the α6-specific rat mAb GoH3 (red) and mouse Abs specific for either the EGFR (A, green) or phosphotyrosine (PY20; B and C, green). Yellow depicts colocalization of two antigens. Arrows indicate colocalization in lamellipodia and ruffles, and small arrows indicate focal adhesion-like structures. Large arrow indicates hemidesmosome area. Bar, 10 μm.
Activation of PKC Redistributes α6β4 from Hemidesmosomes to Cell Protrusions and Induces Phosphorylation of the β4 Subunit on Serine Residues

The above findings indicated that EGF activates a serine protein kinase that is involved in β4 phosphorylation and that could also be involved in the redistribution of α6β4 from hemidesmosomes to lamellipodia and membrane ruffles. We hypothesized that a likely candidate for this kinase is PKC because its activation by EGF is well documented (2). As an initial test of this hypothesis, we examined the effect of PMA stimulation on α6β4 localization in A431 cells. PMA stimulation mobilized α6β4 from hemidesmosomes (Fig. 7, left panels), and increases the formation of α6β4-containing lamellipodia and ruffles (data not shown) as assessed by indirect immunofluorescence microscopy. These data were substantiated biochemically by analyzing the amount of β4 that remains associated with the cytokeratin fraction after stimulation with PMA, using the detergent extraction procedure described above. PMA stimulation markedly reduced the amount of α6β4 in the cytokeratin fraction in comparison to unstimulated cells (Fig. 8 C). Consistent with a role of PKC-dependent phosphorylation in the redistribution of α6β4, we found that PMA stimulation itself increased the phosphorylation of the β4 subunit significantly as assessed by 32P-orthophosphate labeling (Fig. 8 A). The fact that we detected no tyrosine phosphorylation of β4 in response to PMA stimulation using the phosphotyrosine-specific antibodies (data not shown) indicates that the increase in 32P-orthophosphosphate labeling can be attributed to serine phosphorylation.

If PKC activation is required for the mobilization of α6β4 from hemidesmosomes, inhibition of PKC activity should inhibit this process. To establish this causality, we used G06976, an inhibitor of the conventional isoforms of PKC (α, β, γ) (33, 42). The effects of this inhibitor on α6β4 localization were profound. As shown in Fig. 7, G06976 blocked the release of α6β4 from hemidesmosomes on the ventral surface of A431 cells in response to either EGF or PMA stimulation. Consistent with a role for serine phosphorylation in the release of α6β4 from hemidesmosomes, we found that G06976 reduced the EGF-stimulated phosphorylation of the β4 subunit by ∼50% (Fig. 8 B), a reduction that corresponds to the level of β4 phosphorylation observed in the absence of EGF stimulation (see Fig. 6 B). Moreover, G06976 inhibited the EGF-induced dissociation of α6β4 from the cytokeratin fraction as assessed by the detergent extraction approach described above (Fig. 8 C). This inhibition was evident for both EGF and PMA-stimulated cells (Fig. 8 C).

The above data suggested the participation of a conventional PKC isoform in the disassembly of the hemidesmosome and mobilization of α6β4 integrin. To obtain additional evidence for PKC involvement, we examined the possibility that activation of PKC-α, a widely distributed conventional PKC isoform, was sufficient to disassemble hemidesmosomes in the absence of EGF stimulation. For this purpose, we constructed a constitutively active PKC-α cDNA that contained the Src myristoylation site at its amino terminus. This myristoylated PKC-α exhibited a high level of in vitro kinase activity relative to the wild-type enzyme (Fig. 9). Subsequently, we expressed both the myristoylated and wild-type PKC-α cDNAs in A431 cells and analyzed the effect of these cDNAs on hemidesmosome structure. As shown in Fig. 10, A431 cells that expressed myristoylated PKC-α showed a striking reduction in hemidesmosomes. More specifically, expression of both HD-1 and α6β4 was markedly reduced on the basal surface of these cells. In contrast, the cells that expressed the
wild-type PKC-α, showed little change in the formation of hemidesmosomes. These results suggest that activation of PKC-α is sufficient to cause redistribution of the α6β4 integrin and other components of the hemidesmosome.

**PKC Is Essential for α6β4-mediated Chemotaxis**

A key implication of the above findings is that PKC activity is required for EGF-stimulated chemotaxis of A431 cells because the PKC-dependent redistribution of α6β4 is a necessary event in the mechanism of chemotaxis. We tested this implication by analyzing the effect of Gö6976 on the chemotactic response of A431 cells to EGF. The results in Fig. 8D reveal that Gö6976 inhibited EGF-stimulated chemotaxis by >80%. It is important to note that Gö6976 at the concentrations used did not inhibit the attachment or spreading of A431 cells (see Fig. 7). These results support the involvement of PKC in the chemotactic signal elicited by EGF, and they substantiate the importance of a regulated and dynamic redistribution of the α6β4 integrin in chemotactic migration.

**Discussion**

The data we present provide insight into the mechanism of cell migration, especially the migration of epithelial and carcinoma cells. Recent work by our group has established that α6β4 participates in the chemotactic migration of carcinoma cells by interacting with F-actin at their leading edges and regulating essential signaling pathways (39, 44, 51). This function underlies the contribution of this integrin to carcinoma invasion (reviewed in 43). An issue that needed to be resolved, however, is the relationship between α6β4 function in the hemidesmosomes of epithelial-derived cells and its ability to promote the migration of these cells. Epithelial cells use hemidesmosomes to anchor to the basal lamina. These multi-protein structures connect the substratum with cytokeratins to form rigid adhesion complexes. The α6β4 integrin is an essential component of hemidesmosomes and it is necessary for mediating their adhesive function (4, 16, 23). Given this ability of α6β4-containing hemidesmosomes to generate stable adhesive contacts, it is reasonable to assume that their func-
Figure 8. Protein kinase C mediates the effects of EGF on β4 serine phosphorylation, release of αβ4 from a cytokeratin fraction, and chemotaxis. (A) A 431 cells plated on laminin-1 were labeled with 32PO₄ as described in Materials and Methods. These PKC proteins were immunoprecipitated using a FLAG-specific Ab and this information was used to normalize PKC-α expression for the kinase assays (data not shown). Immune complex kinase assays were performed using MBP as the substrate as described in Materials and Methods. 

Figure 9. A analysis of wild-type and myristoylated PKC-α kinase activity. 293T cells were transfected with either the pCMV5 vector alone, the PKC-α-FLAG cDNA, or the myristoylated PKC-α-FLAG cDNA as described in Materials and Methods. These PKC proteins were immunoprecipitated using a FLAG-specific A. B. Expression of PKC-α was confirmed by immunoblotting these precipitates with a PKC-α specific Ab and this information was used to normalize PKC-α expression for the kinase assays (data not shown). Immune complex kinase assays were performed using MBP as the substrate as described in Materials and Methods.

PKC-mediated Mobilization of the αβ4 Integrin

Most likely, the conventional PKC isoform, PKC-α, is involved in the redistribution of αβ4 and the disassembly of hemidesmosomes. G6976, a specific inhibitor of the conventional PKC isoforms (α, β, γ), was able to impede the mobilization of αβ4 from hemidesmosomes and inhibit the EGF-stimulated phosphorylation of the β4 subunit. Indeed, hemidesmosomes were well preserved in EGF-stimulated A431 cells that had been pretreated with G6976. Consistent with the notion that the preservation of hemidesmosomes impedes cell migration, G6976 also inhibited the chemotactic response of A431 cells to EGF. In addition to these data, we observed that activation of PKC-α by expression of a constitutively active, myristoy-
lated form of the enzyme, was sufficient to induce the redistribution of α6β4 and disassembly of hemidesmosomes.

A n issue that remains to be addressed is the nature of the signaling pathway that links the EGF receptor to activation of PKC-α and the mobilization of α6β4 from hemidesmosomes. As mentioned above, PLC-γ is a likely intermediary (12, 25, 38). However, we observed that the PLC-γ inhibitor U 73122 (1 μM) had rather drastic effects on the morphology of A 431 cells and could not be used to assess the involvement of this phospholipase in the dynamic behavior of α6β4. Similarly, other widely used inhibitors such as wortmannin induced morphological changes in A 431 cells even in the absence of EGF stimulation. We did observe, however, a partial inhibition of the EGF-induced redistribution of α6β4 with the MEK inhibitor PD 98059 (data not shown). Although this observation needs to be established more rigorously, it does suggest the possible involvement of the MAP kinase pathway in the EGF-induced mobilization of α6β4 from hemidesmosomes. Interestingly, PD 98059 can abrogate EGF-induced focal adhesion disassembly and cell motility in fibroblasts (59).

A though we cannot exclude a role for tyrosine phosphorylation of the β4 subunit in the EGF-induced chemotaxis of A 431 cells, the data we obtained argue strongly against such a role primarily because we did not detect tyrosine phosphorylation of β4 using EGF at concentrations that promote optimal chemotaxis. In fact, when we used much higher concentrations of EGF (100 ng/ml) we detected an additional increase in β4 serine phosphorylation, but only a marginal increase, at best, in β4 tyrosine phosphorylation. These results contrast with a previous study by Mainiero et al. (31) that reported a striking increase in the tyrosine phosphorylation of the β4 subunit in A 431 cells stimulated with high concentrations of EGF (10–250 ng/ml). At this point, we are unable to explain the discrepancies between our findings and those of Mainiero et al. (31). It is worth noting, however, that in our study, the use of such high concentrations of EGF to stimulate A 431 cells failed to induce chemotaxis and caused the cells to round-up, making it difficult to correlate phosphorylation of β4 with cell migration under these conditions.

The EGF-stimulated phosphorylation of the β4 subunit on serine residues that we identified is novel and it provides an impetus for investigating the nature of this phosphorylation and its role in regulating the cytoskeletal interactions of the α6β4 integrin in more detail. For example, the extremely large β4 cytoplasmic domain contains multiple consensus motifs for PKC phosphorylation based on our analysis of the human β4 cDNA sequence using Prosite (data not shown). The presence of these motifs supports the possibility that PKC may phosphorylate α6β4 directly. It is also worth considering the possibility that PKC may activate another downstream serine kinase that is involved in β4 phosphorylation because there are also consensus sites in the β4 sequence for other serine kinases such as casein kinase II. A further important issue to be studied is whether phosphorylation of the β4 subunit is essential for either its mobilization from hemidesmosomes or its recruitment into lamellipodia and ruffles. The data we provide in this study provide a strong correlation of serine phosphorylation with these events. Definitive proof of involvement will require identification of the specific serine residue(s) in the β4 subunit that are phosphorylated by EGF stimulation and the subsequent mutational analysis of these sites. The possibility that PKC-dependent

Figure 10. Activation of PKC-α is sufficient to induce disassembly of the hemidesmosome and release of α6β4 integrin. A 431 cells were transfected with the PKC-α-FLAG cDNA (A, B, E, and F) or the myristoylated PKC-α-FLAG cDNA (C, D, G, and H) as described in Materials and Methods. After 24 h, the cells were fixed and the presence of hemidesmosomes was assessed by double immunofluorescence using either mAb 121 (HD-1; A and C) and an anti-FLAG mAb (B and D), or GoH3 mAb (α6β4; E and G) and an anti-FLAG mAb (F and H). Arrows indicate FLAG-positive cells. Note the absence of hemidesmosomes in cells that express the myristoylated PKC-α. Bar, 10 μm.
serine phosphorylation also influences other components of hemidesmosomes should be considered. For example, there is evidence that PKC can mobilize BPA G-2 from hemidesmosomes (27), and that it can phosphorylate HD1/plexin and weaken its interaction with intermediate filaments (20).

The EGF-induced release of α6β4 from hemidesmosomes and its association with F-actin in lamellipodia and membrane ruffles are probably independent events. This idea is derived from our finding that G6 6976, an inhibitor of the conventional PKC isozymes, did not block the EGF-induced formation of α6β4-containing membrane ruffles and lamellipodia (data not shown), even though it prevented α6β4 mobilization from hemidesmosomes (Fig. 7). This observation suggests that the PKC-dependent mobilization of α6β4 from hemidesmosomes is independent of the recruitment of α6β4 into the lamellipodia and ruffles that are formed in response to EGF stimulation. As we have shown, however, G6 6976 did inhibit EGF-induced chemotactic migration. Collectively, these findings underscore the hypothesis that the increased formation of cell protrusions in the form of ruffles and lamellipodia is not enough to generate movement and that the destabilization of hemidesmosome function mediated by PKC is an essential component of the migration process.

An interesting issue that arises is the role that EGF plays in the recruitment of α6β4 to the lamellipodia and ruffles. Most likely, EGF stimulates the formation of new cell protrusions that contain α6β4 rather than promoting the preferential incorporation of α6β4 into such structures. This assumption is based on our finding that the few lamellipodia that form in the absence of EGF do incorporate α6β4, and that they have a similar intensity of α6β4 expression as those lamellipodia that are formed in response to EGF stimulation (for example, see Fig. 1). Thus, we suggest that α6β4 is transported to and concentrated at the leading edges by mechanisms intrinsic to lamellipod formation, and that EGF increases the number of protrusions while providing a pool of α6β4 liberated from hemidesmosomes. One possible mechanism by which α6β4 could be recruited to the leading edge is exemplified by the transient association of α6β4 with the actin cytoskeleton that occurs in neuronal growth cones. These aggregates of β1 integrins are transported on the dorsal surface in a directed way to the leading edge (48). A though we do not have direct data to support a transient association of α6β4 with the actin cytoskeleton, the fact that only a fraction of the α6β4 in lamellipodia is resistant to extraction with a Triton X-100 buffer (e.g., compare the extracted lamellipodia of Fig. 4 to the unextracted ones of Fig. 5) may be the reflection of a dynamic equilibrium attained by the constant association and dissociation of α6β4 with F-actin. A novel model for the recruitment of membrane proteins into motile structures involves the concentration of recycling proteins in ruffles by directed exocytosis induced by EGF through a Rac-dependent pathway (7). In this model, the ability of membrane proteins to be recycled is essential for the recruitment into ruffles. This model is of particular interest to our findings because there is evidence that α6β4 is recycled on the cell surface (6), and we have implicated Rac in the α6β4-dependent migration and invasion of carcinoma cells (51).

Moreover, the recycling of the population of α6β4 that is released from hemidesmosomes by EGF could provide a pool for newly forming ruffles and lamellipodia.

If the mobilization of α6β4 from hemidesmosomes constitutes one essential component of the EGF-stimulated migration of A 431 cells, the second component of migration in which α6β4 participates is the actual process of migration itself. Indeed, a function for α6β4 in A 431 migration is supported by its localization in lamellipodia and ruffles and, more directly, by our finding that an α6-specific mAb inhibited both chemotactic migration and lamellae formation on laminin-1. These results are consistent with our previous work on colon carcinoma cells that established a role for α6β4 in the formation and stabilization of lamellae and filopodia (44). In this study, we observed that protruding filopodia, which anchor to the substrate using α6β4, are frequently followed by the extension of the lamella towards the anchoring point. This function of α6β4 may relate to the concept of a molecular clutch that anchors actin bundles to the substrate providing traction tracks for myosin II motors (36). Because a retrograde flow of actin filaments from the periphery towards the nucleus occurs in most motile cells, the net effect of this clutch action on actin polymerization would also be to generate a forward protrusive force, as has been recently shown in a growth cone model (56). In addition to these mechanical functions, we have defined at least two distinct signaling pathways regulated by α6β4 that are essential for lamellae formation, chemotactic migration and invasion of carcinoma cells. These pathways involve PI3-kinase/Rac (51) and a AMP-specific phosphodiesterase (39). It is also worth noting that we have observed apparent ligand-indepen

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above, suggests cooperation in their signaling of chemotactic migration.

In summary, our findings describe a mechanism for the chemotactic migration of carcinoma cells that assemble hemidesmosomes. A new important implication of our findings is that the mobilization of the α6β4 integrin from hemidesmosomes by a PK C-dependent mechanism is an essential step in the migration process, presumably because it releases the strong and stable adhesion mediated by hemidesmosomes and allows for the dynamic adhesive interactions that are required for migration. Importantly, we also demonstrate that the α6β4 integrin can associate with F-actin in lamellipodia and membrane ruffles, and participate in the migration process itself. Collectively, our results explain how this integrin can mediate both stable adhesion and cell migration. They also suggest that growth and motility factors that are known to promote tumor progression may function, in part, by changing the cytoskeletal interactions and localization of this unique receptor.

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