Inhibitory Role of α6β4-associated erbB-2 and Phosphoinositide 3-Kinase in Keratinocyte Haptotactic Migration Dependent on α3β1 Integrin

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Abstract. Keratinocytes and other epithelial cells express two receptors for the basement membrane (BM) extracellular matrix component laminin-5 (Ln-5), integrins α3β1 and α6β4. While α3β1 mediates adhesion, spreading, and migration (Kreidberg, J.A. 2000. *Curr. Opin. Cell Biol.* 12:548–553), α6β4 is involved in BM anchorage via hemidesmosomes (Borradori, L., and A. Sonnenberg. 1999. *J. Invest. Dermatol.* 112:411–418). We investigated a possible regulatory interplay between α3β1 and α6β4 in cell motility using HaCaT keratinocytes as a model. We found that α6β4 antibodies inhibit α3β1-mediated migration on Ln-5, but only when migration is haptotactic (i.e., spontaneous or stimulated by α3β1 activation), and not when chemotactic (i.e., triggered by epidermal growth factor receptor). Inhibition of migration by α6β4 depends upon phosphoinositide 3-kinase (PI3-K) since it is abolished by PI3-K blockers and by dominant-negative PI3-K, and constitutively active PI3-K prevents haptotaxis. In HaCaT cells incubated with anti–α6β4 antibodies, activation of PI3-K is mediated by α6β4-associated erbB-2, as indicated by erbB-2 autophosphorylation and erbB-2/p85 PI3-K coprecipitation. Furthermore, dominant-negative erbB-2 abolishes inhibition of haptotaxis by anti–α6β4 antibodies. These results support a model whereby (a) haptotactic cell migration on Ln-5 is regulated by concerted action of α3β1 and α6β4 integrins, (b) α6β4-associated erbB-2 and PI3-K negatively affect haptotaxis, and (c) chemotaxis on Ln-5 is not affected by α6β4 antibodies and may require PI3-K activity. This model could be of general relevance to motility of epithelial cells in contact with BM.

Key words: erbB-2 • integrin • keratinocytes • laminin-5 • phosphoinositide 3-kinase

Introduction

Epithelial cells are separated from the connective tissue by the basement membrane (BM),[1] a network of extracellular matrix (ECM) polymers consisting of several laminin isoforms and type IV collagen, and connected by glycoproteins such as nidogen (Timpl, 1996; Burgeson and Christiano, 1997). Keratinocytes are the dominant epithelial cell type in the epidermis, a complex squamous epithelium that forms the outer surface of the skin (Priestley, 1993) and that is separated from the underlying dermis by the BM. Contact of basal keratinocytes with the BM and their cell–cell interactions are essential for proper function by modulating cell polarity, proliferation, migration, and differentiation (Adams and Watt, 1993; Burgeson and Christiano, 1997; Fuchs et al., 1997).

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[1]*Abbreviations used in this paper:* BM, basement membrane; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; Ln-5, laminin-5; MAP kinase, mitogen-activated protein kinase; ON, overnight; PI3-K, phosphoinositide 3-kinase; RT, room temperature.

Cell–ECM or cell–cell adhesion are mediated by integrins, α/β-heterodimeric transmembrane glycoprotein receptors (Hynes, 1992). To reach high mechanical stability and resist the frictional stresses the skin is subjected to, the epidermal BM contains specialized anchoring complexes, in addition to conventional integrin-mediated cell–ECM linkages. Such anchoring complexes consist of hemidesmosomes, anchoring fibrils, and anchoring filaments with laminin-5 (Ln-5) as a major component (Burgeson and Christiano, 1997). Basal keratinocytes express two Ln-5 integrin receptors, α3β1 and α6β4, which are recruited to distinct cell adhesion structures (Carter et al., 1990; Fuchs et al., 1997). α6β4 is a component of hemidesmosomes, linking Ln-5 anchoring filaments on the outside of the cell with the keratin filament network inside the cell (Borradori and Sonnenberg, 1999), thus anchoring keratinocytes to the BM. In contrast, α3β1 is recruited to focal contacts and thereby links the ECM to components of the actin cytoskeleton, mediating cell spreading and migration (Carter et al., 1990; Fuchs et al., 1997).

These two types of integrin-mediated adhesive junctions are likely to transmit distinct molecular signals to cells.

Since integrins are not equipped with enzymatic activity, they need to associate with signaling molecules at the cell surface (Schwartz et al., 1995; Giancotti, 1997; Porter and Hogg, 1998; Giancotti and Ruoslahti, 1999, Schwartz and Baron, 1999). α3β1 is a typical integrin in terms of its structure, containing a short (50 amino acid) cytoplasmic β1 tail (Sastry and Horwitz, 1993) implicated in activation of focal adhesion kinase (FAK) (Schlaepfer et al., 1999; Sieg et al., 2000). This latter event is coupled to the turnover of focal adhesions and modifications of the cytoskeleton (Giancotti and Ruoslahti, 1999; Schlaepfer et al., 1999; Ren et al., 2000), both critical in cell migration (Horwitz and Parsons, 1999). Furthermore, α3β1 is associated with transmembrane-4 superfamily proteins such as CD81- or CD151-forming complexes, which may regulate cell migration (Yauch et al., 1998; Testa et al., 1999).

In contrast, α6β4 contains a unique β4 cytoplasmic domain (~1,000 amino acids) with no homology to other known β subunits, which mediates association with the hemidesmosome cytoskeleton (Gil et al., 1994; Spinardi et al., 1995) and contains a tyrosine activation motif that upon phosphorylation can act as docking site for signaling molecules containing Src homology 2 domains. In primary keratinocytes, ligation of α6β4 caused tyrosine phosphorylation of this motif, which recruited the adapter proteins Shc and Grb2 and sequentially activated mitogen-activated protein kinase (MAP kinase) pathways, indicating a role for α6β4 in the regulation of keratinocyte proliferation (Mainiero et al., 1995, 1997). Furthermore, in breast and colon carcinoma cells, α6β4 was shown to activate phosphoinositide 3-kinase (PI3-K), leading to increased Matrigel invasion (Shaw et al., 1997).

Integrins not only use adapter proteins to interact with signaling pathways, but they are also in direct physical interaction with growth factor receptors (Miyamoto et al., 1996). For example, αvβ3 integrin was reported to be associated with activated insulin and PDGFβ receptors (Schneller et al., 1997), and with vascular endothelial growth factor receptor-2 (Soldi et al., 1999). Furthermore, coimmunoprecipitation between β1 integrin and the receptor for epidermal growth factor was demonstrated (Moro et al., 1998). Concerted action of integrins and growth factor receptors may be crucial to tightly control many biological processes, including cell motility during wound repair, inflammation, and organogenesis. Cell migration triggered by adhesion receptors is referred to as haptotaxis/haptokinesis, whereas cytokine and growth factor receptor-controlled motility is defined as chemotaxis/chemokinesis. Ligands for these receptors may occur either in a gradient (-taxis) or at a constant concentration (-kinesis) (Wells, 2000).

During re-epithelialization of wounds, keratinocytes dissolve their stable attachment with the underlying BM and migrate over a provisional matrix, continuously expressing and depositing Ln-5 (Larjava et al., 1993; Yamada et al., 1996). These observations suggested a role for Ln-5 as a migratory substrate for keratinocytes. On the other hand, Ln-5 was also reported to inhibit keratinocyte migration and to promote establishment of cell anchoring hemidesmosomal complexes in quiescent BM zones (Yamada et al., 1996; O'Toole et al., 1997; Goldfinger et al., 1999). How can the same ECM component mediate two such different cell behaviors like migration and anchorage? In spite of a large body of information gathered by many laboratories (Carter et al., 1990; Xia et al., 1996; DiPersio et al., 1997; Fuchs et al., 1997; Mainiero et al., 1997; De Arcangelis et al., 1999; Goldfinger et al., 1999; Nguyen et al., 2000; Raghavan et al., 2000), there is still no satisfactory answer to this question. Investigating this problem may shed light on important processes such as wound healing and may also provide insight on how cells in general regulate static adhesion versus migration.

In this study, we attempted to characterize the signaling network that may regulate migration versus anchorage of keratinocytes on Ln-5, via the two Ln-5 binding integrins, α3β1 and α6β4. As a model, we used the nontumorigenic, spontaneously immortalized human keratinocyte cell line, HaCaT. We report that integrin α3β1 mediates both haptotactic and chemotactic migration on Ln-5 in HaCaT keratinocytes. However, integrin α6β4 may inhibit haptotaxis on Ln-5, but not chemotaxis, via a pathway that involves erbB-2 and PI3-K. Our results define distinct types of keratinocyte migration on Ln-5, and point to possibly general mechanisms whereby α3β1 and α6β4 are predominantly a migratory or an anchoring integrin, respectively, for epithelia in contact with Ln-5.

Materials and Methods

Cell Lines, Constructs, and Retroviral Infections

HaCaT (Boukamp et al., 1988) and A431 cells (American Type Culture Collection) were cultured in DMEM (4.5 g/liter glucose) containing 10% FCS. Primary human keratinocytes were purchased from Clonetics and cultured in completely defined medium (KGMD) according the manufacturer's protocol. Passages 3 and 4 were used for migration assays. Constructs encoding dominant-negative PI3-K (p85ΔiSH2-N and p85ΔSH2-C; Rodriguez-Viciana et al., 1997) were from J. Downward (Imperial Cancer Research Fund, London, UK) and the cDNA for the constitutive-active PI3-K (MMΔ72cp3kFL; Jiang et al., 2000) was a gift from P.K. Vogt (The Scripps Research Institute). Construct HER2VEK753A (Messerle et al., 1994) encoding a dominant-negative erbB-2 variant was from N.E. Hynes (Friedrich Miescher Institute, Basel, Switzerland). All cDNAs were subcloned into the retroviral vector pLNCX (CLONTECH Laboratories, Inc.). Virus production in PT67 packaging cells (CLONTECH Laboratories, Inc.) and infection of HaCaT cells was performed as described in the manufacturer's protocol. A retroviral vector encoding enhanced green fluorescent protein was used to assess infection efficiency, which was at least 95% in each experiment.

Antibodies, Extracellular Matrix Molecules, and Reagents

The anti–CD151 monoclonal antibody (mAb) 1A5 (Testa et al., 1999) was provided by J.P. Quigley (The Scripps Research Institute). mAbs 5C11 (anti–CD151; Yauch et al., 1998), TS2/16 (anti–β1; Hemler et al., 1984), and A3-X8 (anti–α3; Weitzman et al., 1993) were gifts from M.E. Hemler (Dana-Farber Cancer Institute, Boston, MA). mAb 12F1 (anti–α2; Pischel et al., 1987) was provided by V.L. Woods, Jr. (University of California, San Diego, San Diego, CA). Anti–β4 mAbs AA3 and S3-41 and rabbit anti–α6 IgG 6845 were produced in our laboratory (Tamura et al., 1990; Domanico et al., 1997). Commercially available integrin mAbs were ASC-1 (anti–α3; Chemicon), P1B5 (anti–α3; GIBCO BRL), GoH3 (anti–α6; BD PharMingen), and P4C10 (anti–β1; GIBCO BRL). Rabbit anti–FAK IgG (BD PharMingen) was used for immunoprecipitations and mAb anti–FAK and anti–P-Tyr mAb PY20 (Transduction Laboratories) for Western blotting. PI3-K subunit p110α was immunoprecipitated with mAb N-20 (Santa Cruz Biotechnology, Inc.) and mAb to PI3-K p85 subunit for Western blotting was purchased from Transduction Laboratories. Goat anti–AKT1 IgG (C-20) was from Santa Cruz Biotechnology, Inc. and rabbit IgG to phosphorylated AKT was from New England Biolabs, Inc.

erbB-2 was immunoprecipitated with mAb c-neu (Ab-2; Oncogene Research Products) and analyzed in Western blots with mAb erbB-2 (Transduction Laboratories). Rabbit anti–ERK1/2 IgG was from Santa Cruz Biotechnology, Inc. and mAb to phosphorylated ERK1/2 was from New England Biolabs, Inc. Anti–FLAG mAb M2 was from Sigma-Aldrich. Fab fragments were generated by digestion of mAbs with 0.02 mg/ml papain (Sigma-Aldrich). Human collage IV and bovine fibronectin were from Sigma-Aldrich and Ln-5 deposited by the rat bladder carcinoma cell line 804G was purified in our laboratory. LY294002, PD98059, and tyrphostin AG 825 were from Calbiochem and EGF was from Sigma-Aldrich.

Migration and Adhesion Assays

In Transwell migration assays, the underside of the filters (8.0 μm, pore size; Costar) was coated at 4°C overnight (ON) with 0.25 μg/ml Ln-5, 1 μg/ml collagen IV, or 10 μg/ml fibronectin in PBS. Filters were washed twice with PBS containing 0.2% Tween-20 (PBST), and then blocked with 5% dry milk in PBST at room temperature (RT) for 2 h. Cells (HaCaT: 1.2×10^5 cells/filter; A431: 6×10^4 cells/filter, primary keratinocytes: 8×10^4 cells/filter) in migration medium, MM (culture medium without FCS) were preincubated with antibodies, reagents or vehicle for 30 min at RT before plating on filters that were washed twice with PBS after blocking. EGF was added to the lower chamber only, whereas antibodies, reagents, or vehicle were present in both chambers. Cells were maintained at 37°C for 5 h (primary keratinocytes for 15 h), and were then fixed and stained using the LeukoStat kit (Fisher Scientific). The uncoated side of each filter was wiped with a cotton swap to remove cells that had not migrated through the filter. Filters were viewed under bright-field optics and stained cells were counted in eight fields (using a 20× objective) from each of two filters for each condition, determining the mean number of cells counted per field. Each experiment was done at least three times and results are expressed as mean ± SD of relative cell migration with nonstimulated cells set as 1.

Scratch assays were performed in 24-well plates coated ON at 4°C with 0.25 μg/ml Ln-5 in PBS. HaCaT cells (6×10^5/well) in MM were seeded and incubated at 37°C for 2 h. Then, cell layers were wounded with a plastic pipet tip and washed three times with MM. The denuded surfaces were recoated with 0.25 μg/ml Ln-5 in MM for 1 h at 37°C. Cell layers were washed again, TS2/16 (40 μg/ml) and/or EGF (1 ng/ml) were added, and cells were incubated for 14 h at 37°C. Photographs of identical locations within each scratch were taken before, and 14 h after, addition of TS2/16, EGF, or both stimuli.

Adhesion assays were performed as described by Goodwin and Pauli (1995), with minor modifications. Microtiter 96-well plates were coated with 1 μg/ml Ln-5, 1 μg/ml collagen IV, or 10 μg/ml fibronectin ON at 4°C, washed twice with PBS, and blocked with 5% dry milk in PBS for 2 h. Cells (6×10^4 cells/100 μl per well) in MM were preincubated with antibodies for 30 min at RT before plating in wells that were washed twice with PBS after blocking. EGF was added at the time of plating. Cells were maintained at 37°C for 30 min, and then 2×100 μl of Percoll floatation medium [73 ml Percoll (density 1.13 g/ml; Amersham Pharmacia Biotech), 27 ml distilled water, and 900 mg NaCl] were added to each well. Adherent cells were fixed for 15 min with 50 μl/well of 25% glutaraldehyde (Sigma-Aldrich), washed with PBS, and stained with crystal violet (0.5% in 20% MeOH) for 10 min. Excess dye was washed off with water and absorbance was measured at 595 nm. Bars represent mean absorbance ± SD of each condition tested in triplicates. All values have had background substracted that represents cell adhesion to wells blocked with milk. Experiments were done three times.

Biochemical Methods

Analysis of AKT-, erbB-2-, ERK1/2-, and FAK Phosphorylation, and erbB-2/p85 Coimmunoprecipitation. Cell culture dishes (6 cm) were coated with 1 μg Ln-5/dish in PBS at 4°C ON, and then prewarmed to 37°C for 1 h. Serum-starved cells ($2–4 \times 10^6$) in MM were preincubated with antibodies for 30 min at 37°C in suspension before plating onto ligand-coated dishes. (If cells were costimulated with TS2/16 and a second antibody, the latter was added 5 min before TS2/16). After 30–60 min at 37°C, the attached cells were rinsed in PBS and lysed for 1 h on ice in 0.5 ml lysis buffer containing 40 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 6 mM EDTA, 100 mM NaF, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM PMSF, 10 μg/ml leupeptin, and one tablet/50 ml of complete protease inhibitor cocktail (Roche Diagnostics Corp.). When FAK was analyzed, the lysis buffer was supplemented with 10% glycerol, 1% sodium deoxycholate, and 0.1% SDS. Lysates were centrifuged at maximum speed for 10 min in a microcentrifuge and antibodies were added to the supernatant for 3 h at 4°C on a rotatory shaker. Then, antibodies were collected with protein-G Sepharose for 1 h at 4°C on a rotatory shaker. The protein complexes were washed three times with ice-cold lysis buffer before boiling in SDS-PAGE loading buffer. Proteins were separated on acrylamide SDS-PAGE gels, blotted, and blots were incubated with the primary antibodies diluted 1:2,000 in 5% milk in PBS containing 0.1% Tween-20. For PY20, milk was replaced by 4% BSA. Each sample was divided in two and analyzed for total AKT, FAK, or erbB-2 content or for phosphorylation or p85 content using the ECLplus system (Amersham Pharmacia Biotech) and a STORM 860 Fluorimeter.

Integrin-erbB-2 Coimmunoprecipitations. Per sample, 3×10^7 cells were lysed in lysis buffer as described above. Lysates were centrifuged at maximum speed for 10 min and supernatants were precleared for 1 h with protein-G Sepharose. Precleared supernatants were subjected to immunoprecipitation, SDS-PAGE, and Western blotting as described above.

PI3-K Enzyme Assay. PI3-K activity was measured according to Jiang et al. (1998). In brief, cells and cell culture dishes were treated as described above. Per dish, 4×10^6 cells were seeded and incubated for 1 h at 37°C. Then, cells were washed with PBS and lysed in lysis buffer for 30 min on ice. Lysates were cleared by centrifugation and total protein concentration was determined. Equal amounts of protein were incubated with precoupled protein-G Sepharose (precoupling: 2 μg anti–p110α antibody were incubated with 50 μl protein-G Sepharose slurry for 1 h at RT) for 2 h at 4°C. Protein complexes were washed twice with lysis buffer and three times with 50 mM Hepes, pH 8.0, containing 160 mM NaCl and 10 mM EDTA. Immunoprecipitates were resuspended in 50 μl kinase buffer [50 mM Hepes, pH 8.0, 5 mM $MgCl_2$, 0.2 mg/ml phosphatidylinositol (1 mg/ml stock, sonicated), and 60 μM ATP] and kinase reactions were started by adding 20 μCi γ-P^{32} ATP. After 10 min at RT, 60 μl of 1 N HCl were added to stop the reactions, and lipids were extracted twice with 160 μl of chloroform:methanol (1:1). Pooled extracts were evaporated in a Speedvac, resuspended in 10 μl chloroform, and subjected to thin layer chromatography for 4 h at RT, using as running solvent a mixture of 65 ml MeOH, 48 ml chloroform, 36 ml pyridine, 60 μl ethoxyquin, 6 ml H_2O, 2.4 ml formic acid, 9.6 g boric acid, and 300 mg BHT. Before sample application, thin layer chromatography plates were pretreated for 10 s in a mixture of 81 ml H_2O, 3 ml 5 N NaOH, 165 ml EtOH, and 2.27 g CDTA, and then incubated at RT for 30 min and at 100°C for 10 min. Assays were analyzed using a PhosphorImager.

Results

Integrin α3β1 Drives Haptotactic and Chemotactic Keratinocyte Migration on Ln-5

To study whether there is an interplay between α3β1 and α6β4 integrins in regulating keratinocyte motility on Ln-5, we first set up conditions under which haptotaxis and chemotaxis of keratinocytes could be analyzed. In Transwell chamber assays (Fig. 1 A), HaCaT cells showed spontaneous migration towards Ln-5 that was small but highly reproducible (Fig. 1 A). We consider this spontaneous migration haptotactic since it appears to be dependent on adhesion receptor/substrate interaction, with no soluble factor added. Addition of TS2/16, an "activating" anti–β1 integrin antibody (Humphries, 1996) caused a fivefold increase in migration (Fig. 1 A). We consider this effect an enhancement of haptotactic migration and used TS2/16 in most subsequent measurements of haptotaxis since it amplifies signal-to-noise ratio in the assay.

With EGF (1 ng/ml), a well-documented chemoattractant (Wells, 2000), there was an ~25-fold increase in migration (Fig. 1 A). By definition, this increase is due to chemotaxis. Combined exposure to TS2/16 and EGF resulted in an additive effect (Fig. 1 A).

We further tested TS2/16 and EGF in the scratch assay (Fig. 1 B), considered to be an in vitro model for kerati-

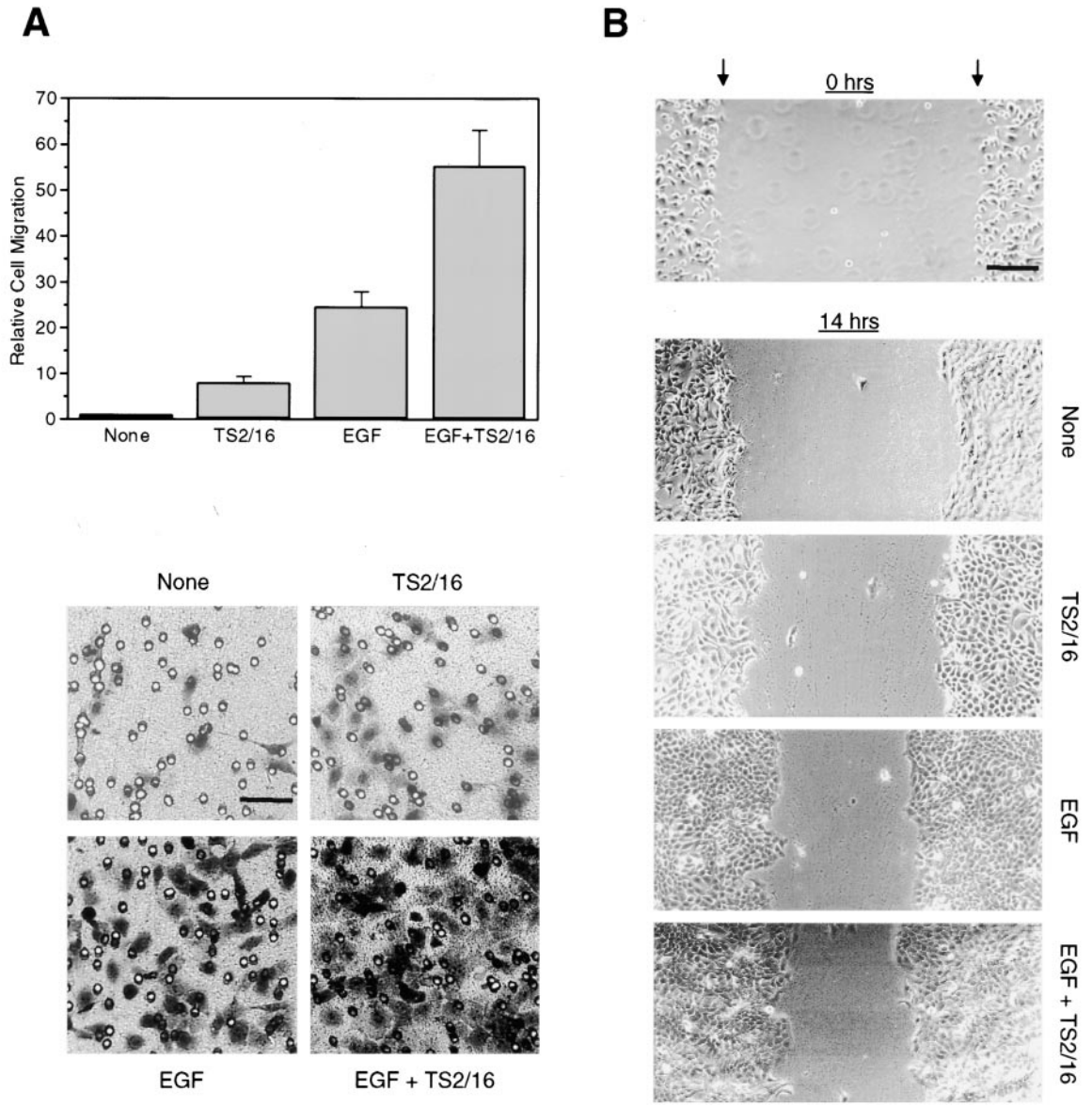

Figure 1. Haptotactic migration of HaCaT cells on Ln-5 can be enhanced by the stimulatory anti–β1 integrin antibody TS2/16. (A) Cell migration assays were performed in Transwell chambers coated with 0.25 μg/ml Ln-5. HaCaT cells were incubated in suspension at RT for 30 min in serum-free culture medium with or without TS2/16 (anti–β1, 40 μg/ml). Then, aliquots were seeded and the plates were incubated at 37°C for 5 h. EGF (1 ng/ml) was present in the lower chamber only, whereas TS2/16 was in both chambers. Migration was quantified by counting cells migrated through filters (eight microscopic fields on each of two filters for each condition). In absence of any stimuli, ~12 cells were counted per microscopic field. In the bar graph, results are expressed as mean ± SD (n = 3) of relative cell migration with nonstimulated cells set at 1. (Bottom) Micrographs of fixed and stained HaCaT cells migrated onto the lower surfaces of Transwell filters (bar, 50 μm). (B) Effects of TS2/16 and EGF in scratch assays. In this assay, cells migrate from the edges of "scrape-wounded" monolayers (arrows) to cover the denuded surface (space between arrows). Scrape wounds were made in serum-free HaCaT cultures plated on Ln-5. Denuded surfaces were recoated with Ln-5 and the wounded cultures were allowed to re-epithelialize for 14 h at 37°C in the presence of TS2/16 (anti–β1, 40 μg/ml), EGF (1 ng/ml), or both stimuli. Micrographs of wounded, nonfixed cell layers at 0 and 14 h after treatment (bar, 100 μm).

nocyte migration occurring during wound healing (Wells, 2000). Scratch closure after 14 h was enhanced by treatment with either TS2/16 or, more markedly, EGF (Fig. 1 B). With both agents together, an additive enhancing effect was detectable on keratinocytes migrating from the edges (Fig. 1 B). These results support a distinction between TS2/16-enhanced and EGF-induced migration, indicated by the Transwell assay.

In the Transwell assay, both TS2/16- and EGF-induced HaCaT migration on Ln-5 required integrin α3β1 since migration was inhibited by antibodies to α3 (P1B5, A3-X8) and β1 (P4C10) integrins, but not by control anti–α2 (12F1) antibody (Fig. 2, A and B). While the effect of P1B5 and P4C10 antibodies may be indirect (i.e., a consequence of adhesion inhibition; Fig. 2 C), A3-X8 antibody is known to block migration but not adhesion (Weitzman

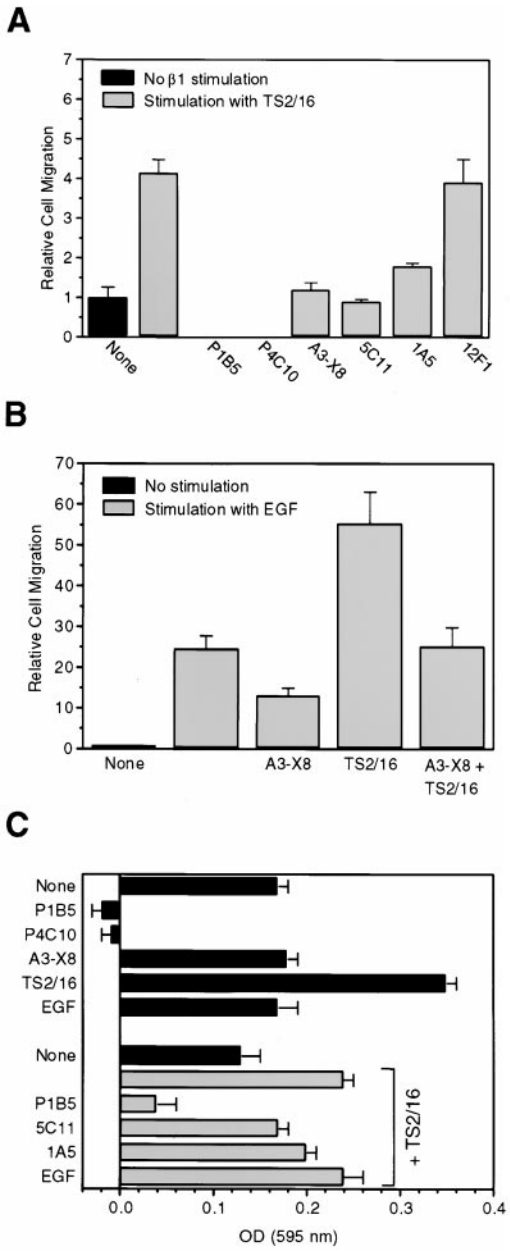

Figure 2. Integrin α3β1 mediates both TS2/16-stimulated haptotaxis and EGF-stimulated chemotactic migration in HaCaT keratinocytes. (A) Haptotactic migration of HaCaT cells was stimulated with the anti–β1 antibody TS2/16, as in Fig. 1 A. Where indicated, cells were preincubated at RT for 15 min with P1B5 (anti-α3, 10 μg/ml), P4C10 (anti–β1, 1:50 dilution), A3-X8 (anti–α3, 10 μg/ml), 5C11 (anti–CD151, 20 μg/ml), 1A5 (anti–CD151, 50 μg/ml), or 12F1 (anti–α2, 40 μg/ml) antibodies. Migration was quantified as described in Fig. 1 A. (B) Inhibitory effect of A3-X8 antibody (anti–α3, 10 μg/ml) on HaCaT cell migration stimulated either by EGF alone or by EGF and TS2/16 together. Migration assays were performed as described in Fig. 1 A. (C) Adhesion assays of HaCaT cells pretreated, as indicated, either with antibodies (as described in A) or with EGF (1 ng/ml added at the time of seeding). (Bottom, gray bars) Cells were TS2/16 stimulated in addition to treatment with indicated antibodies or EGF. Tests were performed with aliquots seeded in triplicates in Ln-5–coated (1 μg/ml) 96-well plates. Cells were allowed to adhere for 30 min at 37°C, and then nonadherent cells were removed, and adherent cells were fixed and stained. Results of no less than three representative experiments are expressed as mean absorbance at 595 nm ± SD.

et al., 1993), indicating that HaCaT migration on Ln-5, under our conditions, is carried out by integrin α3β1. Furthermore, antibodies to CD151, a tetraspanin stoichiometrically associated with α3β1 (Yauch et al., 1998; Testa et al., 1999) also blocked migration (Fig. 2 A), but had little effect on adhesion (C).

Preincubation with TS2/16 increased adhesion to Ln-5 approximately twofold, indicating that TS2/16 induces an increase in α3β1 avidity for Ln-5. Furthermore, an antibody to α3 (P1B5) almost completely blocked this enhanced adhesion (Fig. 2 C), supporting α3β1 dependence. On the other hand, no effect of EGF in adhesion assays was observed (Fig. 2 C), suggesting no EGF influence on α3β1 avidity.

To further characterize TS2/16-induced migration, we tested the involvement of two possible α3β1 effectors, FAK and extracellular signal-regulated kinase (ERK) MAP kinase. All β1 integrins share the ability to promote assembly of focal adhesions and to activate FAK (Giancotti, 1997). Indeed, in cells plated on Ln-5, tyrosine phosphorylation of FAK was increased when compared with cells plated on plastic (Fig. 3 A). This phosphorylation was amplified in the presence of TS2/16, correlating with integrin activation and stimulation of adhesion and migration by this antibody. TS2/16-induced FAK phosphorylation was also seen in cells kept in suspension. This is consistent with the finding that TS2/16 is an activating antibody that induces changes in integrin shape in a ligand-independent manner (Humphries, 1996). As a control, antibodies to α6β4 had no influence on FAK phosphorylation (Fig. 3 A). A3-X8, the anti–α3 antibody that blocked migration but had no influence on adhesion, was without effect on FAK phosphorylation, neither when added alone nor together with TS2/16. Therefore, inhibition of migration by A3-X8 was not due to decreased phosphorylation of FAK.

MAP kinases, such as ERK1 and ERK2, are known to play a stimulatory role in regulation of cell migration (Lauffenburger and Horwitz, 1996; Klemke et al., 1997). Therefore, we tested for the involvement of these enzymes in our system. The ERK kinase (MEK)-specific inhibitor PD98059 prevented TS2/16-stimulated HaCaT migration on Ln-5 (Fig. 3 B), suggesting that ERK1 and ERK2 are possible mediators of haptotaxis. In contrast, PD98059 had no effect in adhesion assays (data not shown), indicating that ERK1/2 are not involved in regulating cell adhesion to Ln-5. Next, ERK1/2 phosphorylation was analyzed in HaCaT cells plated on plastic or on Ln-5 in the presence of anti–integrin antibodies. An increase in ERK1/2 phosphorylation was detected on Ln-5 when compared with the plastic control (Fig. 3 C). Addition of TS2/16 enhanced phosphorylation of ERK1/2 further, whereas A3-X8 had a slightly inhibitory effect. If cells were treated with TS2/16 and A3-X8 together, the stimulatory effect of TS2/16 was blocked by A3-X8. Therefore, inhibition of TS2/16-induced migration by A3-X8 may be due to decreased ERK1/2 activation in the presence of A3-X8. As a control, antibodies to α6β4 had no influence on ERK1/2 phosphorylation, neither when added alone nor in the presence of TS2/16.

These results suggest an involvement of ERK1/2, in the regulation of α3β1 controlled keratinocyte haptotaxis on Ln-5.

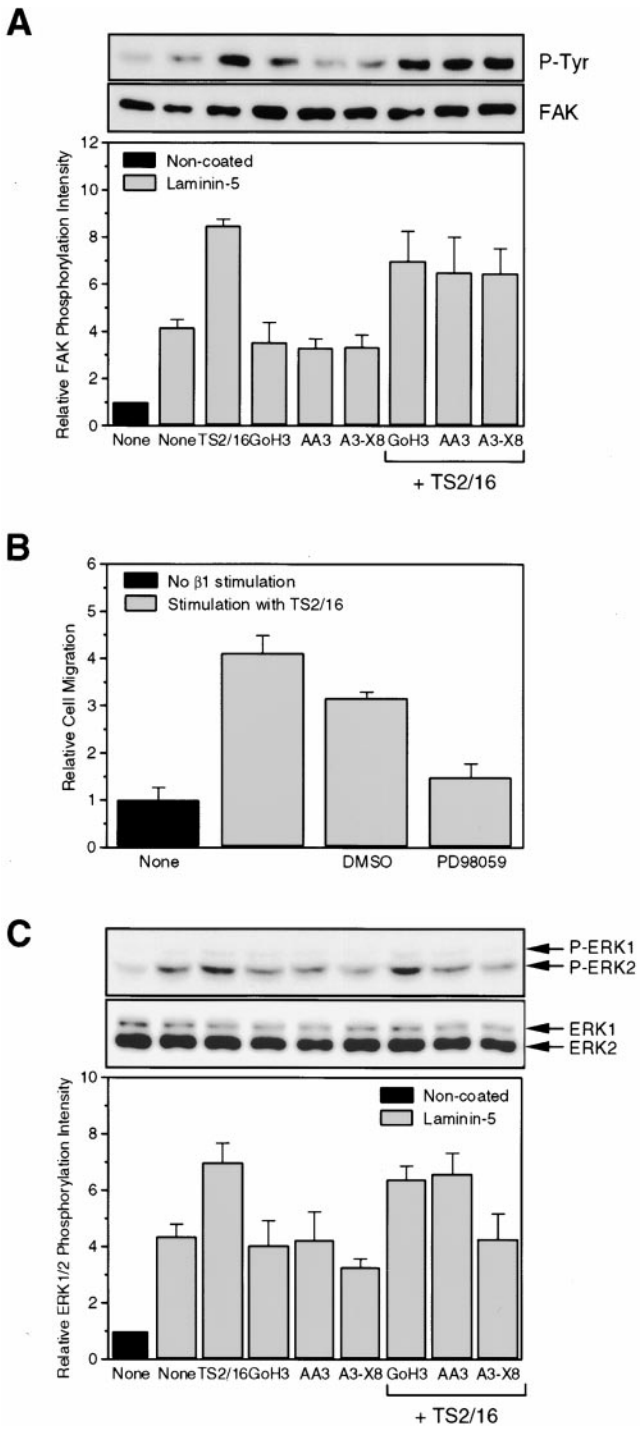

Figure 3. Analyses of FAK and ERK1/2 as candidate downstream signaling molecules in α3β1-dependent haptotaxis. (A) FAK phosphorylation. Changes in FAK phosphorylation state relative to cells plated on Ln-5 are observed after TS2/16, but not A3-X8 or anti–α6β4 antibody treatment. Cells were incubated at 37°C in suspension for 30 min with TS2/16 (anti–β1, 40 μg/ml) and/or GoH3 (anti–α6, 15 μg/ml), AA3 (anti–β4, 40 μg/ml), and A3-X8 (anti–α3, 10 μg/ml) before seeding in noncoated or coated (1 μg Ln-5/dish) dishes and incubation for 30 min at 37°C. Total cell lysates were subjected to immunoprecipitation with an anti–FAK antibody and Western blotting with antibodies to phosphotyrosine (P-Tyr) or FAK. Ratios of phosphorylated FAK to total FAK protein is quantified in the bar graph below. Mean ± SD (n = 3) of relative FAK phosphorylation intensity and one representative experiment is depicted. (B) Inhibition of TS2/16-induced (gray bars) HaCaT haptotaxis by MEK inhibitor PD98059 (PD) on Ln-5–coated filters. Cells were pretreated with PD (50 μM) or vehicle (DMSO). Migration assays were performed and results expressed as in Fig. 1 A. (C) Analysis of ERK1/2 phosphorylation. ERK1/2 phosphorylation relative to cells plated on Ln-5 alone is increased by TS2/16, and this stimulation is blocked by A3-X8. Antibodies to α6β4 have no influence on ERK1/2 activation. Phosphorylated ERK1/2 bands (P-ERK1/2) are shown above total ERK1/2 protein bands (ERK1/2), and their ratio is quantified in the bar graph below. Mean ± SEM (n = 3) of relative ERK1/2 phosphorylation intensity and one representative experiment is depicted. Assay conditions as in A, except that anti–ERK1/2 or anti–phospho-ERK1/2 antibodies were used.

α6β4 Inhibits Haptotactic Cell Migration via Stimulation of PI3-K

We then tested the role of α6β4 in haptotactic keratinocyte migration, using chemotactic migration as a comparison. Spontaneous haptotactic migration on Ln-5 was readily blocked by antibodies to the integrin subunits α6 (GoH3) or β4 (AA3) (Fig. 4 A). TS2/16-enhanced migration was equally inhibited by antibodies to α6β4, including S3-41, which recognizes the α6β4 heterodimer. This inhibition was induced also by the Fab fragments of S3-41 and AA3, suggesting it did not require α6β4 clustering but simply binding of the antibodies. This α6β4 inhibitory effect was Ln-5 specific since TS2/16 was also able to enhance migration on collagen IV, but in this case it was not inhibited by GoH3 (Fig. 4 A).

In contrast to haptotaxis, EGF-induced chemotactic migration was not affected by GoH3 (Fig. 4 B). If both EGF and TS2/16 were added, GoH3 showed partial inhibition (Fig. 4 B), presumably corresponding to that part of migration that was TS2/16 induced. Thus, α6β4 can influence α3β1 controlled migration, but only when it is haptotactic.

Neither anti–α6β4 antibodies (AA3 and GoH3) had any influence on adhesion to Ln-5, nor did they inhibit the increased adhesiveness induced by TS2/16 (Fig. 4 C). Therefore, like for A3-X8, the observed decrease in migration may be a direct effect of α6β4 on signals regulating motility in HaCaT cells.

Next, we were therefore interested in identifying a candidate signaling molecule responsible for α6β4-linked downmodulation of haptotaxis on Ln-5. PI3-K is one such likely candidate because, in breast carcinoma cells, PI3-K was shown to be activated by the anti–α6 antibody GoH3 (O'Connor et al., 1998). Wortmannin, a PI3-K blocker, abolished inhibition of Ln-5 migration by anti–α6β4 antibodies (data not shown). LY294002, a more stable and specific PI3-K blocker, showed stronger effects (Fig. 5 A) and was preferred in further experiments. This finding suggests that downstream of α6β4, PI3-K may mediate inhibition of α3β1-dependent haptotactic migration. In contrast, a decrease in migration by A3-X8 is not PI3-K dependent since LY294002 did not overcome the inhibitory effect of this antibody (Fig. 5 A).

To confirm that α6β4 is capable of activating PI3-K in HaCaT cells, we used a lipid kinase assay to detect α6β4-induced PI3-K activation. Endogenous PI3-K isolated with

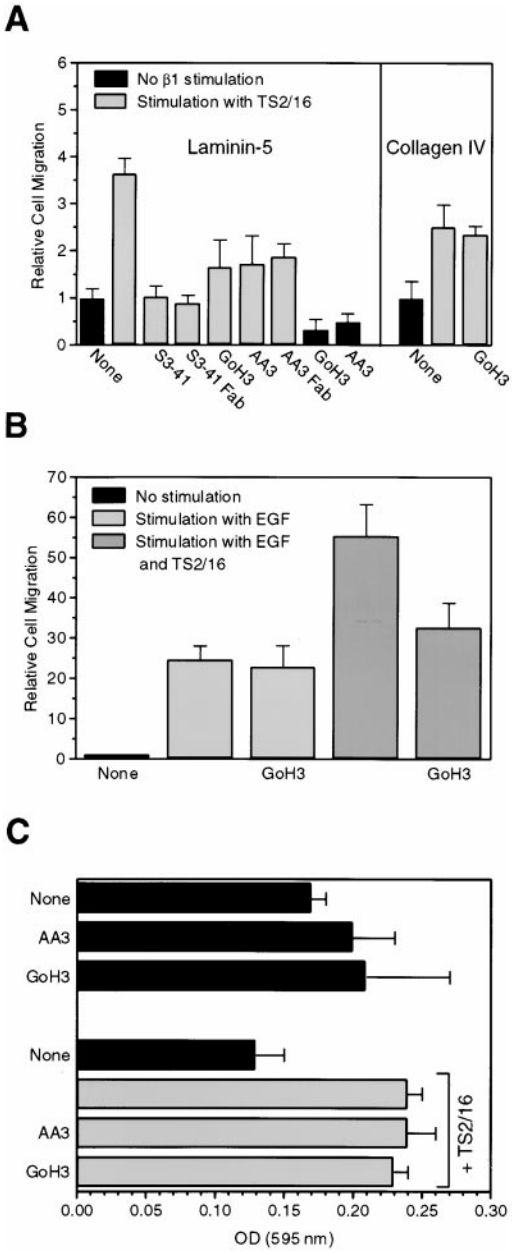

Figure 4. Integrin α6β4 antibodies inhibit α3β1-dependent haptotactic, not chemotactic migration, and have no effect on adhesion in HaCaT cells. (A) HaCaT haptotaxis was stimulated with TS2/16 as described in Fig. 1 A. Where indicated, cells were also pretreated as in Fig. 2 A with S3-41 antibody (anti–α6β4, 40 μg/ml), S3-41 Fab fragments (13.4 μg/ml), GoH3 (anti–α6, 15 μg/ml), AA3 (anti–β4, 40 μg/ml), or AA3 Fab fragments (13.4 μg/ml). Transwell filters were coated with either 0.25 μg/ml Ln-5 or 1 μg/ml collagen IV. Migration assays performed and results expressed as in Fig. 1 A. (B) Anti–α6 antibody GoH3 (15 μg/ml) has no effect on HaCaT chemotactic migration stimulated by EGF (1 ng/ml), but inhibits partially migration of cells stimulated with a combination of EGF and TS2/16 (40 μg/ml). Migration assays performed and results expressed as in Fig. 1 A. (C) Anti–α6β4 antibodies (GoH3, 15 μg/ml, or AA3, 40 μg/ml) have no effect on HaCaT cell adhesion. (Bottom, gray bars) Cells pretreated with TS2/16 in addition to indicated antibodies (as in Fig. 2 C). Antibody treatment of cells, adhesion assays, and result analyses were performed as described in Fig. 2 C.

a p110α-specific antibody showed increased enzymatic activity in cells plated on anti–α6 (GoH3) or anti–β4 (AA3) antibodies, but not on anti–CD151 antibody 1A5, anti–α3 (ASC-1), or anti–β1 (TS2-16) integrin antibodies (Fig. 5 B), suggesting α6β4 integrin-specific PI3-K activation. Production of PI3-P was also increased in cells plated on Ln-5 compared with cells in suspension (Fig. 5 B).

As additional proof that binding of AA3 or GoH3 to α6β4 results in activation of PI3-K, we tested activation of the downstream effector of PI3-K, AKT (Kandel and Hay, 1999). Indeed, phosphorylation of AKT immunoprecipitated from cells plated on Ln-5 was higher than in cells plated on plastic, and was further increased in the presence of AA3 or GoH3 (Fig. 5 C).

These results suggest that, in our system, PI3-K is an effector for α6β4 inhibition of haptotactic migration. To confirm this conclusion, dominant-negative and constitutive-active PI3-K variants were transiently overexpressed in HaCaT cells. To this end, we used a retroviral expression system, since other methods, like calcium phosphate coprecipitation or liposome-mediated transfection, failed because HaCaT cells were not able to migrate after these treatments. In HaCaT cells infected with retrovirus encoding a dominant-negative regulatory subunit p85ΔiSH2-N, migration could still be stimulated by TS2/16. However, inhibition of migration by AA3 was no longer possible (Fig. 6). Thus, the anti–β4 antibody can only act as inhibitor if a functional PI3-K is available in these cells. (Similar results were found with p85ΔiSH2-C and GoH3, respectively, data not shown.) In contrast, overexpression of a constitutive-active catalytic subunit p110myr abolished the stimulatory effect of TS2/16 on migration (Fig. 6).

Taken together, these results support the concept that, in HaCaT cells, α6β4-dependent inhibition of haptotactic migration operates via a class IA PI3-K pathway, with p110α as the responsible catalytic subunit.

PI3-K Inhibits Haptotactic Migration, but Plays a Stimulatory Role in Chemotactic Migration

In apparent disagreement with our findings, PI3-K has been invariably associated with a stimulatory role in migration (Derman et al., 1997; Keely et al., 1997; Gamballetta et al., 2000). To the best of our knowledge, though, most reports referred to chemotactic migration (for review, see Wells, 2000), rather than haptotaxis. Indeed, in our system, PI3-K is involved in stimulating HaCaT cell migration when this is of the chemotactic type, as indicated by inhibition of EGF-induced migration by LY294002 (Fig. 7 A). On the other hand, LY294002 had no effect on haptotactic, TS2/16-induced migration (Fig. 7 B). On collagen IV, another ECM component, HaCaT cells stimulated with TS2/16 behaved exactly as on Ln-5. In summary, PI3-K can play an alternative role in HaCaT motility: if migration is chemotactic, then PI3-K plays a stimulatory role; if migration is haptotactic, then PI3-K plays an inhibitory role.

To ensure that these findings are general to keratinocytes, rather than HaCaT specific, migration experiments were also performed with primary keratinocytes and with A431, an epidermoid squamous carcinoma cell line. Similar to HaCaT, primary keratinocytes showed in-

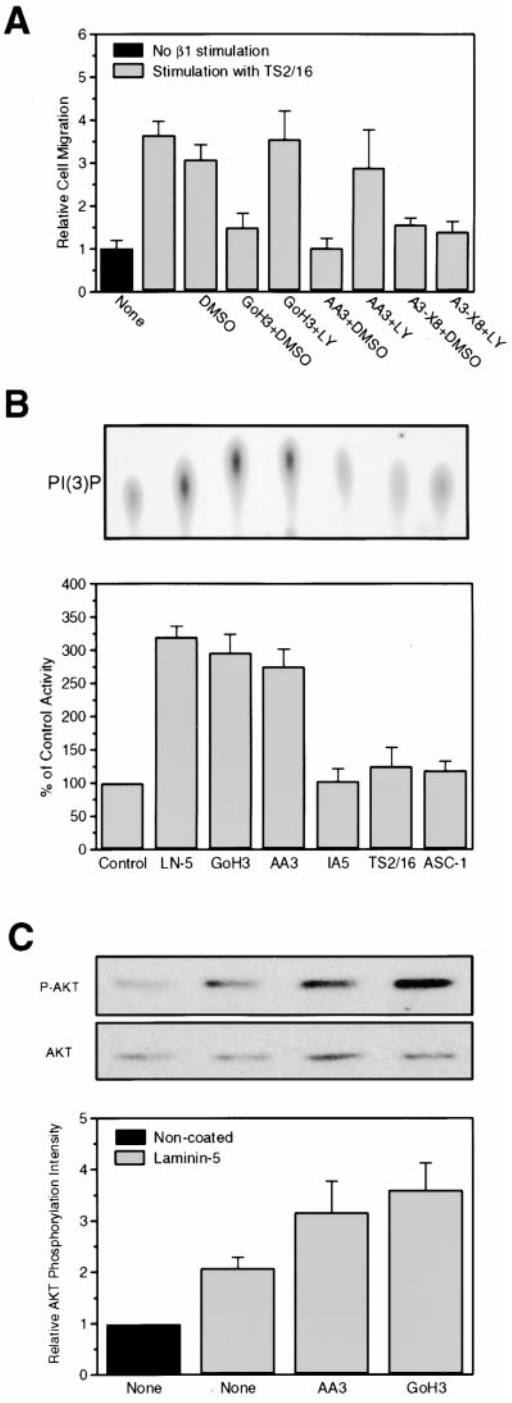

Figure 5. PI3-K blocker LY294002 releases inhibition of HaCaT haptotaxis by anti–α6β4 antibodies. Anti–α6β4 antibodies activate PI3-K in HaCaT cells. (A) Haptotactic migration was stimulated in HaCaT cells by treatment with TS2/16 (as in Fig. 1 A). Where indicated, cells were pretreated with the PI3-K blocker LY294002 (LY) (20 μM) or vehicle (DMSO), GoH3 (anti–α6, 15 μg/ml), AA3 (anti–β4, 40 μg/ml), or A3-X8 (anti–α3, 10 μg/ml). Migration assays performed and results expressed as in Fig. 1 A. (B) PI3-K activity assay. HaCaT cells were allowed to adhere to dishes coated with Ln-5 (1 μg) or indicated antibodies (GoH3, 30 μg; AA3, 50 μg; 1A5, 50 μg; TS2/16, 50 μg; ASC-1, 30 μg) or were kept in suspension (control) for 1 h. Total cell lysates were prepared and equivalent amounts of total proteins were subjected to immunoprecipitation and kinase assay. Phosphorylated lipids were resolved by thin layer chromatography. Mean ± SEM (n = 3 or 4) of percentage of control activity and one representative chromatogram are depicted. PI(3)P, phosphatidylinositol 3-phosphate. (C) AKT phosphorylation. After pretreatment with antibodies (GoH3, 15 μg/ml, or AA3, 40 μg/ml) for 30 min, cells were seeded in dishes coated with Ln-5 (1 μg/dish). After incubation for 30 min at 37°C, total cell lysates were prepared and subjected to immunoprecipitation with an anti–AKT antibody and Western blotting with antibodies to phosphorylated AKT (P-AKT) or AKT (AKT). Mean ± SEM (n = 3) of relative AKT phosphorylation intensity and one representative experiment is shown.

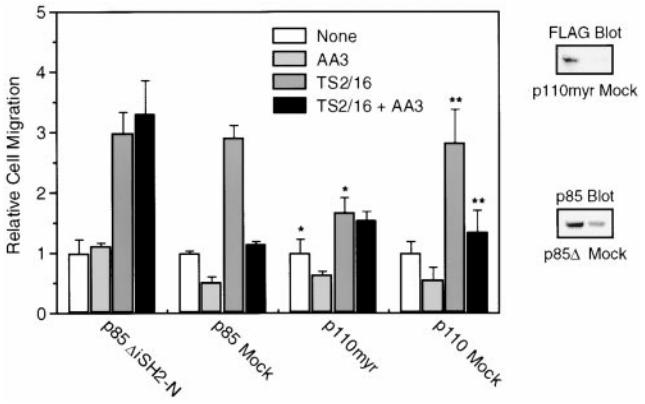

Figure 6. Dominant-negative PI3-K p85 subunit abolishes inhibition of haptotactic migration by α6β4, while constitutive active PI3-K p110 subunit abolishes stimulation of haptotactic migration by TS2/16. HaCaT cells infected with retrovirus encoding a dominant-negative regulatory PI3-K domain (p85 ΔiSH2-N) or a constitutive-active catalytic PI3-K domain (p110myr) or mock infectants were subjected to haptotactic migration assays as described in Fig. 1 A. Data are mean ± range of two duplicate experiments. Nonstimulated cells were set as 1. *Not significantly different values, $P > 0.05$; Student's t test. **Significantly different values, $P < 0.05$. (Right) Expression of infected cDNAs confirmed by immunoblotting of equal amounts of total cell lysates (antibodies indicated above panels).

creased migration in the presence of TS2/16 (Fig. 8 A). This stimulation was inhibited by antitetraspanin antibody 1A5 and by GoH3 (anti–α6). A431 cells showed an ~17-fold higher basal migration than HaCaT (data not shown). Nonetheless, similar to HaCaT, TS2/16 stimulated A431 migration on Ln-5 approximately twofold, and this increase was inhibited by antitetraspanin antibody 1A5, anti–α6β4 integrin antibody S3-41, and by GoH3 (anti–α6) (Fig. 8 B). The α6β4-mediated inhibition of A431 motility also appeared to be linked to a PI3-K pathway since LY294002 abolished it (Fig. 8 B). (Note that the effect of LY294002 could not be analyzed in primary keratinocytes since they did not survive treatment with this reagent.) Furthermore, like HaCaT, A431 migration in the presence of TS2/16 increased on collagen IV (Fig. 8 B).

These results showed that our findings on HaCaT cells are likely to be of general applicability to keratinocytes. We then carried out further investigations on possible links between integrin α6β4 and PI3-K that may inhibit α3β1-dependent haptotactic migration.

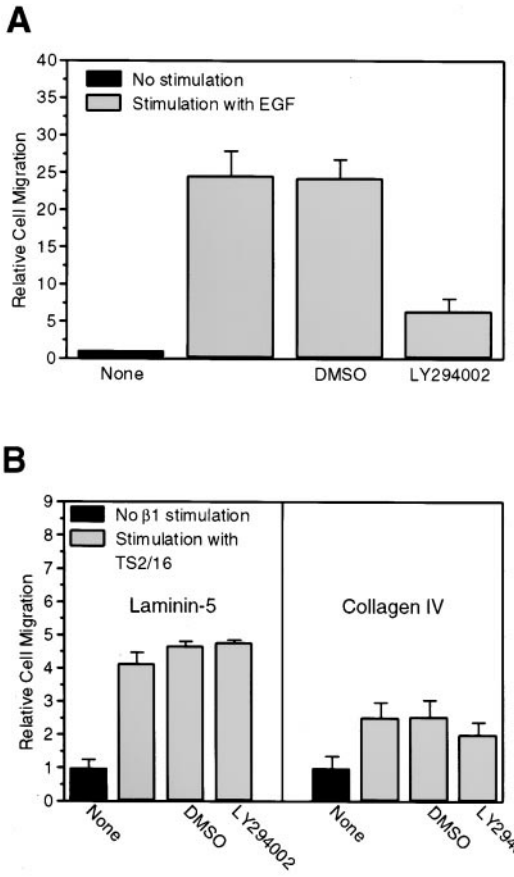

Figure 7. PI3-K blocker LY294002 inhibits chemotaxis but not haptotaxis in HaCaT cells. (A) Chemotactic migration was stimulated with EGF, and cells were pretreated with PI3-K blocker LY294002 or vehicle (DMSO) as in Figs. 1 A and 3 B, respectively. (B) Haptotactic migration was performed as in Fig. 1 A on Transwell filters coated with Ln-5 (0.25 μg/ml) or collagen IV (1 μg/ml). HaCaT cells were treated (gray bars) with TS2/16 (anti–β1, 40 μg/ml) to stimulate haptotaxis. Where indicated, cells were pretreated as in A. Results are expressed as mean ± SD ($n = 3$) of relative cell migration with nonstimulated cells set as 1.

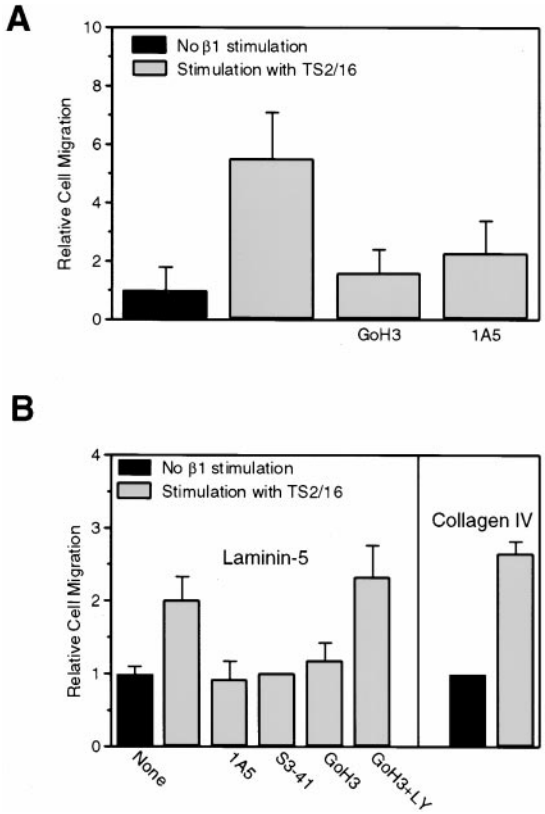

Figure 8. Primary keratinocytes and A431 epidermoid carcinoma cells behave like HaCaT cells: TS2/16 enhances haptotaxis, anti–α6β4 antibodies inhibit TS2/16-induced haptotaxis, and PI3-K blockers abolish the anti-α6β4 antibody-mediated inhibition of haptotaxis. (A) Primary keratinocytes were stimulated to migrate haptotactically with TS2/16 antibody (gray bars) as in Fig. 1 A. Where indicated, they were pretreated with antibodies to α6 (GoH3, 15 μg/ml) or CD151 (1A5, 50 μg/ml). Migration was performed on filters coated with Ln-5 (0.5 μg/ml) and quantified after 15 h at 37°C as in Fig. 1 A. Results pooled from two experiments are expressed as in Fig. 1 A. (B) A431 cells were tested in haptotactic migration assays performed as in Figs. 1 A and 4 A, except that half the number of cells/well was seeded (6×10^5 cells/ml).

erbB-2 May Be a Signaling Link between α6β4 and PI3-K

Class IA PI3-K enzymes are stimulated by receptors with intrinsic protein tyrosine kinase activity (Wymann and Pirola, 1998). Integrin α6β4 has no such activity, but it was shown to be associated with the EGF receptor family member erbB-2 in human mammary and ovarian carcinoma cell lines (Falcioni et al., 1997). This interaction may provide a signaling link between α6β4 and PI3-K. To address this possibility, we first tested whether α6β4 is physically associated with erbB-2 in HaCaT cells. Indeed, in coimmunoprecipitation experiments, erbB-2 was precipitated with the anti–α6 antibody GoH3 and with AA3 (anti–β4), but not with the anti–β1 antibody TS2/16 (Fig. 9 A). In addition, the presence of α6β4/erbB-2 complexes was supported by the fact that an antibody to erbB-2 precipitated integrin subunit α6 (Fig. 9 A).

If erbB-2 is necessary for α6β4-mediated PI3-K activation, stimulation with AA3 or GoH3 should result in erbB-2 autophosphorylation, leading to subsequent recruitment of the PI3-K regulatory domain p85. Phosphorylation of erbB-2 was higher in cells plated on Ln-5 than on uncoated dishes. This effect was further increased when cells were treated with AA3 or GoH3, whereas TS2/16 was without effect (Fig. 9 B). Treatment with GoH3 was also effective in the absence of ECM ligand, as seen in cells stimulated in suspension.

Next, we investigated physical interactions between erbB-2 and PI3-K. Coimmunoprecipitation experiments showed that stimulation with GoH3 leads to increased association of p85 with erbB-2 (Fig. 9 C), providing a means for triggering increased PI3-K activity.

To substantiate the role of erbB-2 in our system, the effect of the erbB-2–specific inhibitor Tyrphostin AG 825 (Tsai et al., 1996) was investigated in migration assays. This compound abolished the inhibitory effect of AA3 and GoH3 on TS2/16-stimulated migration on Ln-5 (Fig. 10 A), indicating that the presence of functional erbB-2 is required for α6β4-mediated inhibition of TS2/16-induced haptotaxis. Finally, the involvement of erbB-2 was also demonstrated by the finding that TS2/16-stimu-

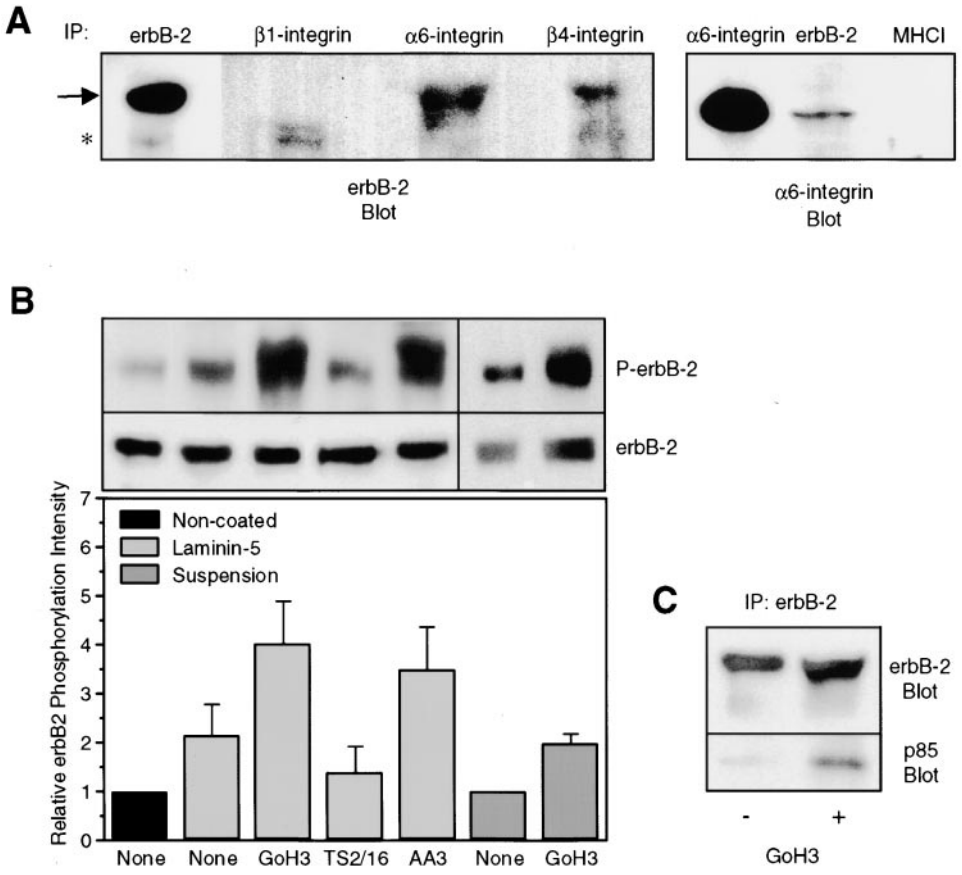

Figure 9. α6β4-associated erbB-2 becomes phosphorylated and binds PI3-K p85 subunit in the presence of anti–α6β4 antibodies. (A) Coimmunoprecipitation of erbB-2 with α6β4. (Left) erbB-2 (arrow) is present in lanes corresponding to HaCaT lysates subjected to immunoprecipitation with antibodies to erbB-2, α6-integrin (GoH3), or β4-integrin (AA3), but not to β1-integrin (TS2/16). Immunocomplexes were analyzed by Western blotting with an antibody to erB-2. *Background band present in all lanes. (Right) α6 reactivity is present in cell lysates subjected to immunoprecipitation with antibodies to erbB-2 or α6-integrin (GoH3), but not with control anti-MHCI antibody W6/32. Immunocomplexes were analyzed by Western blotting with an antibody to the α6-integrin. (B) erbB-2 phosphorylation in response to Ln-5 and α6β4 antibodies (GoH3 and AA3), but not anti–β1 antibody TS2/16. Ratio of phosphorylated bands (P-erbB-2) versus protein bands (erbB-2) is represented in the bar graph below (mean ± SD, $n = 3$). Assay conditions are as in Fig. 3, except that an antibody to erbB-2 was used for immunoprecipitations and Western blots. (C) Coimmunoprecipitation of erbB-2 with p85 PI3-K. The PI3-K subunit p85 is coprecipitated by anti–erbB-2 only in cells that were GoH3 treated. Cells were pretreated with GoH3 (15 μg/ml) for 30 min at 37°C, and then seeded on dishes coated with Ln-5 (1 μg/dish). After incubation for 60 min, total cell lysates were prepared and subjected to immunoprecipitation with an anti–erbB-2 antibody and Western blotting with antibodies to p85 PI3-K or erbB-2. Representative of three experiments.

lated HaCaT cells overexpressing a dominant-negative erbB-2 variant could no longer be blocked with AA3 when migrating on Ln-5 (Fig. 10 B).

In summary, we provide evidence that erbB-2 mediates α6β4-controlled stimulation of PI3-K in HaCaT cells.

Discussion

In this paper, we investigated integrin-dependent signaling that regulates haptotactic migration of keratinocytes on one of their natural substrates, Ln-5. We obtained results that may be useful to understand the haptotactic component of migration in epithelial cells in general (e.g., during tissue remodeling and regeneration) or in BM crossing by transformed epithelial cells.

Our conclusions can be summarized as follows: (a) one of two integrins that bind Ln-5, α3β1, drives haptotactic as well as chemotactic migration of keratinocytes; (b) the other Ln-5–binding integrin, α6β4, inhibits haptotactic, but not chemotactic migration; (c) α6β4 interferes with keratinocyte haptotaxis via stimulation of PI3-K; (d) PI3-K inhibits only haptotactic migration, whereas it has a stimulatory role in chemotactic migration; (e) erbB-2 is a signaling link to PI3-K for the inhibition of α3β1-dependent haptotaxis by α6β4; and (f) the interplay between integrins α3β1 and α6β4 affects migration, but not adhesion.

These conclusions are based on results obtained in Transwell migration assays, in which HaCaT cells showed haptotactic migration spontaneously (to a low level) or after stimulation with the integrin-activating antibody TS2/16 (to a higher level), as well as chemotactic migration after exposure to EGF. In all cases, α3β1 was the integrin-mediating migration as concluded from antibody blocking experiments. Of particular importance to this conclusion were data accumulated with anti–CD151 and A3-X8 antibodies, which interfere with α3β1-dependent migration, not adhesion (Weitzman et al., 1993; Yauch et al., 1998; Testa et al., 1999). Interestingly, we found that A3-X8 antibody may block migration by inhibiting α3β1-dependent ERK stimulation, but not FAK phosphorylation.

The distinction between haptotactic (i.e., controlled by adhesion receptors) and chemotactic (i.e., controlled by growth factor receptors) migration is physiologically relevant, but it is sometimes overlooked. In physiopathological situations such as wound healing and inflammation (Martin, 1997; Wells, 2000), chemotaxis induced by chemokine gradients may dominate. On the other hand, haptotaxis may be more relevant when tumor cells traverse the BM (Damsky and Werb, 1992). In this study, we took

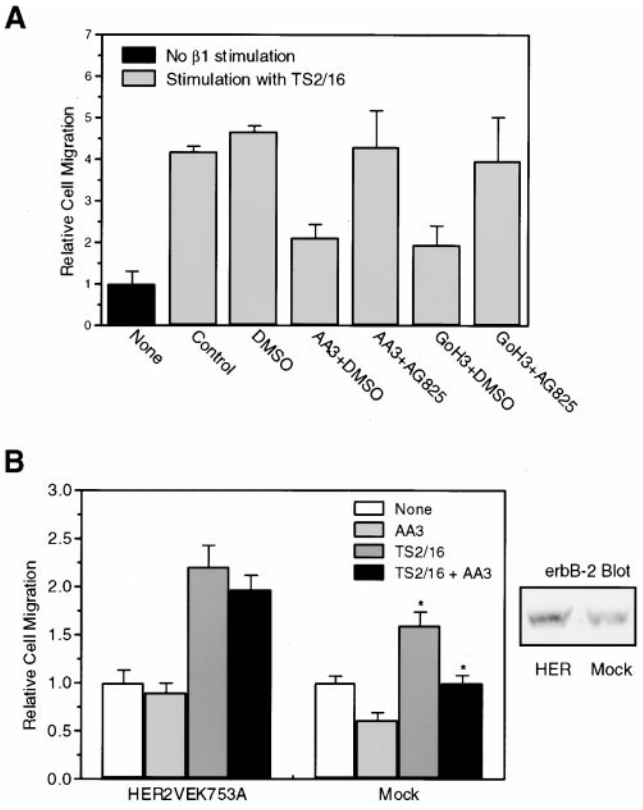

Figure 10. Analyses of erbB-2 as a link between α6β4 and PI3-K for inhibiting α3β1-dependent haptotaxis. (A) HaCaT haptotaxis, induced as in Fig. 1 A, is blocked by anti–α6β4 antibodies, but not in the presence of AG826 (100 nM), a specific erbB-2 inhibitor. Cells were treated with antibodies, AG826 or vehicle (DMSO) as in Fig. 3 B. Assays performed and result analyses as in Fig. 1 A. (B) HaCaT cells infected with retrovirus encoding dominant-negative erbB-2 (HER2VEK753A), but not mock infectants, fail to display inhibition of haptotaxis by anti–α6β4 antibodies. Migration assays with TS2/16 stimulation performed as in Fig. 6. Data shown are mean values ± range of two duplicate experiments, with nonstimulated cells set at 1. *Significantly different values, $P < 0.1$, Student's t test. (Right) Expression of infected cDNA confirmed by immunoblotting of equal amounts of total cell lysates with an anti–erbB-2 antibody.

advantage of the well-known β1 integrin-activating antibody TS2/16 to enhance spontaneous haptotactic migration of keratinocytes on Ln-5 (as well as collagen IV), producing Transwell assay results with much better signal-to-noise ratio. While the exact mechanisms underlying activation of integrins by TS2/16 are not well defined, this antibody presumably functions by inducing changes in integrin shape, stabilizing a conformation that resembles the ligand-bound conformation of the integrin (Humphries, 1996; Bazzoni and Hemler, 1998). Consequently, avidity for ligand is increased, as measured in adhesion assays.

In contrast, in EGF-treated cells, α3β1 did not appear to be activated, as indicated by unchanged avidity in adhesion assays. Nonetheless, EGF-stimulated cells migrated more efficiently than TS2/16-stimulated ones, consistent with the fact that EGF acts at several levels within cells, possibly lowering their threshold for motility (Wells, 2000). Therefore, it appears that integrin activation may be a regulatory step for haptotactic, not chemotactic migration.

Another indication that TS2/16 and EGF induce two distinct types of migration is that they are differentially affected by various inhibitors. In this regard, the difference most relevant to this work, and perhaps keratinocyte biology, concerns sensitivity to anti–α6β4 antibodies. Thus, we found that antibodies to α6β4 inhibited TS2/16-induced migration, but not EGF-stimulated migration. This finding suggests a role for α6β4 in downregulating α3β1-dependent haptotactic migration on Ln-5. Indeed, α6β4 is well known to play a major role in protein complexes called hemidesmosomes, which anchor basal epidermal cells to the underlying basement membrane (Borradori and Sonnenberg, 1999). Therefore, it is not surprising that α6β4 may favor immobilization of cells to the substrate. This seems to be accomplished by inhibiting α3β1-dependent haptotactic migration at the level of signaling rather than by stabilizing adhesion, since anti–α6β4 antibodies did not increase cell adhesion to Ln-5. Furthermore, anti–α6β4 antibodies block migration via a different pathway than A3-X8, because they do not inhibit ERK stimulation.

Recent studies described α6β4 as the integrin-mediating carcinoma cell migration on Ln-1 and in Matrigel invasion assays (Rabinovitz and Mercurio, 1997; Shaw et al., 1997; Gambaletta et al., 2000). These results seemingly contradict our findings, as well as the anchoring role of α6β4 in epidermis. However, in those assays, α6β4-dependent motility was only observed in chemotactic, but not in haptotactic migration assays (O'Connor et al., 1998). Furthermore, it depended strictly upon the overexpression of α6β4 (Shaw et al., 1997; O'Connor et al., 1998; Gambaletta et al., 2000). In our system, we found no evidence that α6β4 supported motility. Instead, keratinocyte migration (either type) on Ln-5 could be entirely accounted for by α3β1. Differences in cell types and ECM substrates may be responsible for these discrepancies, which need to be solved by further studies.

As mentioned above, the interference by α6β4 in α3β1 migration appears to occur at the level of signaling. The phenomenon that occupancy of one integrin, here α6β4, can suppress the function of another integrin, here α3β1, has been observed in several other cell systems and is a concept appreciated as trans-dominant inhibition (Diaz-Gonzalez et al., 1996). For example, anti–αvβ3 antibodies suppress α5β1-dependent phagocytosis (Blystone et al., 1994), ligation of α4β1 inhibits α5β1-dependent expression of metalloproteinases (Huhtala et al., 1995), and α3β1 inhibits fibronectin and collagen IV receptor functions (Hodivala-Dilke et al., 1998). In general, trans-dominant inhibition involves changes in integrin conformation and requires integrin-linked signal transduction cascades (Sastry and Horwitz, 1993; Diaz-Gonzalez et al., 1996, Hughes et al., 1997). It remains to be seen which mechanisms apply to the α6β4-initiated inhibition of α3β1 migration.

As an initial attempt to identify such mechanisms, we began to analyze signaling pathways downstream of α6β4 responsible, in our system, for the inhibition of α3β1 migration. Several lines of evidence implicated PI3-K. First, LY294002, a specific inhibitor of PI3-K, abolished the inhibitory effect of anti–α6β4 antibodies on TS2/16-induced haptotaxis in HaCaT cells, as well as A431. Second, consti-

tutive-active PI3-K prevented TS2/16-induced haptotaxis, and dominant-negative PI3-K prevented inhibition of haptotaxis by anti–α6β4 antibodies. Third, phosphatidylinositol 3-phosphate production by PI3-K, identified as class IA p110α isoform, was exclusively increased in cells stimulated with anti–α6β4 antibodies, whereas anti–α3β1 antibodies had no effect. Fourth, phosphorylation of AKT, a downstream effector of PI3-K (Kandel and Hay, 1999), was increased by anti–α6β4 antibodies in cells plated on Ln-5. Together, these results strongly indicate that PI3-K mediates α6β4-initiated inhibition of HaCaT haptotactic migration on Ln-5.

In recent literature, PI3-K was reported to play a stimulatory role in growth factor–initiated cell migration (for review, see Giancotti and Ruoslahti, 1999; Wells, 2000). How can the same PI3-K enzyme, in the same cell system, mediate both inhibition and stimulation of migration at the same time? This apparent inconsistency may actually not be difficult to account for, because of the complexity and redundancies of the signaling pathways in which PI3-K may be involved (Ren and Schwartz, 1998; Wymann and Pirola, 1998; Rameh and Cantley, 1999; Vanhaesebroeck and Waterfield, 1999; Nebl et al., 2000). Thus, there are many hypothetical possibilities for envisioning PI3-K operating in pathways that have distinct effects on HaCaT migration. Distinct PI3-K isoforms may also be in play (Zhang et al., 1998; Arcaro et al., 2000). Distinguishing among these possibilities will have to await further characterization of PI3-K signal transduction pathways.

We detected association of α6β4 with a class IA PI3-K isoform. Activation of this isoform generally requires translocation to the plasma membrane, mediated by the adapter subunit (50, 55, or 85 kD) that links the p110 catalytic subunit to a cell surface receptor with tyrosine kinase domains (Wymann and Pirola, 1998). Integrin α6β4 has no such kinase domain, but was shown to be physically associated with a receptor tyrosine kinase, erbB-2 or Her2/neu (Falcioni et al., 1997), a member of the EGF receptor family (Hynes and Stern, 1994; Alroy and Yarden, 1997). Here, we demonstrate that binding to Ln-5 induces tyrosine phosphorylation of erbB-2 and that this effect is amplified by anti–α6β4 antibodies. Furthermore, we detected increased association of p85 with this activated erbB-2. Complexing of erbB-2 with p85 has been shown to lead to increased p110 activity (Ram and Ethier, 1996; Olayioye et al., 1998). These data corroborate the idea that erbB-2 may act as a signaling link between α6β4 and PI3-K. Moreover, we also found that blockage of endogenous erbB-2 by a specific inhibitor or by dominant-negative erbB-2 abolished the inhibitory effect of anti–α6β4 antibodies on α3β1-linked haptotaxis. These are independent indications that α6β4 cooperates with erbB-2 to trigger downstream signaling pathways regulating integrin-linked functions.

Recent data on breast cancer cells revealed a stimulatory role for erbB-2 in migration (Spencer et al., 2000) that may superficially seem in conflict with the negative role we describe here. However, there are several critical differences between the study by Spencer et al. (2000) and ours, including the fact that cell migration was stimulated by EGF-related peptides and was therefore chemotactic, while we studied haptotaxis and the nature of the ECM substrate. The difference in cell type may also be relevant. It will be interesting, in future studies, to analyze possible distinctive parameters of erbB-2 signaling in these different migration systems.

Results with Fab fragments suggest that clustering of α6β4 is not required for induction of the downstream signaling we detected. On the other hand, addition of antibodies appeared to amplify signals occurring when α6β4 engages with Ln-5, similar to amplification of FAK and ERK1/2 phosphorylation by antibody TS2/16. The receptor–ligand interactions between integrins and Ln-5 are not well understood at the structural level, and it is therefore difficult to interpret these antibody effects at this time. Nonetheless, it is tempting to propose that antibodies may modify binding of α3β1 or α6β4, respectively, to Ln-5, thus shifting the balance of cellular responses to Ln-5.

Taken together, our data support a model whereby a quiescent cell may be stably attached to Ln-5 via α3β1 and α6β4. Upon selective activation of α3β1, the cell begins to migrate haptotactically over Ln-5 until α6β4 engagement, when stimulation of a PI3-K pathway may slow the cell down. An important issue is, which molecules or structures are responsible for this shift in integrin dominance? Ln-5 is such a candidate itself since it exists in several proteolytically cleaved fragments that can either stimulate or inhibit cell migration (Giannelli et al., 1997; Goldfinger et al., 1998, 1999) and that might bind to α3β1 and α6β4 with different affinities, thereby favoring adhesion or migration. A future challenge is to identify factors that, in vivo, may produce the same effects as TS2/16 on haptotaxis.

In summary, our keratinocyte migration system provides a model for studying signaling pathways that control haptotactic migration in cells that are α6β4 positive. These include epithelial cells from the gastrointestinal tract, the genitourinary tract, and breast gland. Furthermore, α6β4 is overexpressed or expressed de novo in many carcinoma cell types. Further studies are necessary to clarify how α6β4 signaling may relate to α3β1 functions when these cell types come in contact with Ln-5 containing BM. A crucial challenge is to identify signaling molecules downstream of the α6β4/erbB-2/PI3-K complex that are responsible for interference with α3β1 dependent migration.

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