Anchorage Mediated by Integrin α6β4 to Laminin 5 (Epiligrin) Regulates Tyrosine Phosphorylation of a Membrane-associated 80-kD Protein

Yuping Xia,* Susana G. Gil,* and William G. Carter*‡

Departments of *Cell Biology, Fred Hutchinson Cancer Research Center, and ‡Pathobiology, University of Washington, Seattle, Washington 98104

Abstract. Detachment of basal keratinocytes from basement membrane signals a differentiation cascade. Two integrin receptors α6β4 and α3β1 mediate adhesion to laminin 5 (epiligrin), a major extracellular matrix protein in the basement membrane of epidermis. By establishing a low temperature adhesion system at 4°C, we were able to examine the exclusive role of α6β4 in adhesion of human foreskin keratinocyte (HFK) and the colon carcinoma cell LS123. We identified a novel 80-kD membrane-associated protein (p80) that is tyrosine phosphorylated in response to dissociation of α6β4 from laminin 5. The specificity of p80 phosphorylation for laminin 5 and α6β4 was illustrated by the lack of regulation of p80 phosphorylation on collagen, fibronectin, or poly-L-lysine surfaces. We showed that blocking of α3β1 function using inhibitory mAbs, low temperature, or cytochalasin D diminished tyrosine phosphorylation of focal adhesion kinase but not p80 phosphorylation. Therefore, under our assay conditions, p80 phosphorylation is regulated by α6β4, while motility via α3β1 causes phosphorylation of focal adhesion kinase. Consistent with a linkage between p80 dephosphorylation and α6β4 anchorage to laminin 5, we found that phosphatase inhibitor sodium vanadate, which blocked the p80 dephosphorylation, prevented the α6β4-dependent cell anchorage to laminin 5 at 4°C. In contrast, adhesion at 37°C via α3β1 was unaffected. Furthermore, by in vitro kinase assay, we identified a kinase activity for p80 phosphorylation in suspended HFKs but not in attached cells. The kinase activity, α6β4, and its associated adhesion structure stable anchoring contacts were all cofractionated in the Triton-insoluble cell fraction that lacks α3β1. Thus, regulation of p80 phosphorylation, through the activities of p80 kinase and phosphatase, correlates with α6β4-SAC anchorage to laminin 5 at 4°C in epithelial cells of the skin and intestine. Transmembrane signaling through p80 is an early tyrosine phosphorylation event responsive to and possibly required for anchorage to laminin 5 by HFK and LS123 epithelial cells.

CELL interactions with extracellular matrix (ECM)[1] are mediated by the integrin family of cell adhesion receptors (Hynes, 1992). Integrin interactions with ECM have profound effects on several aspects of epithelial cell biology (Juliano and Haskill, 1993; Damsky and Werb, 1992; Lin and Bissell, 1993; Hay, 1993; Carter et al., 1994). Wounding of the epidermis activates basal cells from an anchored phenotype to a dynamic motile state (Grinnell, 1992; Falanga et al., 1994). Detachment of epidermal basal cells from the basement membrane initiates a differentiation cascade and signals cell movement through the multiple stratified cell layers (Adams and Watt, 1993). Detachment from laminin 5 has been shown to regulate differentiation (Symington and Carter, 1995; Gil et al., 1994). Furthermore, changes in tyrosine phosphorylation accompany keratinocyte differentiation (Filvaroff et al., 1991, 1992; Zhao et al., 1992).

Basal keratinocytes are the least differentiated cells in the epidermis and are the only epidermal cells capable of self-renewal (for review see Adams and Watt, 1993; Fuchs, 1990; Lin and Bissell, 1993; Hay, 1993; Carter et al., 1994). Three major integrins, α2β1, α3β1, and α6β4, are expressed in the normal basal cell layer (Carter et al., 1990*a*, *b*; Wayner et al., 1988; Adams and Watt, 1993). The latter two receptors are involved in the attachment of the basal cells to laminin 5, a major basement membrane adhesive ligand synthesized by epithelial cells from several different

Address all correspondence to W.G. Carter, Department of Pathobiology, University of Washington, 1124 Columbia St., Seattle, WA 98104. Tel.: (206) 667-4478. Fax: (206) 667-6877.

1. *Abbreviations used in this paper*: BPA II, bullous pemphigoid antigen II; ECM, extracellular matrix; FA, focal adhesion; HD, hemidesmosome; HFF, human foreskin fibroblast; HFK, human foreskin keratinocyte; HFM, human foreskin melanocyte; KGM, keratinocyte growing medium; pp125^{FAK}, focal adhesion kinase; SAC, stable anchoring contact.

tissues, including intestine, lung, and epidermis (Carter et al., 1991; Sonnenberg et al., 1993; Gil et al., 1995[2]). Laminin 5 is also referred to as epiligrin (Gil et al., 1994; Ryan et al., 1994), and is indistinguishable from proteins termed BM600/GB-3 antigen/Nicein (Domloge-Hultsch et al., 1992; Verrando et al., 1987, 1991) and kalinin (Rousselle et al., 1991). The integrin receptors α3β1 and α6β4 bind to laminin 5 via two different cell junctions (Carter et al., 1990*a*, *b*, 1991; Wayner et al., 1993; Sonnenberg, 1993; Gil et al., 1995[2]): α3β1-focal adhesions (FA) (for review see Burridge et al., 1992), and α6β4-hemidesmosomes in tissue (HD) (for review see Jones and Green, 1991), or hemidesmosome-like stable anchoring contacts (SAC) in cell culture (Carter et al., 1990*a*).

Integrins α6β4- and α3β1-mediated interactions with laminin 5 are physiologically relevant adhesion events. These two adhesion receptors have functionally distinct roles in the epidermis. In homeostatic epidermis, HDs are the predominant adhesion structures associated with cytoplasmic intermediate filaments and use α6β4 as the adhesion receptor (Stepp et al., 1990; Carter et al., 1990*a*; Kurpakus et al., 1991; Sonnenberg et al., 1991; Jones et al., 1991). Inherited defects in expression of either laminin 5 or β4 result in insufficient HD formation, which causes severe blistering of the epidermis (Gil et al., 1994). In contrast, motile human foreskin keratinocytes (HFK) in wounded epidermis upregulate the synthesis of laminin 5 (Gil et al., 1994; Ryan et al., 1994) and use α3β1 for interaction with laminin 5 via actin-containing FAs (Carter et al., 1991; Ryan et al., 1994; Gil et al., 1995[2]).

Recent studies suggest that integrins transduce extracellular signals across the plasma membrane in addition to providing a mechanical function in cell adhesion (Hynes, 1992; Damsky and Werb, 1992; Miyamoto et al., 1995). Integrin binding to its extracellular ligand modulates intracellular pH (Ingber et al., 1990), increases protein tyrosine phosphorylation (Kornberg et al., 1992), and induces gene expression in fibroblasts (Werb et al., 1989), monocytes (Sporn et al., 1990; Lin et al., 1994), and mammary epithelial cells (Streuli et al., 1991). The identification of focal adhesion kinase (pp125^{FAK}) (Guan et al., 1991; Kornberg et al., 1991; Shaller et al., 1992) provided the first evidence for the activation of an intracellular signaling molecule by integrins. The pp125^{FAK} becomes phosphorylated on tyrosine residues in the process of FA formation. The phosphorylation is regulated by β1-containing integrins. Several other known kinases are also activated in this process, including Src kinase, phosphatidylinositol-3 kinase (PI3K), MAP kinase, and Csk (for review see Zachary and Rozengurt, 1992; Schaller and Parsons, 1993; Clark and Brugge, 1995). The possible role of α6β4 in intracellular signaling was clarified in a recent report on signal transduction associated with α6β4. Mainiero et al. (1995) demonstrated the interaction of the integrin β4 subunit with the known signaling molecules Shc and Grb 2, which potentially links α6β4 to the *ras* pathway.

We distinguished the anchorage activity (cell adhesion without spreading or migration) of α6β4 from the motility function of α3β1 and other β1 integrins by cell adhesion to laminin 5 at 4°C (Gil et al., 1995[2]). We described, in this paper, a novel 80-kD phosphoprotein (p80) that is specifically regulated by α6β4-mediated anchorage to laminin 5. At 4°C, the function of β1 integrins is suppressed, leaving α6β4 as the primary adhesion receptor for laminin 5 (see Fig. 1). In addition, we observed that treatment of HFKs with the phosphatase inhibitor sodium vanadate blocked α6β4-mediated adhesion to laminin 5 at 4°C, but not the adhesion at 37°C via α3β1. We established an in vitro kinase assay to demonstrate the association of p80 kinase with α6β4. We found that the kinase activity and α6β4-SAC, but not α3β1-FA, are both present in the Triton-insoluble fraction of suspended HFKs.

Materials and Methods

Cells and Cell Culture

Normal neonatal HFKs and human foreskin melanocytes (HFM) were prepared as described by Boyce and Ham (1985). HFKs were maintained in serum-free keratinocyte growing medium (KGM) (Clonetics Corp., San Diego, CA) containing insulin, EGF (0.1 ng/ml), hydrocortisone (0.5 mg/ml), and bovine pituitary extract (~50 μg/ml of protein). HFMs were grown in melanocyte growth medium (Clonetics Corp.). Human colon carcinoma cell lines Caco-2 and LS123 were purchased from American Type Culture Collection (Rockville, MD). They were maintained in KGM with 10% FBS and RPMI with 10% FBS, respectively. FEPE1L8 and FEP1811-T1 are HFKs that have been transfected with transforming genes E6 and E7 from human papilloma virus 16 (Kaur et al., 1989) and were maintained in KGM. Primary cultures of human foreskin fibroblasts (HFF) were prepared by collagenase digestion of dermis from neonatal foreskins and were grown in RPMI 1640 containing 10% FBS. HT1080 human fibrosarcoma cells were grown in RPMI containing 10% FBS.

Antibodies

mAbs to integrin receptor α3β1 (P1B5), α2 (P1H5), and β1 (P4C10) have been described (Wayner and Carter, 1987; Wayner et al., 1988; Carter et al., 1990*a*, *b*). C2-9 is an inhibitory mAb against laminin 5 (Gil et al., 1994; Gil et al., 1995[2]), and P3E4 is an mAb against laminin 5 (Wayner et al., 1993). Rat mAb anti-α6 (GoH3) was a generous gift from Dr. A. Sonnenberg, (The Netherlands Cancer Institute, Amsterdam, The Netherlands) and is also commercially available from Amac, Inc. (Westbrook, ME). mAb to integrin β4 subunit, Um-A9, was a generous gift from Dr. Carter Van Waes (University of Michigan, Ann Arbor). Antiphosphotyrosine mouse mAb Py20 was obtained from ICN Pharmaceuticals, Inc. (Irvine, CA). Rabbit anti–mouse IgG and rabbit anti–mouse IgG–conjugated peroxidase were obtained from Zymed Laboratories, Inc. (South San Francisco, CA) and Dako, Corp. (Santa Barbara, CA), respectively. Biotin-conjugated antiphosphotyrosine mAb 4G10, mAb anti-PI3K, and anti-pp125^{FAK} mAbs were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). mAb to plakoglobin was generously provided by Dr. Pam Cowin (New York University).

Laminin 5 ECM Ligands

Laminin 5 ECM was prepared as follows: subconfluent HFK cultures were grown for 2–3 d in KGM on tissue culture plastic dishes. The cells were removed from their matrix by 5–10-min treatment with 0.05% trypsin (No. 840-7073IM; Gibco Laboratories, Grand Island, NY) and 1 mM EDTA in PBS. The laminin 5 ECM was then treated with 0.1 mg/ml soybean trypsin inhibitor, blocked with 1 mg/ml BSA in PBS, and used for cell adhesion studies. Laminin 5 is the major adhesive ligand present on these plastic surfaces. The possible contribution of other ligands to cell adhesion on laminin 5 ECM was evaluated and eliminated by inhibition studies with anti–laminin 5 mAbs (Gil et al., 1995[2]). The laminin 5 ECM prepared in this way was more effective at inducing cell adhesion than surfaces prepared by differential extraction or by coating with purified laminin 5 (Carter et al., 1991). Plasma fibronectin and collagen type I were prepared as described (Wayner and Carter, 1987), and each was used at 10 μg/ml in PBS. Poly-L-lysine was purchased from Sigma Chemical Co. (St. Louis, MO), and used at 0.5 mg/ml.

2. Gil, S.G., Y.-P. Xia, and W.G. Carter, manuscript submitted for publication.

Adhesion Surface Coated with Purified Laminin 5

Purified laminin 5 surface is prepared by first coating tissue culture dishes with affinity-purified anti-laminin 5 antibody P3E4 (2 μg/ml) (Wayner et al., 1993) overnight at 4°C, followed by incubation with heat-denatured BSA (0.5 mg/ml) to block uncoated area. Conditioned culture supernatant collected from HFKs was used as source of laminin 5 and added to the immobilized laminin 5–specific antibody. The adhesion surface was washed three times with PBS to remove unbound proteins.

ECM Ligands Immobilized on Culture Dish

Petri dishes were coated at 4°C with fibronectin (10 μg/ml in PBS), collagen type I (10 μg/ml in PBS), or poly-L-lysine (0.5 mg/ml in PBS) overnight. Plates were washed three times with PBS to remove unbound ligands and blocked with 0.25% heat-denatured BSA in PBS solution overnight at 4°C.

Immunofluorescence Staining

Trypsin-suspended cells were either extracted in suspension with 1% vol/vol Triton X-100 for 10 min and fixed with 2% formaldehyde in 0.1 M sodium cacodylate, 0.1 M sucrose, pH 7.2, for 20 min, or fixed before the treatment with Triton X-100 and then treated with 1 mM $NaIO_4$ for 5 min at 4°C. Cells treated under these conditions were attached to coverslips that were coated with polyacrylhydrazido-agarose, washed with PBS, blocked with 1% wt/vol heat-denatured BSA, and stained with the indicated antibodies. The coverslips were then incubated with dilutions of affinity-purified, species-specific, FITC-conjugated secondary antibodies, washed with PBS, and mounted with 25 mg/ml 1,4-diazobicyclo-(2,2,2)-octane in glycerol, pH 8.6. Cells were examined with a fluorescence microscope (Zeiss, Inc., Thornwood, NY) equipped with a narrow band FITC filter using an ×63 oil Neofluor antiflex objective.

Cell Adhesion Assays

Cell adhesion assays were performed as previously described (Wayner and Carter, 1987; Wayner et al., 1988; Carter et al., 1990*a*, 1991). Briefly, 24- or 48-well tissue culture plates coated with purified laminin 5 were blocked with 0.25% heat-denatured BSA to minimize nonspecific bindings. Cells were labeled with $Na_2{}^{51}CrO_4$ (50 μCi/ml for 2–4 h), washed, and preincubated with the inhibitory antibodies for 15 min before and during the adhesion at either 37°C or 4°C. The nonadherent cells were removed by washing with PBS, and adherent cells were fixed with 2% formaldehyde, 100 mM sodium cacodylate, 100 mM sucrose, pH 7.2, for 20 min. Subsequently, the adherent labeled cells were dissolved in 0.5% SDS, 0.25 N NaOH, and radioactivity quantified in a gamma counter.

Cell Extracts and Immunoblotting

Cells were rinsed twice with PBS before lysis. Protein extracts were made by incubating cells with lysis buffer (1% vol/vol Triton X-100, 1 mM PMSF, 2 mM *N*-ethyl maleimide, 1 mM sodium vanadate in PBS) on ice for 15 min, followed by brief vortexing. Samples were microcentrifuged at 4°C, and the clear supernatants were collected for subsequent analysis. Protein extracts were fractionated by SDS-PAGE (Laemmli, 1970), transferred to nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH), and immunoblotted with antiphosphotyrosine mAb.

Subcellular Fractionation

Cells were homogenized with a tight-fitting dounce homogenizer in 2 M urea in PBS. Cell lysates were ultracentrifuged at 100,000 *g* to separate the cytosolic fraction from the insoluble pellet. Membrane components were solubilized by suspending the pellet with buffer containing 1% Triton X-114 detergent in PBS at 4°C and centrifuged to remove the insoluble nuclear and cytoskeletal components. The detergent-soluble extracts were fractionated by phase partitioning at 37°C (Bordier, 1981). The temperature increase results in formation of a detergent phase that contains membrane components with dominant hydrophobic character and an aqueous phase with dominant hydrophilic character. The two phases were separated by centrifugation at room temperature.

Immunoprecipitation

Protein extracts were incubated at 4°C with primary antibody overnight with rocking. Rabbit anti–mouse IgG conjugated with protein A agarose was used as secondary antibody and incubated with antigen–primary antibody complex at 4°C overnight. The immune complex was washed with buffer (20 mM Tris-HCl, pH 7.5, 400 mM NaCl, 1 mM EDTA, 0.5% NP40, 1 mM PMSF, and 2 mM *N*-ethyl maleimide) three times. Antigen was dissociated from the protein A–agarose beads by boiling for 5 min in Laemmli sample buffer.

In Vitro Kinase Assay

Adherent or suspended HFKs were extracted in lysis buffer (0.5% Triton X-100, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM sodium vanadate, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin). Cell lysate was used directly in the kinase reaction containing 10 μCi of [γ-^{32}P]ATP (10 Ci/mmol; DuPont Instruments, Wilmington, DE), 25 mM Tris-HCl, pH 7.2, 1 mM Na_3VO_4, and 10 mM magnesium acetate. The kinase reaction was incubated for 20 min at 30°C and terminated by adding SDS sample buffer to the reactions. The labeled proteins were electrophoresed on SDS-PAGE and visualized by autoradiography.

Results

HFKs Use Both α6β4 and α3β1 for Adhesion to Laminin 5

The distinct function of α3β1 in mediating HFK motility and α6β4 in mediating anchorage is summarized in Fig. 1 (point A in legend). We have previously shown that laminin 5 adhesion surfaces prepared from either laminin 5 ECM or purified soluble laminin 5 provide comparable adhesion results (Gil et al., 1995[2]). For the following adhesion studies, we have used laminin 5 surfaces prepared with both protocols yielding similar adhesion and signaling results.

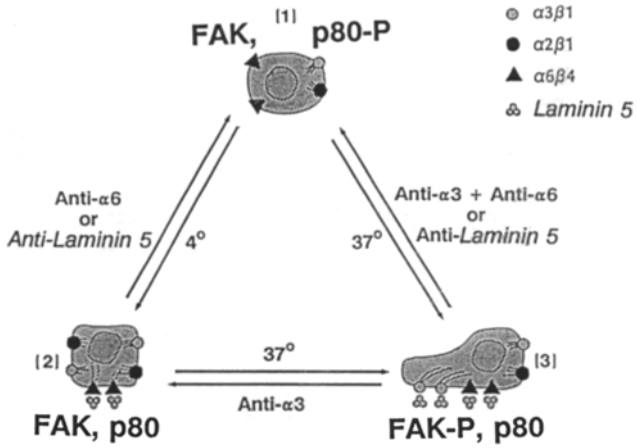


Figure 1. Integrins α6β4 and α3β1 mediate anchorage and motility on laminin 5, respectively, and induce distinct signaling events. (Point A) Suspended HFKs (*1*) interact with laminin 5 via α3β1 for motility (attachment, spreading, and migration) and α6β4 for anchorage at 37°C (*3*). At 4°C, HFKs attach to laminin 5 via α6β4 without spreading (*2*). This adhesion can be blocked with anti-α6 or -β4 antibody. Adhesion at either temperature is blocked with anti–laminin 5 mAbs. 4°C-adherent HFKs (*2*) spread via α3β1 when warmed to 37°C (*3*). This spreading is blocked by anti-α3β1. (Point B) HFKs in suspension (*1*) express tyrosine-phosphorylated p80 and dephosphorylated pp125^{FAK} (*FAK*). Adhesion to laminin 5 at 4°C via α6β4 results in dephosphorylation of p80 (*2*). Adhesion to laminin 5 at 37°C via both α6β4 in SACs and α3β1 in FAs (*3*) results in dephosphorylation of p80 and phosphorylation of pp125^{FAK} (*FAK-P*). Warming of cells attached at 4°C (*2*) to 37°C (*3*) results in cell spreading via α3β1 and phosphorylation of pp125^{FAK} (*FAK-P*).

Integrins that are involved in HFK adhesion to laminin 5 ECM were examined by inhibiting the function of a specific receptor with inhibitory mAbs (Fig. 2). HFKs attached but did not spread on laminin 5 ECM in the presence of anti-α3β1 mAb P1B5 (Fig. 2 *B*). These data showed that α3β1 mediated spreading on laminin 5 ECM and that an additional receptor was also involved in attachment. Anti-α6 antibody GoH3 (Fig. 2 *C*) alone had no obvious effect on either attachment or spreading. However, the combination of anti-α3β1 and anti-α6 completely blocked attachment and spreading (Fig. 2 *D*). Also anti–laminin 5 mAb, C2-9, completely inhibited adhesion and spreading on the ligand (Gil et al., 1995[2]). These data indicate that both integrin receptors are involved in HFK attachment to laminin 5.

Separation of α3β1 and α6β4 Functions in Adhesion to Laminin 5

Formation of focal adhesion via α3β1 is an energy-dependent process. In contrast, adhesion to laminin 5 mediated by α6β4 takes place at 4°C (Gil et al., 1995[2]). To separate HFK adhesion via α3β1 in actin-associated attachments from adhesion via α6β4 in SACs, we compared cell adhesion at both 37 and 4°C. We used laminin 5 that was purified by trapping the protein onto a surface coated with immunopurified laminin 5–specific antibody. HFKs anchored to laminin 5 at 4°C but did not spread. This adhesion was inhibited to basal level by mAb anti-α6, but not by anti-α3β1 (Fig. 3, *white bars*). This result indicated that HFK adhesion to laminin 5 at 4°C was mediated by α6β4 alone. In a confirmatory experiment, anti-β4 antibody Um-A9 was incubated with HFKs, and the adhesion of HFKs to laminin 5 was also inhibited. Adhesion at 37°C was inhibited to a basal level by the combined effect of anti-α6 and anti-α3β1. Incubating HFKs with anti–laminin 5 mAb blocked cell adhesion at both temperatures. In addition, none of the antibodies against receptors for collagen or fibronectin inhibited the adhesion to laminin 5 (Gil et al., 1995[2]). Cell spreading was inhibited by either reducing the temperature to 4°C or by treatment with anti-α3β1 mAb at 37°C, consistent with the role of α3β1 in cell spreading at 37°C and lack of function at 4°C. In a control experiment, mouse Ig showed no inhibition of HFK adhesion to laminin 5 at either temperature. Adhesion of HFKs to fibronectin was prevented with anti-β1 (P4C10) or anti-α5 (P1D6) antibodies but not with any of the mAbs against α3, α6, α2, or laminin 5, which showed the specificity of these mAbs (data not shown). From these studies we concluded that anchorage of HFKs to purified laminin 5 at 4°C is mediated by α6β4, while adhesion at 37°C is dependent on both α6β4 and α3β1, the latter also mediating spreading. Identical adhesion results were observed at both 37 and 4°C with laminin 5 ECM.

Tyrosine Phosphorylation of Proteins in Attached or Detached HFKs

Since integrins α6β4 and α3β1 are two functionally different receptors interacting with laminin 5, we investigated whether there are different signaling processes that are activated by each of the receptors. We examined tyrosine phosphorylation events that are regulated by HFK interaction with laminin 5. HFKs were detached by trypsin/EDTA treatment. Protein extracts were prepared from 37°C-adherent or trypsin-suspended cells, fractionated by

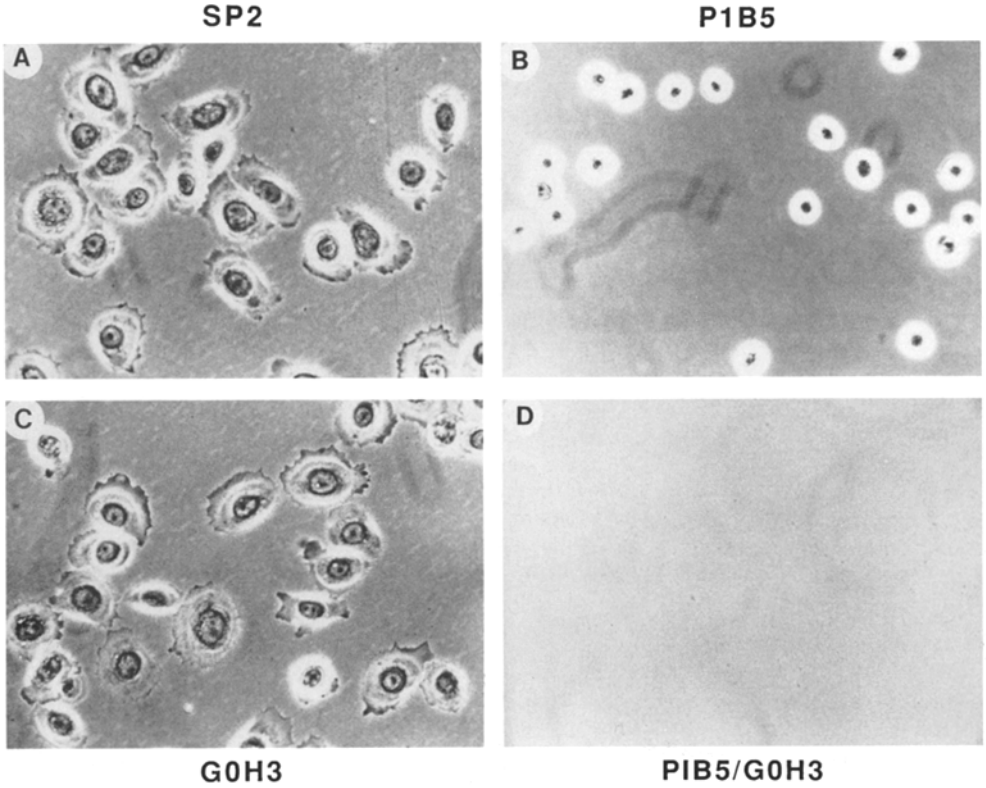


Figure 2. Inhibition of HFK adhesion and spreading on laminin 5 ECM. HFKs were attached on laminin 5 ECM in the presence of (*A*) *SP2*, a conditioned culture supernatant from Fox-NY myeloma cells, (*B*) an inhibitory antibody to α3β1 (*P1B5*), (*C*) α6 (*GoH3*), and (*D*) combined anti-α3β1 and anti-α6 (*P1B5/GoH3*). Each antibody was diluted to 2.5 μg/ml in culture medium KGM. Cells were incubated with the antibodies at 37°C for 1 h, rinsed with PBS to remove nonadherent cells, fixed, and photographed under a light microscope. HFKs attached but did not spread in the presence of anti-α3β1 (*B*). Combined anti-α3β1 and anti-α6 blocked cell adhesion (*D*). Anti-α6 (*GOH3*) had no obvious effect on adhesion or spreading (*C*).

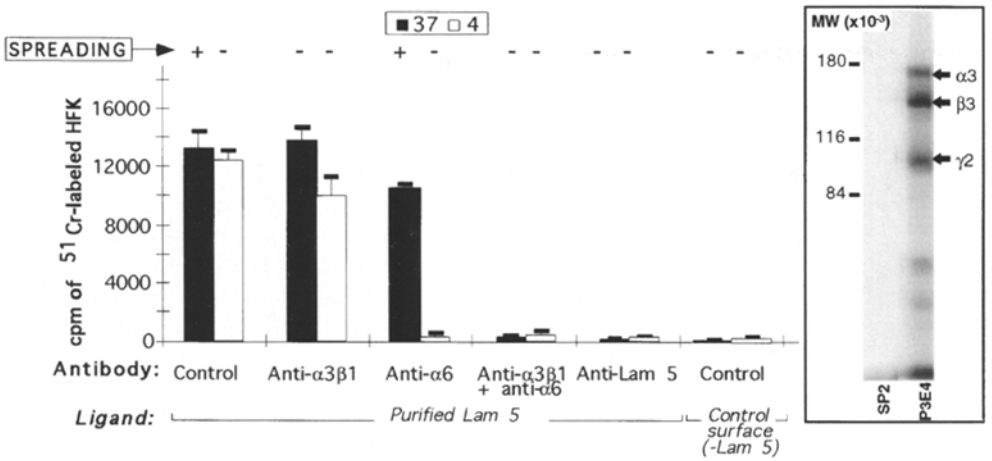


Figure 3. Inhibition of HFK adhesion to purified laminin 5 at 4°C and 37°C by inhibitory mAbs. Adhesion of ^{51}Cr-labeled HFKs to laminin 5, purified by trapping with laminin 5–specific antibody P3E4, was performed in the presence of mAbs at 37°C (*black bars*) or at 4°C (*white bars*). ^{51}Cr-labeled HFKs were suspended by trypsinization and incubated with mAbs before plating onto purified laminin 5 or control surface (tissue culture plate coated with P3E4 unconjugated to laminin 5). The mAbs used were mouse γ-globulins as a control, anti-α3β1 (P1B5), anti-α6 (GoH3), and anti–laminin 5 (C2-9). Each bar, average of three assays. Error bars, SD (n = 3). Adhesion, cpm of the labeled adherent cells. The presence/absence of cell spreading, positive (+) or negative (−) signs, respectively. At 37°C, HFK attachment to laminin 5 is blocked with anti–laminin 5 mAb or the combined antibodies of anti-α3β1 (P1B5) and anti-α6 (GoH3). Spreading but not attachment is inhibited by anti-α3β1. At 4°C, HFK adhesion is inhibited by either anti–laminin 5 or anti-α6 mAbs. (*Inset*) Immunoprecipitation of culture supernatant from [^{35}S]methionine-labeled HFK with laminin 5–specific antibody *P3E4* (lane *2*) and *SP2*, conditioned culture supernatant from Fox-NY myeloma cells (lane *1*).

SDS-PAGE, and immunoblotted with antiphosphotyrosine mAb Py20 (Fig. 4 *A*). In adherent HFKs, a protein that has been identified previously as pp125^{FAK} appeared as the major phosphoprotein (Fig. 4 *A*, lane *1*). The identity of pp125^{FAK} was confirmed by immunoprecipitation with anti-pp125^{FAK} antibody and immunoblotting with biotinylated antiphosphotyrosine mAb 4G10 (Fig. 4 *A*, lanes *3* and *4*). The phosphorylated pp125^{FAK} could only be detected in adherent HFKs (Fig. 4 *A*, lane *3*), and it migrated to the same position as the 125-kD protein detected by antiphosphotyrosine mAb Py20 (Fig. 4 *A*, lane *1*). Detachment of HFKs caused dephosphorylation of pp125^{FAK} to background levels (Fig. 4 *A*, lanes *2* and *4*). The intriguing finding is that detachment also induced tyrosine phosphorylation of an 80-kD (p80) protein. Tyrosine-phosphorylated p80 was the major tyrosine phosphoprotein in detached HFKs (Fig. 4 *A*, lane *2*). An identical result was also observed with purified laminin 5 (Fig. 4 *A*, lanes *5* and *6*). In parallel studies, we immunoprecipitated p80 from [^{35}S]methionine-labeled HFK cells with antiphosphotyrosine antibody Py20. It showed that phosphorylated p80 exists only in detached cells (Fig. 4 *B*, lane *2*). Reattachment of HFKs to laminin 5 reversibly induced dephosphorylation of p80 and rephosphorylation of pp125^{FAK} (data not shown).

To eliminate the possibility that the appearance of phosphorylated p80 was caused by trypsin digestion of a precursor protein or due to mitogenic stimulation by trypsin rather than caused by changes in cell–substrate adhesion, we examined the kinetics of trypsin/EDTA–induced detachment from the substratum (Fig. 5 *A*). Attached HFKs were digested with trypsin for different time periods at room temperature. In the first 8 min of trypsin incubation, the spread HFKs rounded up but remained adherent. The rounded HFKs detached after ~12 min of digestion. Rounded but attached HFKs did not have phosphorylated p80 (Fig. 5 *A*, lanes *1–5*). However, decreased pp125^{FAK} phosphorylation was observed at the end of 8 min (Fig. 5 *A*, lane *5*). This is consistent with the disruption of β1-dependent adhesion structures that control the adhesion, spreading, and migration of pp125^{FAK}, and regulate its phosphorylation (Kornberg et al., 1991). In contrast, p80

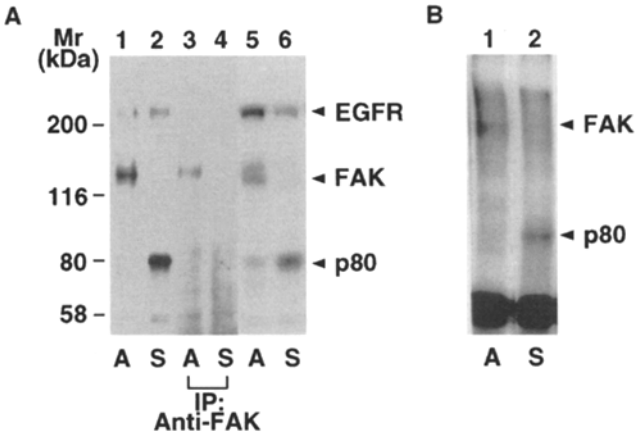


Figure 4. Tyrosine phosphorylation of proteins in adherent and suspended HFKs. Subconfluent HFKs plated onto laminin 5 ECM (*A*, lanes *1–4*; *B*, lanes *1* and *2*) or purified laminin 5 (*A*, lanes *5* and *6*) were either extracted directly as adherent cells (*A*) with 1% Triton X-100 in PBS (*A*, lanes *1*, *3*, and *5*; *B*, lane *1*) or detached from matrix (*S*) by trypsin digestion (*A*, lanes *2*, *4*, and *6*; *B*, lane *2*) before detergent extraction. Equal amounts of each extract were loaded and fractionated by SDS-PAGE. (*A*) Phosphotyrosine-containing proteins were detected by immunoblotting with phosphotyrosine-specific mAb, Py20. In lanes *3* and *4*, cell extracts were immunoprecipitated with anti-pp125^{FAK} mAb (*IP: Anti-FAK*) before immunoblotting with biotinylated 4G10 mAb, an antiphosphotyrosine mAb. The biotin was detected with peroxidase-conjugated strepavidin. (*B*, lanes *1* and *2*) Autoradiographs of [^{35}S]methionine-labeled HFKs immunoprecipitated with Py20. (*Arrowheads*) The positions of EGF receptor (*EGFR*), pp125^{FAK} (*FAK*), and p80. Relative molecular weight markers are indicated in the left margin.

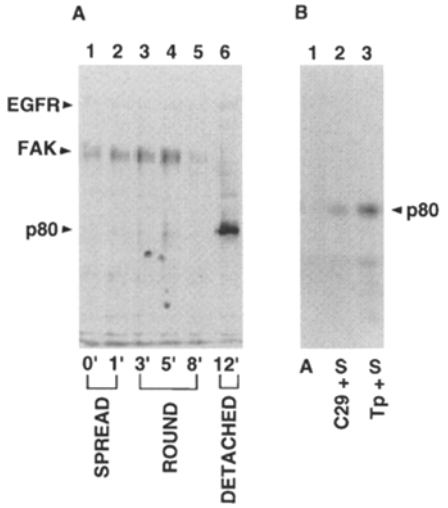


Figure 5. Disruption of HFK adhesion to laminin 5 by trypsin digestion or treatment with inhibitory anti–laminin 5 mAb (C2-9) induces tyrosine phosphorylation of p80. (*A*) Adherent HFKs were digested with trypsin (0.05% trypsin and 1 mM EDTA in PBS) at room temperature for the indicated time periods (*0′, 1′*, etc.). Digestion was terminated by adding soybean trypsin inhibitor. The designations *Spread*, *Round*, and *Detached* represent the morphology of the cells at the indicated time of trypsin digestion. Adherent (lanes *1–5*) or detached cells (lane *6*) from each time point were extracted with 1% Triton X-100 in PBS, and an equal amount of protein from each extract was fractionated by SDS-PAGE. The phosphotyrosine proteins were immunoblotted with antiphosphotyrosine mAb Py20. (*B*) Suspended HFK cells were plated onto laminin 5 at 4°C. Under this condition, cells attach via α6β4 but do not spread (see Fig. 1). The attached (*A*) HFKs (lane *1*) were washed with cold PBS buffer to remove nonadherent cells. Anti–laminin 5 mAb C2-9 (*C29+*; lane *2*) or trypsin (*Tp+*; lane *3*) was added to cells, followed by incubation at 37°C. Cells became rapidly detached at 37°C, and the detached cells were extracted with Triton X-100. Protein extracts were fractionated by SDS-PAGE, followed by immunoblotting with Py20.

phosphorylation was abrupt and was apparent only when the rounded cells detached from the adhesion surface (Fig. 5 *A*, lane *6*). This result suggested that p80 phosphorylation occurred subsequent to FAK dephosphorylation and concurrent with the detachment but not rounding of the cells.

HFK Adhesion to Laminin 5 Regulates p80 Phosphorylation

To determine if p80 phosphorylation resulted from the disruption of HFK adhesion to its endogenous basement membrane ligand laminin 5, HFKs were plated onto laminin 5 at 4°C. Consistent with the results in Fig. 3, HFKs adhered to laminin 5 as round cells and did not spread. When incubated with inhibitory anti-laminin 5 mAb C2-9, HFKs detached within 10–15 min after being shifted from 4 to 37°C or more slowly at 4°C. Immunoblotting of the C2-9–detached cells with Py20 showed enhanced phosphorylation of p80 (Fig. 5 *B*, lane *2*), which comigrated with the 80-kD protein induced by trypsin-induced detachment (Fig. 5 *B*, lane *3*). Thus, p80 phosphorylation can be induced by specific dissociation of α6β4 interaction with laminin 5.

We also examined the regulation of p80 phosphorylation by other adhesion ligands such as collagen, fibronectin, and poly-L-lysine. Suspended HFKs (Fig. 6, lane *1*) were allowed to attach to different substratum in short-term adhesion at 37°C. Protein extracts were prepared when 90% of the cells attached to each ligand. Prolonged incubation was avoided to eliminate the possible deposition of endogenously synthesized laminin 5 onto collagen, fibronectin, and poly-L-lysine surfaces (see Discussion). Analysis of cells adherent to fibronectin, collagen, and poly-L-lysine showed no dephosphorylation of p80 (Fig. 6, lanes *3–5*) as compared to cells in suspension (Fig. 6, lane *1*). In contrast, cells attached to laminin 5 had significant dephosphorylation of p80 (Fig. 6, lane *2*). These data suggest that the rapid regulation of p80 phosphorylation is specific to laminin 5. In contrast, adhesion to laminin 5, fibronectin, and collagen, but not poly-L-lysine, induce $pp125^{FAK}$ phosphorylation (Fig. 6, lanes *2–5*), which is consistent with the specific regulation of $pp125^{FAK}$ by β1 integrin (Kornberg et al., 1992).

Regulation of p80 Phosphorylation Is Functionally Linked to α6β4-mediated Cell Adhesion

To determine if α6β4, a physiologically relevant adhesion receptor for laminin 5, was involved in p80 dephosphorylation, suspended HFK cells were plated at 4°C onto tissue culture dishes coated with different ECM ligands. Under such conditions, adhesion via α3β1 to laminin 5, α2β1 to collagen, and α5β1 to fibronectin was prevented, but α6β4 adhesion to laminin 5 was not (Gil et al., 1995[2]). HFKs attached to laminin 5 at 4°C and assumed a round morphology (Fig. 7 *A*). No spreading was observed even after overnight incubation at 4°C. We then transferred the cells from 4 to 37°C, resulting in rapid cell spreading (Fig. 7 *A*). When protein phosphorylation was analyzed, we found that p80 is efficiently dephosphorylated in HFKs that adhere to laminin 5 ECM at 4°C (Fig. 7 *B*, compare lanes *1* and *2*). However, there was no phosphorylation of $pp125^{FAK}$ upon cell adhesion at this temperature (Fig. 7 *B*, lane *2*). Phosphorylation of $pp125^{FAK}$ occurred only when round HFKs were warmed to 37°C (Fig. 7 *B*, lane *3*), facilitating α3β1 interaction with laminin 5 and resulting in cell spreading. Thus, adhesion to laminin 5 via α6β4 at 4°C in-

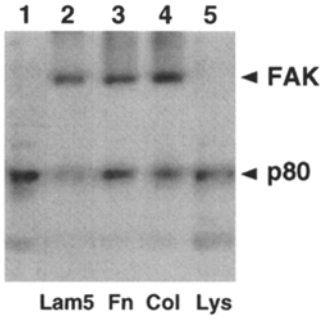


Figure 6. Phosphorylation of p80 is regulated specifically by HFK adhesion to laminin 5. HFKs were kept in suspension (lane *1*) or plated on laminin 5 (lane *2*), fibronectin (lane *3*), collagen type 1 (lane *4*), and poly-L-lysine (lane *5*). Protein extracts were prepared after 90% of HFK attached to each different ligand. Nonadherent cells were removed by gentle rinse with PBS. Cell lysates were prepared by extraction with 1% Triton in PBS. Phosphotyrosyl-containing proteins were detected by immunoblotting with Py20.

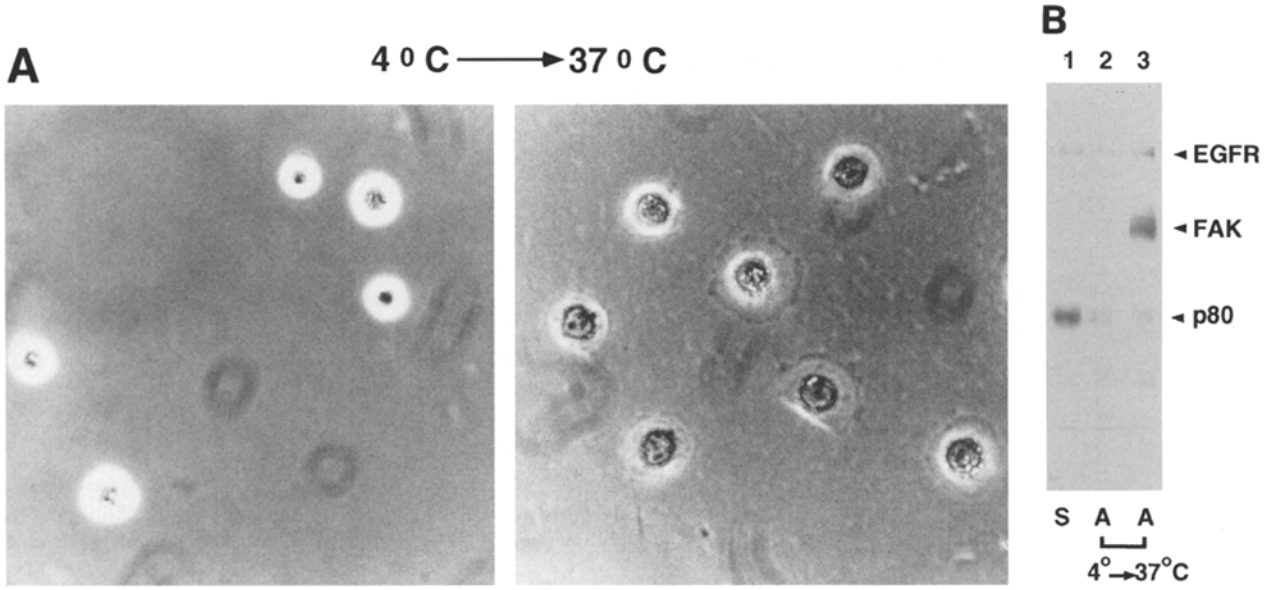


Figure 7. HFK anchorage to laminin 5 at 4°C via α6β4 dephosphorylates p80 while warming to 37°C induces spreading via α3β1 and phosphorylation of pp125^{FAK}. (*A*) HFKs adhere to laminin 5 ECM at 4°C, but do not spread. Warming of the adherent cells to 37°C induces spreading (*4°C→37°C*). (*B*) Aliquot of suspended (*S*) HFKs was either collected directly (lane *1*), or reattached (*A*) to laminin 5 at 4°C (lane *2*) followed by warming up to 37°C (lane *3*). Cells were extracted with Triton X-100 detergent, fractionated by SDS-PAGE, and analyzed for phosphotyrosyl-containing proteins by immunoblotting with Py20. The 80-kD protein was efficiently dephosphorylated upon adhesion (lane *2*) to laminin 5 ECM at 4°C as compared to the control HFKs in suspension (lane *1*). Phosphorylation of pp125^{FAK} was observed when the temperature was raised from 4 to 37°C (lane *3*). (*Arrowheads*) Phosphorylated EGFR, pp125^{FAK}, and p80.

duced dephosphorylation of p80. Under the same conditions, phosphorylation of pp125^{FAK} was not induced. Phosphorylation of pp125^{FAK} correlated with activation of α3β1 functions in cell spreading on laminin 5 at 37°C.

To rule out the possibility that lack of pp125^{FAK} phosphorylation at 4°C was due to inactivation of pp125^{FAK} kinase activity at cold temperature, rather than dysfunctioning of β1 integrin, we performed cell adhesion to laminin 5 at 37°C in the presence of inhibitory anti-α3β1 mAbs to block the function of β1 integrins. Under these conditions, regulation of p80 phosphorylation upon adhesion/detachment occurred normally (Fig. 8, lanes *3* and *4*), whereas the phosphorylation of pp125^{FAK} was prevented (Fig. 8, lane *3*). This result further supported the idea that α6β4 regulates p80 but not pp125^{FAK} phosphorylation. The latter event requires the function of β1 integrin.

Phosphorylation of p80 Does Not Require Intact Actin Stress Fibers

To further confirm the independent role of α6β4 in regulating p80 phosphorylation, cells were treated with cytochalasin D, which depolymerizes the network of actin filaments and inhibits the clustering of β1 in focal adhesions (Rankin and Rozengurt, 1994). Analysis of HFK cells at 37°C, pretreated for 2 h with cytochalasin D (1.25 μM) followed by detachment, showed increased tyrosine phosphorylation of p80 compared to adherent cells (Fig. 9, lanes *3* and *4*). However, regulation of pp125^{FAK} phosphorylation was diminished after cytochalasin D treatment (Fig. 9, lanes *3* and *4*). Thus, unlike pp125^{FAK}, regulation of p80 phosphorylation does not require an intact actin cytoskeleton. The increased phosphorylation of p80 was consistently observed (Fig. 9, lanes *2* and *4*) in cytochalasin D–treated cells, which suggests that α3β1-FA may exert a negative effect on α6β4-mediated regulation of p80 phosphorylation.

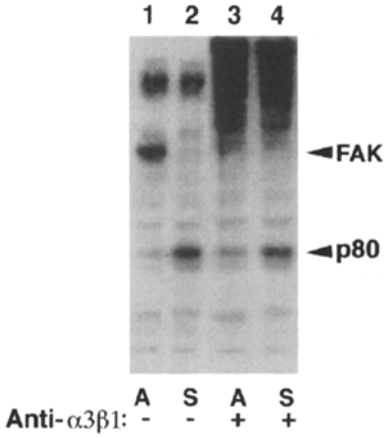


Figure 8. Inhibitory mAbs against α3β1 block the phosphorylation of pp125^{FAK} but not the phosphorylation of p80. Suspended HFKs were plated onto laminin 5 surface in the presence of SP2 (lanes *1* and *2*), or P1B5 (anti-α3β1) plus P4C10 (anti-β1, lanes *3* and *4*). Cells were either detached by trypsin digestion (*S*) or extracted directly from tissue culture plates (*A*) with 1% Triton X-100. Phosphotyrosine-containing proteins were immunoblotted with Py20. (*Arrowheads*) Phosphorylated pp125^{FAK} and p80.

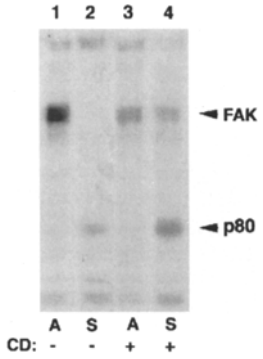


Figure 9. Cytochalasin D treatment of HFK cells diminishes pp125^{FAK} phosphorylation but enhances detachment-induced phosphorylation of p80. HFK cells were preincubated with (lanes *3* and *4*) or without (lanes *1* and *2*) cytochalasin D (*CD*, 1.25 μM) for 2 h at 37°C, followed by detachment (*S*) or readhesion (*A*) to laminin 5 surface. Cell lysates were electrophoresed on polyacrylamide gel, and phosphotyrosine-containing proteins were immunoblotted with Py20. (*Arrowheads*) Phosphorylated pp125^{FAK} and p80.

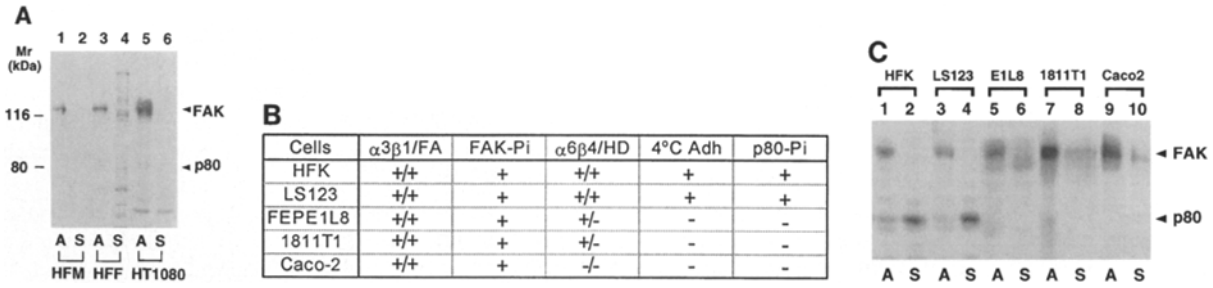
Phosphorylation of p80 in Nonepithelial Cells and in Transformed Epithelial Cells

As described above, 4°C adhesion occurred only in cells that have functional α6β4 and when laminin 5 was provided as adhesion surface. We therefore used binding to laminin 5 at 4°C as an indicator for the adhesive function of α6β4. To further establish the correlation of p80 phosphorylation with α6β4 function, we analyzed several nonepithelial cell populations including HFFs, HFMs, and human fibrosarcoma cells (HT1080). Previous studies had shown that these cells express α3β1, but not α6β4, and they did not adhere to laminin 5 at 4°C (Gil et al., 1995[2]). Phosphorylation of p80 was not detected in extracts of these suspended cells (Fig. 10 *A*, lanes *2*, *4*, and *6*). Nevertheless, pp125^{FAK} phosphorylation was detected in all adherent cells (Fig. 10 *A*, lanes *1*, *3*, and *5*).

We next analyzed p80 phosphorylation in immortalized HFKs, since phosphorylation of p80 is tightly regulated by cell adhesion to substratum, and transformed cells frequently manifest altered adhesion. Human papilloma virus–transformed keratinocytes, FEPE1L8 and FEP-1811T1 (Kaur et al., 1989), were examined. These cells express α6β4 at levels comparable to parental HFKs (Carter et al., 1991). However, they do not assemble SACs (Kaur and Carter, 1992) and do not adhere to laminin 5 at 4°C (Fig. 10 *B* and Gil et al., 1995[2]). Consistently, we did not detect p80 phosphorylation in suspensions of FEPE1L8 and FEP-1811T1 (Fig. 10 *C*, lanes *5–8*). Phosphorylation of p80 was not observed in the colon carcinoma cell line Caco-2 (Fig. 10 *C*, lanes *9* and *10*), which neither expressed β4 nor adhered to laminin 5 at 4°C (Fig. 10 *B*). In contrast, LS123 cells, a colon carcinoma cell line that expresses α6β4 in SACs and adheres to laminin 5 at 4°C (Fig. 10 *B*), retained the ability to regulate p80 phosphorylation (Fig. 10 *C*, lanes *3* and *4*). These data suggest that p80 phosphorylation correlates with the assembly of SACs that contain α6β4 and anchorage at 4°C.

Tyrosine-phosphorylated p80 Is a New Signaling Protein Associated with the Plasma Membrane

To determine the cellular localization of phosphorylated p80, we performed subcellular fractionation on adherent and suspended HFKs. The 80-kD protein was detected in a Triton-soluble membrane fraction in suspended HFKs by immunoblotting with Py20 (Fig. 11, lane *7*). As an internal control, phosphorylated pp125^{FAK} was recovered in the cytosolic fraction of adherent but not suspended HFKs (Fig. 11, lanes *1* and *5*), which is consistent with previous subcellular localization studies (Kornberg et al., 1991). Phosphorylated EGF receptor (EGFR) was detected in Triton-aqueous fractions of both adherent and suspended cells (Fig. 11, lanes *3* and *7*). This fractionation protocol separates β1 integrins and CD44 into the Triton-aqueous and Triton-detergent fractions, respectively (Carter et al., 1988). Additionally, we compared p80 with known 80-kD phosphoproteins, including cortactin (Wu and Parsons, 1993), CD44 (Carter and Wayner, 1988), plakoglobin (Franke et al., 1989), and phosphatidylinositol 3–kinase (PI3-K) (Filvaroff et al., 1992). These proteins were immunoprecipitated with their respective antibodies from extracts of adherent and suspended HFKs, followed by immunoblotting with Py20. In contrast to p80, none of these previously characterized phosphoproteins showed enhanced tyrosine phosphorylation in response to detachment of



Cells	α3β1/FA	FAK-Pi	α6β4/HD	4°C Adh	p80-Pi
HFK	+/+	+	+/+	+	+
LS123	+/+	+	+/+	+	+
FEPE1L8	+/+	+	+/-	-	-
1811T1	+/+	+	+/-	-	-
Caco-2	+/+	+	-/-	-	-

Figure 10. Tyrosine phosphorylation of p80 in nonepithelial cells and in transformed epithelial cells. (*A*) pp125^{FAK}, but not p80, is tyrosine phosphorylated in nonepithelial cells expressing α3β1 but not α6β4. HFM, HFF, and human fibrosarcoma cells (*HT1080*) were plated onto laminin 5 ECM for 30 min at 37°C. Protein extracts of adherent (*A*) or suspended (*S*) cells were made from each cell line. (*B*) p80 is not phosphorylated on tyrosine in transformed HFKs lacking α6β4-SACs. Cultured cells were immunostained, as described in Materials and Methods, with mAb P1F2 (reacting with α3β1 in FA) or GoH3 (reacting with α6 in SAC). Tyrosine phosphorylation of proteins were analyzed by immunoblotting with Py20 (*C*). Transformed cells used were indicated above each lane. Phosphotyrosine-containing proteins were immunoblotted with Py20. (*Arrowheads*) Phosphorylated pp125^{FAK} and p80. *FAK-Pi*, tyrosine-phosphorylated pp125^{FAK}; p80-Pi, tyrosine-phosphorylated p80; 4°C Adh, adhesion of cells to laminin 5 at 4°C.

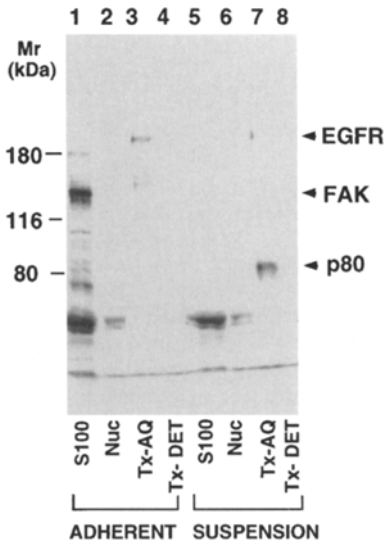


Figure 11. Subcellular fractionation of p80. Adherent HFKs (lanes *1–4*) or suspended HFKs (lanes *5–8*) were subjected to subcellular fractionation as described in Materials and Methods. Cells were homogenized and differentially extracted with buffer containing 2 M urea in PBS to obtain a cytosolic fraction (*S100*; lanes *1* and *5*), a nuclear fraction (*Nuc*; lanes *2* and *6*), and with Triton X-114 to obtain a membrane fraction that was further separated into a Triton-aqueous fraction (*Tx-AQ*; lanes *3* and *7*) and a Triton-detergent fraction (*Tx-DET*; lanes *4* and *8*). The Triton-aqueous fraction contains membrane components with dominant hydrophilic character, and the Triton-detergent fraction contains membrane components that are predominantly hydrophobic. Samples were fractionated by SDS-PAGE and analyzed for phosphotyrosyl-containing proteins by immunoblotting with mAb Py20. (*Arrowheads*) The positions of EGFR, pp125^{FAK}, and p80.

HFKs (data not shown). To the best of our knowledge, p80 is likely a new signaling molecule in epithelial cells.

Requirement for Phosphatase Activity in α6β4 Anchorage to Laminin 5

The above data indicated that p80 phosphorylation is regulated by anchorage to laminin 5 via α6β4. We speculated that the p80 phosphorylation state is controlled by its kinase/phosphatase activities (here we define the kinase that phosphorylates p80 as p80 kinase, and the phosphatase as p80 phosphatase). These activities are probably induced by α6β4 disengagement and conjugation with ligand, respectively. Therefore, we examined the role of p80 phosphatase and kinase activities in α6β4-mediated adhesion. Since we have consistently observed that p80 is a major tyrosine-phosphorylated protein responsive to α6β4-mediated interaction with laminin 5, we asked whether p80 dephosphorylation is required in the adhesion process. In other words, would prevention of p80 dephosphorylation affect α6β4 anchorage to laminin 5? HFKs were treated with sodium vanadate (1 mM, 30 min at 37°C) before plating on purified laminin 5 at 4°C. The rapid anchorage to laminin 5 was inhibited (Fig. 12) along with the inhibition of p80 dephosphorylation. When the same treated cells were attached to laminin 5 at 37°C, adhesion and spreading occurred, suggesting that inhibition of HFK adhesion at 4°C is not due to cytotoxic effect of sodium vanadate. Rather, it is likely due to specific inhibition of tyrosine phosphatase activity. These data indicated that the rapid initial adhesion, which is characteristic of α6β4-mediated binding to laminin 5 (Gil et al., 1995[2]), requires tyrosine dephosphorylation, possibly involving the activity of p80 phosphatase.

The p80 Kinase Activity and α6β4-SAC but Not α3β1-FA Are Present in Triton-insoluble Fraction of Suspended HFKs

Despite the use of various immunoprecipitation conditions with anti-integrin antibodies, we were unable to demonstrate the presence of p80 in the anti-α6β4 immunoprecipitates. The p80 itself may not be a component of α6β4-SACs. Alternatively, the association between p80 and α6β4 may be indirect and sensitive to detergent extraction. We therefore developed an in vitro kinase assay to determine if the p80 kinase activity was associated with α6β4. When the protein extracts from adherent or detached HFKs were analyzed, the p80 kinase activity was detected only in the detached cells but not in the adherent cells (Fig. 13 *A*, lanes *1* and *2*). Furthermore, when the extract of adherent HFKs was added to that of suspended cells, the phosphorylation of p80 was inhibited. Since sodium vanadate (1 mM) was included in the kinase reaction to prevent dephosphorylation of p80, we reasoned that the suppression of p80 phosphorylation is more likely due to the presence of p80 kinase inhibitor in adherent HFKs. Immunoprecipitation of the in vitro phosphorylated proteins with Py20 confirmed that the ^{32}P-p80 in suspended HFK was phosphorylated on tyrosine residues (Fig. 13 *B*).

We have previously reported (Gil et al., 1995[2] and as shown in Fig. 14 *A*) that α6β4 associates with Triton-insoluble SACs in detached HFKs. The Triton extraction depletes HFKs of α3β1 and other β1 integrins. We suggested that these preassembled SACs may play a role in the energy-independent anchorage of HFKs on laminin 5. In the following experiments, we determined that p80 kinase preferentially associates with the Triton-insoluble α6β4-SAC fraction of suspended HFKs. As a first attempt to localize p80 kinase activity, we performed a selective extraction procedure (Fig. 14 *B*): detached HFKs in TBS were sonicated to rupture the plasma membrane, followed by centrifugation at 100,000 *g* for 40 min. The cytosolic fraction (*a*) was collected and the pellet (*b*) was resuspended in TBS containing 1% Triton X-100 to give rise to a suspension (*c*). The suspension was then centrifuged to obtain Triton-soluble fraction (*d*) and Triton-insoluble fraction (*e*). As shown in Fig. 14 *C*, p80 kinase was not detectable in cytosol (lane *1*), Triton-soluble fraction (lane *3*), or Triton-insoluble fraction (lane *4*). Only in Triton suspension was p80 phosphorylation clearly detected (lane *2*). Further, when the Triton-soluble fraction was reconstituted with the insoluble fraction, it restored p80 phosphorylation. This suggested that the kinase was not inactivated during fractionation (data not shown). Since p80 itself was found in the Triton-soluble fraction (Fig. 11, lane *7*, and data not shown), p80 kinase is therefore more likely to associate with the Triton-insoluble cellular structure that contains α6β4 in SACs than to the α3β1-FA. These findings provided biochemical evidence that p80 kinase and

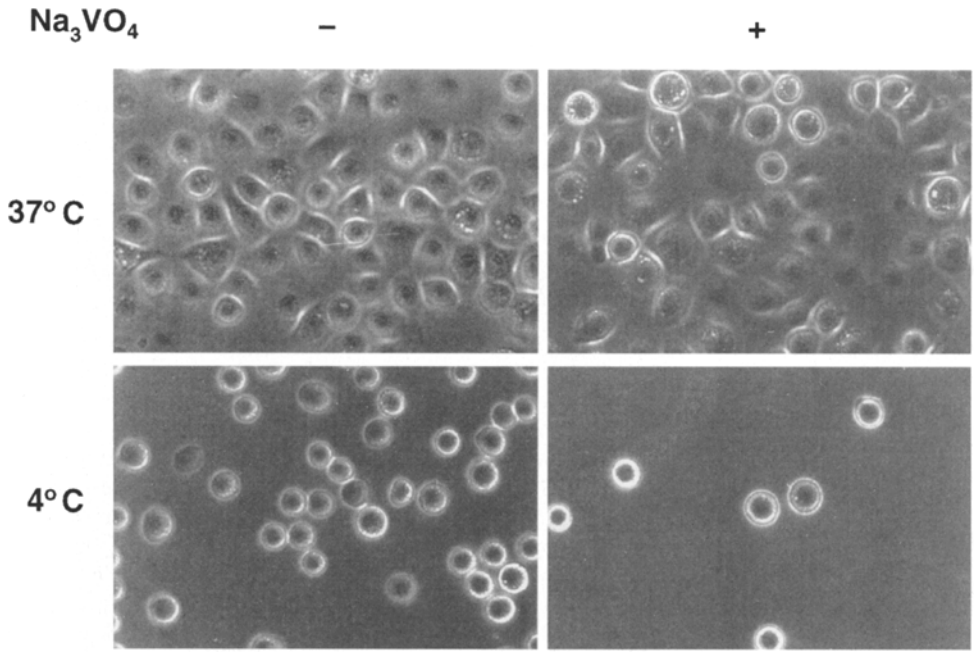


Figure 12. Inhibition of adhesion to laminin 5 at 4°C by sodium vanadate. HFKs were plated on purified laminin 5 at 37 or 4°C for 20 min in the presence or absence of 1 mM sodium vanadate (cells incubated with sodium vanadate had been pretreated with the solution for 30 min before plating on laminin 5). Plates were rinsed with PBS to remove nonadherent cells and then photographed under a light microscope.

α6β4-SAC, but not α3β1-FA, are present in Triton-insoluble fraction of suspended HFKs.

Discussion

In this report, we described a novel 80-kD membrane-associated protein that is phosphorylated on tyrosine residue(s) in response to disruption of α6β4-mediated anchorage of HFKs to laminin 5. The kinase activity for p80 was detected in extracts of suspended HFKs, which can be suppressed by an inhibitory activity from the adherent cells. This kinase activity was found to cofractionate with α6β4 in Triton-insoluble fraction of HFKs. Regulation of p80 phosphorylation does not appear to correlate with changes in cell shape. Disruption of the final linkage of unspread HFKs on laminin 5 is necessary and sufficient to activate p80 kinase. Conversely, binding of α6β4 with laminin 5 at 4°C in round cells is sufficient to activate p80 phosphatase. Therefore, α6β4-dependent interaction with laminin 5 regulates p80 phosphorylation.

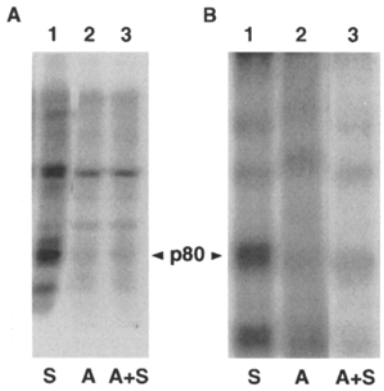


Figure 13. Detached HFKs contain p80 kinase activity that can be suppressed by inhibitor in adherent HFK. (*A*) Triton extract of adherent (*A*) or suspended (*S*) HFKs was assayed either separately (lanes *1* and *2*) or mixed (lane *3*) before kinase reaction in the presence of [γ-^{32}P]ATP. (*B*) Py20 immunoprecipitates of in vitro phosphorylated proteins. Proteins were subjected to SDS-PAGE and visualized by autoradiography.

We compared tyrosine phosphorylation induced by α6β4 with that of the α3β1. A summary of our conclusions is presented in diagrammatic form in Fig. 1 (point B in legend). We have established adhesion conditions for the selective assay of α3β1 and α6β4 functions. Cell anchorage at 4°C is specific for α6β4 binding to laminin 5. When tyrosine phosphorylation was examined, we found that dephosphorylation of p80 occurred upon HFK adhesion to laminin 5 at 4°C via α6β4. Inhibition of HFK adhesion to laminin 5 with anti-α6 (GoH3) or anti–laminin 5 (C2-9) at this temperature prevented dephosphorylation of p80. Conversely, detachment of adherent HFKs with anti–laminin 5 antibody induced phosphorylation of p80. Together these results suggest that p80 phosphorylation is regulated by HFK adhesion to laminin 5 via α6β4. In comparison, HFK adhesion to laminin 5 at 4°C does not induce the phosphorylation of pp125^{FAK}. Phosphorylation of pp125^{FAK} was apparent only when adherent cells were warmed to 37°C inducing cell spreading and migration via α3β1. This is consistent with the report that phosphorylation of pp125^{FAK} is triggered by β1 integrins (Kornberg et al., 1991). Under our assay conditions, the two functionally different integrins α6β4 and α3β1 activate phosphorylation of different signaling proteins p80 and pp125^{FAK}, respectively. Phosphorylation of p80 does not seem to require cell–cell contact, since the level of p80 phosphorylation was the same in actively growing cells with different densities (data not shown).

Phosphorylation of p80 was regulated specifically by laminin 5–coated adhesion surface in short-term adhesion assay. However, prolonged incubation of cells on adhesion ligands other than laminin 5 also resulted in dephosphorylation of p80. Two possible explanations could be: (*a*) HFKs deposit endogenously synthesized laminin 5 rapidly

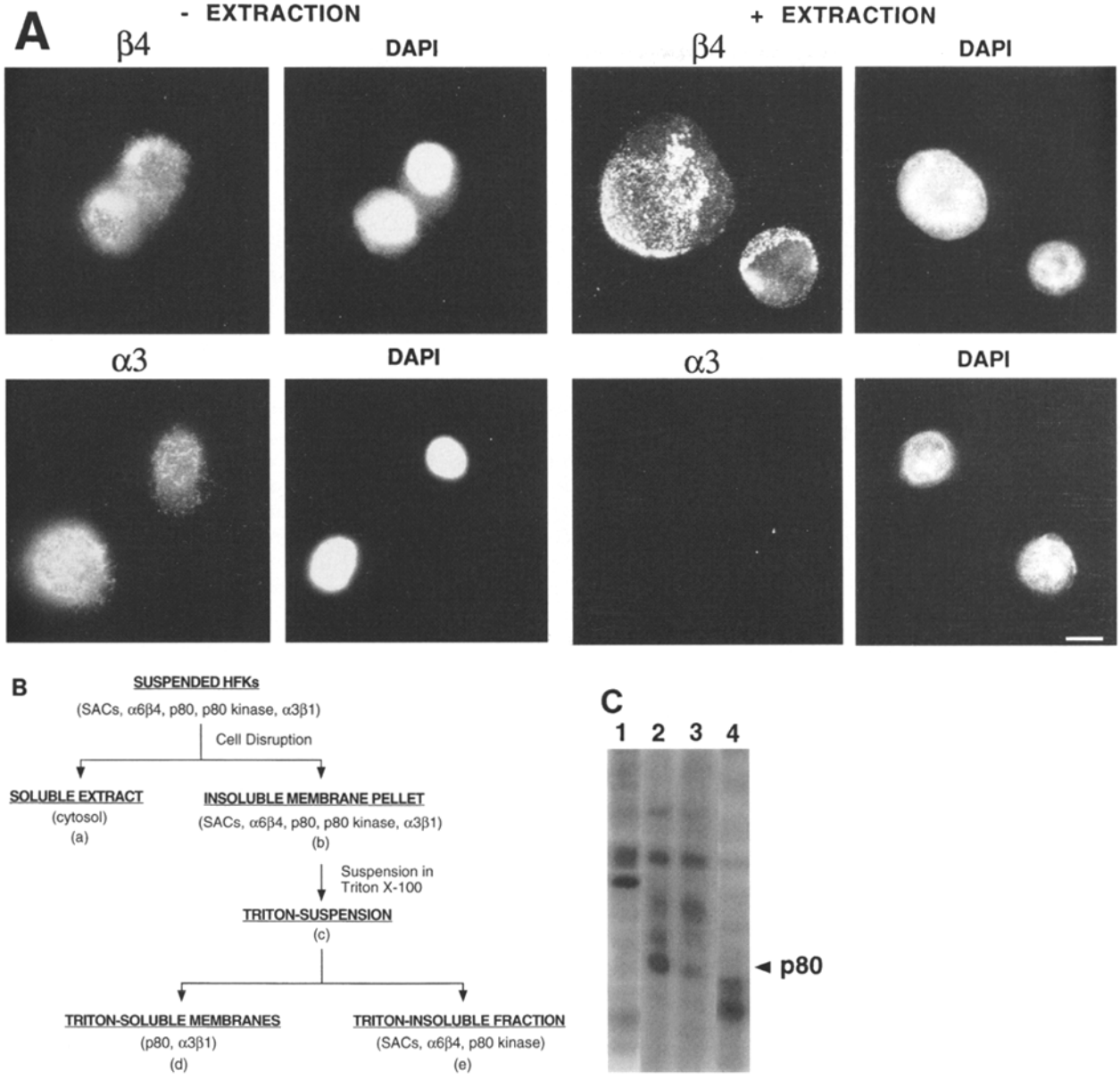


Figure 14. The kinase activity of p80 and α6β4-SACs, but not α3β1-FA, is present in Triton-insoluble fraction of the suspended HFKs. (*A*) HFKs were suspended and then fixed in suspension both before (−*EXTRACTION*) and after extraction (+*EXTRACTION*) with Triton X-100 detergent (1% vol/vol in PBS). The suspended cells were permeabilized with Triton X-100 detergent and then mounted on a nonspecific adhesion surface coated with polyacrylhydrazido-agarose for visualization (Gil et al., 1995[2]). After blocking with 0.5% heat-denatured BSA, the cells were stained for β4 (3E1) and α3β1 (P1B5). The nuclei were visualized by double staining with 4′, 6-diamino-2-phenylindole (*DAPI*). β4 localizes in cell–substrate contact and cannot be extracted. In contrast, α3 is completely removed by Triton extraction. (*B*) Procedure of selective extraction of HFKs. (*C*) Kinase activity of HFK subcellular fractions. Detached HFKs were fractionated according to procedure illustrated in *B*. Kinase assay was performed on cytosolic fraction (*a*) (lane *1*), Triton suspension (*c*) (lane *2*), Triton-soluble membrane fraction (*d*) (lane *3*), and Triton-insoluble fraction (*e*) (lane *4*).

onto substratum upon initial attachment to exogenous ligand, allowing ligand binding of α6β4 and therefore, the regulation of p80 phosphorylation. (*b*) The existence of a shared regulatory step between β1 and α6β4 integrin receptors (Xia, Y.-P., S.G. Gil, and W.G. Carter, manuscript in preparation) may lead to indirect regulation of p80 phosphorylation by β1 integrins. In fact, we have not been able to formally exclude α3β1-dependent regulation of p80 phosphorylation at this point. However, in cells expressing α3β1 but not α6β4 (HFF and HFM), no phosphorylation of p80 was observed. Additionally, anti-α3β1 antibody which blocked HFK spreading on laminin 5 did not change the level of p80 phosphorylation. Furthermore, p80 kinase activity cofractionated with α6β4 in SACs, not β1 integrins. Together these data strongly suggest that α6β4 is more likely the primary regulator of p80 kinase, and if there is any function for α3β1, it probably is indirect. In this study, we did not observe significant tyrosine phosphorylation on the β4 subunit in cells adherent to laminin 5, in contrast to what has been described by Mainiero et al.

(1995). This discrepancy could be due to differences in the time course for our phosphorylation studies. In our short-term adhesion assay, cells adherent on laminin 5 were extracted 10 min after being plated onto the adhesion surface, as opposed to the 60-min incubation that allows the maximum phosphorylation of β4 on laminin 5 (Mainiero et al., 1995). The kinase that phosphorylates the β4 subunit may not be the same kinase that phosphorylates the p80, since the activation of β4 is induced by its ligation. In contrast, dissociation of β4 with extracellular ligand causes p80 phosphorylation.

The disruption of actin stress fibers by cytochalasin D enhanced phosphorylation of p80 upon cell detachment. This finding suggests (*a*) α6β4 is sufficient to regulate p80 phosphorylation in the absence of β1-FAs function. (*b*) β1 integrin may suppress p80 phosphorylation regulated by α6β4 when they coexist as functional receptors. Cross talk between the β1 and α6β4 signaling pathways is an intriguing possibility for the coordinated regulation of motility versus anchorage. As a matter of fact, we observed increased migration and spreading on laminin 5 when α6β4 function is blocked by its inhibitory antibody (Gil et al., 1995[2]). Moreover, a previous report by Riddelle et al. (1992) provided evidence of an interaction between α6β4-associated intermediate filaments and β1-linked microfilaments in rat bladder epithelial cell line 804G cells. Our studies suggested further the existence of a shared regulatory step in the two pathways of α3β1 and α6β4, which would facilitate differential use of FAs and SACs in motile HFKs versus stationary cells. This shared regulatory step could either be at the level of the extracellular interaction of receptors with laminin 5 or via signaling inside cells. It is noteworthy that α3β1-FAs are located in close proximity to α6β4-SACs in a complex adhesion structure (Carter et al., 1991). Furthermore, anti–laminin 5 mAb C2-9 blocks both anchorage via α6β4 and motility via α3β1, suggesting that both receptors may compete with each other for interactions with the same or proximal binding sites on laminin 5.

By in vitro kinase assay and differential Triton extraction of α3β1-FAs (Triton soluble) and α6β4-SACs (Triton insoluble), we showed that most of the p80 kinase activity was associated with the Triton-insoluble fraction rather than the Triton-soluble fraction. These data do not prove a direct association of p80 kinase with α6β4. Rather, they provide biochemical evidence that the p80 kinase is a Triton-insoluble membrane factor. The identification of an inhibitory activity for p80 kinase in adherent HFKs illustrates a possible regulatory mechanism for phosphorylation of p80. Based on the observation that α6β4 binding to laminin 5 induces rapid dephosphorylation of p80, and that the tyrosine phosphatase inhibitor sodium vanadate inhibits HFK anchorage to laminin 5 at 4°C but not the adhesion at 37°C, we postulate that a phosphatase activity is also involved. Therefore, dephosphorylation of p80 is regulated by the functions of both kinase inhibitor and phosphatase. A tyrosine phosphatase activity was also mentioned by Mainiero et al. (1995) in cells treated with β4-specific mAb 3E1. More studies are needed to determine whether this phosphatase is specifically activated by β4 ligation, as has been observed for p80 phosphatase.

Unlike pp125^{FAK} that is expressed in all cell lines examined in this study, p80 phosphorylation was detected in a narrower range of cells. Phosphorylation of p80 correlates with the presence of α6β4 that is expressed primarily in epithelial and Schwann cells (Niessen et al., 1994). In addition, phosphorylation of p80 may require cells to form SACs. Thus, transformed HFKs that express α6β4 but do not form SACs or attach to laminin 5 at 4°C do not phosphorylate p80. In contrast, colon carcinoma cell LS123 that has α6β4-SACs and is able to adhere to laminin 5 at 4°C retained the regulation of p80 phosphorylation. These results, together with the inhibition of HFK adhesion to laminin 5 at 4°C by sodium vanadate, indicated that regulation of p80 phosphorylation may be involved in the stable anchorage that is mediated by α6β4-SACs in normal epithelial cells. Disruption of the anchorage could facilitate invasion of dermal tissue and/or metastasis. Our findings also suggested that signal transduction mediated by integrins may require both the adhesion receptor and the formation of an appropriate adhesion structure. This is consistent with the observation that phosphorylation of pp125^{FAK} occurs during the formation of focal adhesions. The mere occupancy of integrin receptor by monovalent ligand without the formation of new focal adhesions is not sufficient for inducing pp125^{FAK} phosphorylation (Kornberg et al., 1992; Miyamoto et al., 1995).

Based on our results, integrin α3β1 and α6β4 are sufficient to account for HFK and LS123 adhesion to laminin 5 under our assay conditions. Additional adhesion systems such as the uncharacterized bullous pemphigoid antigen II (BPA II) function may contribute but are minor compared to α3β1 and α6β4. However, LS123 cells that do not express BPA II anchored to laminin 5 at 4°C and regulated p80 phosphorylation. This observation excludes a significant function for BPA II in 4°C anchorage to laminin 5 and p80 phosphorylation.

Detachment of keratinocytes from the substratum has also been shown to initiate differentiation (Adams and Watt, 1993). We have demonstrated that anti-α3 induces expression of involucrin, a keratinocyte differentiation marker, and that exogenous laminin 5 can inhibit this differentiation (Symington and Carter, 1995). In light of these observations, we examined changes of p80 phosphorylation in differentiating HFK. Downregulation of p80 was detected both in progressively differentiating cell culture or in cells treated with calcium and 12-*O*-tetradeconylphorbol 13-acetate (data not shown).

The existence of different downstream signaling molecules for α3β1 and α6β4 may explain some functional differences between FAs and SACs (or HDs). FAs are dynamic adhesion structures and are more likely involved in HFK migration in wound tissue. Conversely, HDs are more prevalent in quiescent tissue than in activated cells, cell culture, or wounds (Gipson et al., 1993). HDs represent stable adhesion structures in homeostatic tissue. The anchorage via HDs provides mechanical stability at the dermal-epidermal junction. It may also provide a needed restriction or regulation of the β1-dependent motility. We suggest that regulation of p80 phosphorylation by p80 kinase and phosphatase will play a critical role in the regulation of anchorage versus migration or proliferation versus differentiation.

We thank Drs. Virginia Richmond, Maureen Ryan, Paul Lampe, and Tod Brown for critical review of this manuscript.

This work was supported by grants from the National Institutes of Health (RO1-CA49259 [WGC]). Yuping Xia is a recipient of a stipend award from the Terry Fox Guild.

References

Adams, J.C., and F.M. Watt. 1993. Regulation of development and differentiation by the extracellular matrix. *Development (Camb.).* 117:1183–1198.

Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* 256:1604–1607.

Boyce, S.T., and R.G. Ham. 1983. Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. *J. Invest. Dermatol.* 81:335–405.

Burridge, K., L.A. Petch, and L.H. Romer. 1992. Signals from focal adhesions. *Curr. Biol.* 2:537–539.

Carter, W.G., and E.A. Wayner. 1988. Characterization of the class III collagen receptor (CD44), a phosphorylated, transmembrane glycoprotein expressed in nucleated human cells. *J. Biol. Chem.* 263:4193–4201.

Carter, W.G., P. Kaur, S.G. Gil, P.J. Gahr, and E.A. Wayner. 1990*a*. Distinct functions for integrins α3β1 in focal adhesions and α6β4/bullous pemphigoid antigen in a new stable anchoring contact (SAC) of keratinocytes: relation to hemidesmosomes. *J. Cell Biol.* 111:3141–3154.

Carter, W.G., E.A. Wayner, T.S. Bouchard, and P. Kaur. 1990*b*. The role of integrins α2β1 and α3β1 in cell–cell and cell–substrate adhesion of human epidermal cells. *J. Cell Biol.* 110:1387–1404.

Carter, W.G., M.C. Ryan, and P.J. Gahr. 1991. Epiligrin, a new cell adhesion ligand for integrin α3β1 in epithelial basement membranes. *Cell.* 65:599–610.

Carter, W.G., S.G. Gil, B.E. Symington, T.A. Brown, S. Hattori, and M.C. Ryan. 1994. Integrin receptors and epiligrin in cell-cell and cell-substrate adhesion in the epidermis. *In* Integrins: The Biological Problems. Y. Takada, editor. CRC Press, Inc., Boca Raton, FL. 147–176.

Clark, E.A., and J.S. Brugge. 1996. Integrins and signal transduction pathways: the road taken. *Science (Wash. DC).* 268:233–239.

Damsky, C.H., and Z. Werb. 1992. Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. *Curr. Opin. Cell Biol.* 4:772–781.

Domloge-Hultsch, N., W.R. Gammon, R.A. Briggaman, S.G. Gil, W.G. Carter, and K.B. Yancey. 1992. Epiligrin, the major human keratinocyte integrin ligand, is a target in both an acquired autoimmune and an inherited subepidermal blistering skin disease. *J. Clin. Invest.* 90:1628–1633.

Falanga, V., F. Grinnell, B. Gilchrest, Y.T. Maddox, and A. Moshell. 1994. Workshop on the pathogenesis of chronic wounds. *J. Invest. Dermatol.* 102: 125–127.

Filvaroff, E., D.F. Stern, and G.P. Dotto. 1991. Tyrosine phosphorylation is an early and specific event involved in primary keratinocyte differentiation. *Mol. Cell. Biol.* 10:1164–1173.

Filvaroff, E., E. Calautti, F. McCormick, and G.P. Dotto. 1992. Specific changes of Ras GTPase-activating protein (GAP) and a GAP-associated p62 protein during calcium-induced keratinocyte differentiation. *Mol. Cell. Biol.* 12: 5319–5328.

Franke, W.W., M.D. Goldschmidt, R. Zimbelmann, H.M. Mueller, D.L. Schiller, and P. Cowin. 1989. Molecular cloning and amino acid sequence of human plakoglobin, the common junctional plaque protein. *Proc. Natl. Acad. Sci. USA.* 86:4027–4031.

Fuchs, E. 1990. Epidermal differentiation: the bare essentials. *J. Cell Biol.* 111: 2807–2814.

Gil, S.G., T.A. Brown, M.C. Ryan, and W.C. Carter. 1994. Junctional epidermolysis bullosa: defects in expression of epiligrin/nicein/kalinin and integrin β4 that inhibit hemidesmosome formation. *J. Invest. Dermatol.* 103:31s–38s.

Gipson, I.K., S. Spurr-Michaud, A. Tisdale, J. Elwell, and M.A. Stepp. 1993. Redistribution of the hemidesmosome components α6β4 integrin and bullous pemphigoid antigens during epithelial wound healing. *Exp. Cell Res.* 207:86–98.

Grinnell, F. 1992. Wound repair, keratinocyte activation and integrin modulation. *J. Cell Sci.* 101:1–5.

Guadagno, T.M., M. Ohtsubo, J.M. Roberts, and R.K. Assoian. 1993. A link between cyclin A expression and adhesion-dependent cell cycle progression. *Science (Wash. DC).* 262:1572–1575.

Guan, J.L., J.E. Trevithick, and R.O. Hynes. 1991. Fibronectin/integrin interaction induces tyrosine phosphorylation of 120-kDa protein. *Cell Regul.* 2:951–964.

Hay, E.D. 1993. Extracellular matrix alters epithelial differentiation. *Curr. Opin. Cell Biol.* 5:1029–1035.

Hynes, R.O. 1992. Integrins: versatility, modulation and signaling in cell adhesion. *Cell.* 69:11–25.

Ingber, D.E., D. Prusty, J.V. Frangioni, E.J. Cragoe, C. Lechene, and M.A. Schwartz. 1990. Control of intracellular pH and growth by fibronectin in capillary endothelial cells. *J. Cell Biol.* 110:1803–1811.

Jones, J.C.R., and K.J. Green. 1991. Intermediate filament-plasma membrane interactions. *Curr. Opin. Cell Biol.* 3:127–132.

Jones, J.C.R., M.A. Kurpakus, H.M. Cooper, and V. Quaranta. 1991. A function for the integrin α6β4 in the hemidesmosome. *Cell Regul.* 2:427–438.

Juliano, R.S., and S. Haskill. 1993. Signal transduction from the extracellular matrix. *J. Cell Biol.* 120:577–585.

Kaur, P., and W.G. Carter. 1992. Integrin expression and differentiation in transformed human epidermal cells is regulated by fibroblasts. *J. Cell Sci.* 103:755–763.

Kaur, P., J.K. McDougall, and R. Cone. 1989. Immortalisation of primary human epithelial cells by cloned cervical carcinoma DNA containing human papillomavirus 16 E6/E7 ORFs. *J. Gen. Virol.* 70:1261–1266.

Kornberg, L.J., H.S. Earp, C.E. Turner, C. Prockop, and R.L. Juliano. 1991. Signal transduction by integrins: increased protein tyrosine phosphorylation caused by clustering of beta 1 integrins. *Proc. Natl. Acad. Sci. USA.* 88:8392–8396.

Kornberg, L.J., H.S. Earp, J.T. Parsons, M. Schaller, and R.L. Juliano. 1992. Cell adhesion and integrin clustering increases phosphorylation of a focal adhesion associated tyrosine kinase. *J. Biol. Chem.* 267:23439–23442.

Kurpakus, M.A., V. Quaranta, and J.C.R. Jones. 1991. Surface relocation of alpha6beta4 integrins and assembly of hemidesmosomes in an in vitro model of wound healing. *J. Cell Biol.* 115:1737–1750.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Lin, C.Q., and M.J. Bissel. 1993. Multi-faceted regulation of cell differentiation by extracellular matrix. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 7:737–743.

Lin, T.H., A. Yurochko, L. Kornberg, J. Morris., J.J. Walker, S. Haskill, and R.L. Juliano. 1994. The role of protein tyrosine phosphorylation in integrin-mediated gene induction in monocytes. *J. Cell Biol.* 126:1585–1593.

Mainiero, F., A. Pepe, K.K. Wary, L. Spinardi, M. Mohammadi, J. Schlessinger, and F.G. Giancotti. 1995. Signal transduction by the α6β4 integrin: distinct β4 subunit sites mediate recruitment of Shc/Grb2 and association with the cytoskeleton of hemidesmosomes. *EMBO (Eur. Mol. Biol. Organ.) J.* 14: 4470–4481.

Miyamoto, S., S.K. Akiyama, and K.M. Yamada. 1995. Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. *Science (Wash. DC).* 267:883–885.

Niessen, C.M., O. Cremona, H. Damms, S. Feraresi, A. Sonnenberg, and P.C. Marchisio. 1994. Expression of the integrin α6β4 in peripheral nerves: localization in Schwann and perineural cells and different variants of the β4 subunit. *J. Cell. Sci.* 107:543–552.

Rankin, S., and E. Rosengurt. 1994. Platelet-derived growth factor modulation of focal adhesion kinase($P125^{FAK}$) and paxillin tyrosine phosphorylation in Swiss 3T3 cells. *J. Biol. Chem.* 269:704–710.

Riddelle, K.S., S.B. Hopkinson, and J.C.R. Jones. 1992. Hemidesmosomes in the epithelial cell line 804G: their fate during wound closure, mitosis and drug induced reorganization of the cytoskeleton. *J. Cell. Sci.* 103:475–490.

Rouselle, P., G.P. Lunstrum, D.R. Keene, and R.E. Burgeson. 1991. Kalinin: an epithelium-specific basement membrane adhesion molecule that is a component of anchoring filaments. *J. Cell Biol.* 114:567–576.

Ryan, M.C., R. Tizard, D.R. VanDevanter, and W.G. Carter. 1994. Cloning of the Lan3 gene encoding the α3 chain of the adhesive ligand epiligrin. *J. Biol. Chem.* 269:22779–22787.

Schaller, M.D., and J.T. Parsons. 1993. Focal adhesion kinase: an integrin-linked protein tyrosine kinase. *Trends Cell Biol.* 3:258–261.

Schaller, M.D., C.A. Borgman, B.S. Cobb, R.R. Vines, A.B. Reynolds, and J.T. Parsons. 1992. *Proc. Natl. Acad. Sci. USA.* 89:5192–5196.

Sonnenberg, A., J. Calafat, H. Janssen, H. Daams, L.M.H. van der Raaij-Helmer, R. Falcioni, S.J. Kennel, J.D. Aplin, J. Baker, M. Loizidou, et al. 1991. Integrin α6β4 complex is located in hemidesmosomes, suggesting a major role in epidermal cell–basement membrane adhesion. *J. Cell Biol.* 113: 907–917.

Sonnenberg, A., A.A. de Melker, A.M. Martinez de Velasco, J. Calafat, and C.M. Niessen. 1993. Formation of hemidesmosomes in cells of a transformed murine mammary tumor cell line and mechanisms involved in adherence of these cells to laminin and kalinin. *J. Cell Sci.* 106:1083–1102.

Sporn, S.A., D.F. Eirman, C.E. Johnson, J. Morris, G. Martin, M. Ladner, and S. Haskill. 1990. Monocyte adherence results in selective induction of novel genes sharing homology with mediators of inflammation and tissue repair. *J. Immunol.* 144:4434–4441.

Stepp, M.A., S. Spurr-Michaud, A. Tisdale, J. Elwell, and I.K. Gipson. 1990. α6β4 integrin heterodimer is a component of hemidesmosomes. *Proc. Natl. Acad. Sci. USA.* 87:8970–8974.

Streuli, C.H., N. Bailey, and M.J. Bissell. 1991. Control of mammary epithelial differentiation: basement membrane induces tissue-specific gene expression in the absence of cell–cell interaction and morphological polarity. *J. Cell Biol.* 115:1383–1395.

Symington, B.E., and W.G. Carter. 1995. Modulation of epidermal differentiation by epiligrin and integrin α3β1. *J. Cell Sci.* 108:831–838.

Verrando, P., B.L. Hsi, C.J. Yeh, A. Pisani, N. Serieys, and J.P. Ortonne. 1987. Monoclonal antibody GB3, a new probe for the study of human basement membranes and hemidesmosomes. *Exp. Cell Res.* 170:116–128.

Verrando, P., C. Blanchet-Bardon, A. Pisani, L. Thomas, F. Cambazard, R.A.J. Eady, O. Schofield, and J.P. Ortonne. 1991. Monoclonal antibody GB3 defines a widespread defect of several basement membranes and a keratinocyte dysfunction in patient with lethal junctional epidermolysis bullosa. *Lab. Invest.* 64:85–92.

Wayner, E.A., and W.G. Carter. 1987. Identification of multiple cell adhesion receptors for type VI collagen and fibronectin in human fibrosarcoma cells possessing unique α and common β subunits. *J. Cell Biol.* 105:1873–1884.

Wayner, E.A., W.G. Carter, R.S. Piotrowicz, and T.J. Kunicki. 1988. The func-

tion of multiple extracellular matrix receptors in mediating cell adhesion to extracellular matrix: preparation of monoclonal antibodies to the fibronectin receptor that specifically inhibit cell adhesion to fibronectin and react with platelet glycoproteins Ic-IIa. *J. Cell Biol.* 107:1881–1891.

Wayner, E.A., S.G. Gil, G.F. Murphy, M.S. Wilke, and W.G. Carter. 1993. Epiligrin, a component of epithelial basement membranes, is an adhesive ligand for $\alpha 3\beta 1$ positive T lymphocytes. *J. Cell Biol.* 121:1141–1152.

Werb, Z., P.M. Tremble, O. Behrendtsen, E. Crowley, and C.H. Dambsky. 1989. Signal transduction through the fibronection receptor induces collagenase and stromelysin gene expression. *J. Cell Biol.* 109:877–889.

Wu, H., and J.T. Parsons. 1993. Cortactin, an 80/85-kilodalton $pp60^{src}$ substrate, is a filamentous actin-binding protein enriched in the cell cortex. *J. Cell Biol.* 120:1417–1426.

Zachary, I., and E. Rozengurt. 1992. Focal adhesion kinase ($p125^{FAK}$): a point of convergence in the action of neuropeptides, integrins, and oncogenes. *Cell.* 71:891–894.

Zhao, Y., M. Sudol, H. Hanafusa, and J. Krueger. 1992. Increased tyrosine kinase activity of c-Src during calcium-induced keratinocyte differentiation. *Proc. Natl. Acad. Sci. USA.* 89:8298–8302.