Phosphorylation of a Novel Site on the $\beta 4$ Integrin at the Trailing Edge of Migrating Cells Promotes Hemidesmosome Disassembly

Emily C. Germain, Tanya M. Santos, and Isaac Rabinovitz

Division of Cancer Biology and Angiogenesis, Department of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA 02115

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Hemidesmosomes (HDs) are multiprotein structures that anchor epithelial cells to the basement membrane. HD components include the $\alpha 6 \beta 4$ integrin, plectin, and BPAGs (bullous pemphigoid antigens). HD disassembly in keratinocytes is necessary for cells to migrate and can be induced by EGF through $\beta 4$ integrin phosphorylation. We have identified a novel phosphorylation site on the $\beta 4$ integrin: S₁₄₂₄. Preventing phosphorylation by mutating S $\rightarrow$ A₁₄₂₄ results in increased incorporation of $\beta 4$ into HDs and resistance to EGF-induced disassembly. In contrast, mutating S $\rightarrow$ D₁₄₂₄ (mimicking phosphorylation) partially mobilizes $\beta 4$ from HDs and potentiates the disassembly effects of other phosphorylation sites. In contrast to previously described sites that are phosphorylated upon growth factor stimulation, S₁₄₂₄ already exhibits high constitutive phosphorylation, suggesting additional functions. Constitutive phosphorylation of S₁₄₂₄ is distinctively enriched at the trailing edge of migrating keratinocytes where HDs are disassembled. Although most of this S₁₄₂₄-phosphorylated $\beta 4$ is found dissociated from HDs, a substantial amount can be associated with HDs near the cell margins, colocalizing with plectin but always excluding BPAGs, suggesting that phospho-S₁₄₂₄ might be a mechanism to dissociate $\beta 4$ from BPAGs. S₁₄₂₄ phosphorylation is PKC dependent. These data suggest an important role for S₁₄₂₄ in the gradual disassembly of HDs induced by cell retraction.

INTRODUCTION

Epithelial cells attach to the basal lamina using a variety of anchoring junctions to stabilize their position and regulate their mobility (Hynes, 1992; Litjens *et al.*, 2006). One of these stabilizing junctions, the hemidesmosome (HD), is a transmembrane multiprotein complex that connects the basement membrane to the intracellular cytokeratin network providing the cell with tensile strength to resist mechanical loads and shear stress (Green and Jones, 1996; Nievers *et al.*, 1999; Litjens *et al.*, 2006). HDs in keratinocytes are dynamic structures that need to disassemble when cells migrate, for example, during wound closure or carcinoma invasion (Gipson *et al.*, 1993; Herold-Mende *et al.*, 2001). The regulation of HD disassembly has recently received much attention because of its implications in carcinoma invasion (Rabinovitz *et al.*, 1999, 2004; Dans *et al.*, 2001; Mariotti *et al.*, 2001; Litjens *et al.*, 2006; Wilhelmsen *et al.*, 2007). The mechanisms of HD disassembly are incompletely understood, although recent studies indicate that phosphorylation of one of its components, the $\alpha 6 \beta 4$ integrin, plays an important role in this process.

Classical HDs (type I) are composed of several proteins: the $\alpha 6 \beta 4$ integrin, the bullous pemphigoid antigens (BPAG) 1 and 2, HD1-plectin, and the tetraspanin CD151 (Nievers *et al.*, 1999; Litjens *et al.*, 2006). These are commonly found in epithelia such as skin and most glands. HDs type II lack the BPAG antigens and are common in intestinal epithelia (Uematsu *et al.*, 1994). Growth factors are elevated during wound healing and carcinoma invasion and have been shown to induce HD disassembly (Mainiero *et al.*, 1996; Rabinovitz *et al.*, 1999). Epidermal growth factor (EGF) and MSP (macrophage-stimulating protein) have been shown to induce HD disassembly and have been used as tools to unravel the mechanisms involved in this process (Mainiero *et al.*, 1996; Rabinovitz *et al.*, 1999; Santoro *et al.*, 2003). These factors activate different signaling pathways that result in the phosphorylation of several HD components including the $\alpha 6 \beta 4$ integrin, which is the central organizer of HDs. The $\alpha 6 \beta 4$ integrin is a transmembrane heterodimer that binds extracellularly to the laminins of the basal lamina and intracellularly to plectin and BPAG1 and BPAG2, all of which mediate the link with the cytokeratin network (Mercurio, 1995; Koster *et al.*, 2003; Litjens *et al.*, 2003). Growth factors induce the phosphorylation of the $\beta 4$ integrin subunit on serine and tyrosine residues (Mainiero *et al.*, 1996; Rabinovitz *et al.*, 2004). Serine phosphorylation on the $\beta 4$ integrin induced by EGF is dependent on a functional protein kinase C (PKC) pathway, and results in the disassembly of the HD and mobilization of the $\beta 4$ integrin to actin-rich protrusions (Rabinovitz *et al.*, 2004; Wilhelmsen *et al.*, 2007). A similar mobilization of $\beta 4$ through PKC is observed downstream the Ron receptor (MSP), including its association with 14-3-3 proteins (Santoro *et al.*, 2003). Inhibition of PKC pathway

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+ Address correspondence to: Isaac Rabinovitz (irabinov@bidmc.harvard.edu).

Abbreviations used: BPAG, bullous pemphigoid antigen; EGF, epidermal growth factor; HD, hemidesmosome; OKA, okadaic acid; PKC, protein kinase C; pp, phosphopeptide; TLC, thin-layer chromatography; TLE, thin-layer electrophoresis.

prevents $\beta 4$ phosphorylation and HD disassembly (Rabinovitz *et al.*, 1999, 2004; Santoro *et al.*, 2003).

There are around 15 different phosphorylation sites on the $\beta 4$ integrin, and ~95% of the phosphorylation induced by EGF occurs on serine residues (Rabinovitz *et al.*, 1999, 2004). We have previously identified a serine cluster in the connecting segment $\beta 4$ integrin (S_{1356} , S_{1360} , and S_{1364}) that is phosphorylated after EGF stimulation in a PKC-dependent manner (Rabinovitz *et al.*, 2004). This triad constitutes ~50% of the total phosphorylation of $\beta 4$. Substitution of these serines with alanine impedes phosphorylation and reduces the mobilization of $\beta 4$ induced by EGF or activated PKC in transfected cells. In contrast, substitution with aspartate, which mimics constitutive phosphorylation, results in the partial loss of HD-like structures, suggesting an important role in HD disassembly. Wilhelmssen *et al.* (2007) have recently corroborated the phosphorylation of these residues and determined their importance in regulating $\beta 4$ interaction with plectin and HD disassembly in keratinocytes. Other studies have shown that phosphorylation of tyrosine residues are involved in HD disassembly, and four sites have been identified that are dependent on Fyn (Dans *et al.*, 2001; Mariotti *et al.*, 2001).

A common finding in all these studies is that preventing phosphorylation of individual tyrosine or serines residues on the $\beta 4$ subunit produces only partial resistance to HD disassembly, and therefore several events of phosphorylation might be needed to complete the process (Dans *et al.*, 2001; Rabinovitz *et al.*, 2004; Wilhelmssen *et al.*, 2007). Several phosphorylation sites remain to be identified as well as their intimate mechanism of collaboration toward HD disruption. In this article we report the identification of a novel phosphorylation site in the connecting segment of the $\beta 4$ integrin: S_{1424} . We show that mutation of S_{1424} to alanine increases the incorporation of $\beta 4$ into HDs and resists mobilization induced by EGF, suggesting that phosphorylation of this site destabilizes the HD. Moreover, mutation of S_{1424} to aspartate, a mutation that mimics constitutive phosphorylation, reduces $\beta 4$ incorporation into HDs. In contrast to the other identified sites of phosphorylation, S_{1424} already shows high levels of constitutive phosphorylation in nonstimulated cells, suggesting a different role in HD regulation. A possible function of this site is provided by immunofluorescence and video microscopy analyses that show S_{1424} is mostly phosphorylated at the trailing edge of randomly migrating cells, suggesting that S_{1424} may contribute to the disassembly of the rear HDs as the cell moves forward. Importantly, S_{1424} -phosphorylated $\beta 4$ may be colocalized with plectin but not with BPAG1 or 2, indicating that S_{1424} may regulate the binding of the latter components. Our results also show that in the absence of growth factors S_{1424} can be phosphorylated by PKC α , indicating that these two different mechanisms of phosphorylation share the final steps of a common pathway. S_{1424} shows increased phosphorylation in several carcinoma cell lines. Altogether these findings indicate an important role for S_{1424} in the regulation of HDs.

MATERIALS AND METHODS

Cells and Reagents

HaCat cells, immortalized human keratinocytes, were obtained from Dr. S. La Flamme (Albany Medical College, Albany, NY). Cos-7 monkey kidney fibroblast cells were provided by Dr. J. P. Kinet (Beth Israel Deaconess Medical Center, Boston, MA). 804G rat bladder carcinoma cells were provided by Dr. A. M. Mercurio (UMass Med, Worcester, MA). All cells were maintained in DMEM with 10% fetal calf serum, at 37°C in a humidified atmosphere containing 5% CO₂. The following antibodies were used in this study: mouse mAb 3E1 (integrin $\beta 4$ -specific, Chemicon, Temecula, CA); rat mAb 439-9B

(integrin $\beta 4$ -specific) provided by Dr. R. Falcioni (Regina Elena Cancer Institute, Rome, Italy; Falcioni *et al.*, 1988); and rat mAb GoH3 (integrin $\alpha 6$, Chemicon). A peptide-specific antiserum elicited against the last 20 amino acids of the COOH-terminus of the $\beta 4$ subunit was prepared commercially. Mouse monoclonal antibodies specific for BPAG1 (R815) and BPAG2 (1D1) were a gift of Dr. Owaribe (Nagoya University, Japan; Hieda *et al.*, 1992). The HD1/plectin Ab was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) or Becton Dickinson (Mountain View, CA). Cy3- and Cy2-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratory (West Grove, PA). An affinity-purified phospho-specific $\beta 4$ rabbit polyclonal Ab (anti-pS₁₄₂₄ Ab) raised against a synthetic peptide [TRDYN(pS)LTRSE] was produced by Quality Controlled Biochemicals (Hopkinton, MA). Collagen type I was purchased from Collagen Corp (Palo Alto, CA). Human recombinant EGF and α -chymotrypsin were purchased from Sigma Chemical (St. Louis, MO). Go6976, OKA, H89, and PD98059 were purchased from Axxora (San Diego, CA).

Metabolic Radiolabeling

Cells were plated on laminin-1-coated dishes for 2 h and then were washed several times with phosphate-free DMEM and starved for 1 h before adding ³²PO₄ (1.0 mCi/ml; NEN, Boston MA) and incubating for 3 more hours. The cells were then stimulated with EGF (100 ng/ml) for 15 min, washed several times with PBS, and extracted in RIPA buffer containing 0.1% SDS, 1% Triton X-100, 0.5% deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 50 mM sodium pyrophosphate, 100 mM sodium fluoride, 1 mM sodium vanadate, 1 mM PMSF, 10 μ g/ml each of leupeptin, pepstatin A, and aprotinin, and 50 mM Tris-HCl, pH 7.5. The samples were immunoprecipitated using the 439-9B Ab, resolved by SDS-PAGE, and transferred to PVDF membranes (Immobilon-P, Millipore, Bedford, MA).

Analysis of the Radiolabeled $\beta 4$ Integrin Subunit

The PVDF membranes containing the $\beta 4$ immunoprecipitates were exposed to a phosphor screen (Bio-Rad, Richmond, CA), and the area of the membrane that contained the $\beta 4$ subunit was excised with a razor. For phosphoamino acid analysis the excised band (or eluted phosphopeptide; see below) was acid-hydrolyzed and resolved using 2D thin-layer electrophoresis (TLE) and exposed to a phosphor screen following standard techniques (Boyle *et al.*, 1991; Sefton, 1997). For phosphopeptide mapping, the band was digested with trypsin as described previously (Boyle *et al.*, 1991; Sefton, 1997) and run on a thin-layer chromatography (TLC) plate (cellulose) using a pH 1.9 buffer during the electrophoresis separation in the first dimension (TLE) and then a standard chromatography buffer in the second dimension (TLC), followed by exposure of the plate to a phosphor screen. The phosphopeptide profile of $\beta 4$ was compared with a prediction profile generated by PhosphoPeptort4 (www.genesteam.org) to identify candidate sequences. The cellulose area corresponding to the spots of interest was scraped off and the phosphopeptides eluted for further analysis. Manual Edman degradation was performed using standard techniques (Boyle *et al.*, 1991; Sefton, 1997). The eluted phosphopeptides were subject to two to five cycles of degradation, then resolved using TLE, and exposed to a phosphor screen. Diagnostic secondary digestion from the phosphopeptides was performed as described (Boyle *et al.*, 1991; Sefton, 1997) by digesting the eluted phosphopeptides with α -chymotrypsin (1 μ g/ml in ammonium bicarbonate buffer, pH 8). The digest was inactivated and resolved using TLE alongside undigested controls.

Site-directed Mutagenesis and cDNA Transfections

Single or multiple amino acid substitutions on human wild-type $\beta 4$ cDNA were made by generating PCR fragments into which the desired mutations had been introduced by means of appropriately designed primers following standard techniques (Cormack, 2003). The resulting point mutations were confirmed by dideoxy sequencing. The vectors (pcDNA4 plasmid, Invitrogen, Carlsbad, CA) containing the mutant $\beta 4$ cDNAs (1 μ g) were transfected into the 804G cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Indirect Immunofluorescence

Cells were stained as described previously (Rabinovitz and Mercurio, 1997; Rabinovitz *et al.*, 1999). Briefly, cells were fixed with a buffer containing 2% paraformaldehyde, 100 mM KCl, 200 mM sucrose, 1 mM EGTA, 1 mM MgCl₂, 1 mM PMSF, and 10 mM PIPES at pH 6.8 for 15 min. In some cases, the cells were extracted before fixation with a buffer containing 0.2% Triton X-100, 100 mM KCl, 200 mM sucrose, 10 mM EGTA, 2 mM MgCl₂, 1 mM PMSF, and 10 mM PIPES at pH 6.8 for 1 min. After fixation, the cells were rinsed with PBS and incubated with a blocking solution that contained 1% albumin in TBS for 20 min. Cells that were to be stained for BPAG1 or 2, were fixed with methanol. Primary antibodies in blocking solution were added to the fixed cells separately or in combination for 30 min. The cells were rinsed three times with PBS and either a Cy2- or Cy3-conjugated Ab against indicated species were used separately or in combination to stain the cells for 30 min. Cells were rinsed with PBS and mounted in a mixture (8:2) of glycerol and PBS (pH 8.5) containing 1% propylgallate. The slides were analyzed using fluorescence

(SQVD₁₀₂₅YR had no changes and is not shown). The arrow indicates the disappearance of pp_f. (F) The fourth serine of the pp_f that was mutated.

microscopy. When using the detergent extraction protocol, complete extraction of membranes was monitored using an anti-MHC antibody (Sigma-Aldrich) as the primary, and the preservation of the cytoskeleton was corroborated using Alexa 350-phalloidin (Invitrogen). Analysis of HDs: 804G cells transfected with the $\beta 4$ wt and mutant constructs were processed and stained as described above using 3E1 anti-human $\beta 4$ Ab that does not recognize rat $\beta 4$. The preparations were analyzed using fluorescence microscopy. A quantitative analysis was performed by counting the number of cells showing hemidesmosomal plaques as a percentage of the total number cells expressing similar levels of $\beta 4$. Videomicroscopy analysis of HaCat cells: HaCat cells grown on 60-mm plates were filmed digitally using time-lapse videomicroscopy on a Nikon Eclipse2000 inverted microscope (Melville, NY) equipped with a heated stage. The coordinates of the cells were registered before permeabilization, fixation, and staining for indirect immunofluorescence analysis as described above. The phase-contrast and fluorescent images were merged, and frame sequences were animated using image analysis software.

In Vitro PKC Kinase Assay

After immunoprecipitation of the $\alpha 6\beta 4$ integrin with anti- $\alpha 6\beta 4$ Ab and anti-rat agarose in RIPA buffer, the immunocomplexes were washed several times with RIPA buffer and resuspended in alkaline phosphatase buffer (New England Biolabs, Beverly, MA; no. 2 buffer) to be digested with alkaline phosphatase for 30 min at 37°C. The beads were washed with kinase buffer (50 mM Tris/HCl, pH 7.5, 10 mM MgCl₂) and resuspended in 50 μ l of kinase buffer containing 1 μ g of a PKC α (Calbiochem), 1 mM ATP, 140 μ M phosphatidylserine, 4 μ M DOG (Avanti, Birmingham, AL). The reaction was carried out for 30 min at 30°C. The samples were centrifuged, washed, and eluted in sample buffer before PAGE and electrotransfer onto a PVDF membrane. The membrane was blotted with anti-pS₁₄₂₄ Ab and visualized by chemiluminescence.

Biochemical Analyses

Whole Cell Extractions. Cells subjected to treatments indicated in the figures were extracted directly using sample buffer, and aliquots were processed for Western analysis.

Immunodepletion. HaCat cells were lysed in RIPA buffer and immunoprecipitated with anti-pS₁₄₂₄ Ab and anti-rabbit agarose for four sequential rounds. The remaining $\beta 4$ was concentrated using a Microcon filter (Millipore) and denatured in sample buffer. Aliquots of each step were processed for Western analysis.

Detergent Extractions. HaCat cells (0.3×10^6) were incubated overnight on two tissue culture dishes. One of the dishes was directly lysed using 200 μ l of sample buffer, which contained the total $\beta 4$ (T _{$\beta 4$}). A second dish was extracted twice using 1 ml of buffer containing 0.2% Triton X-100, 100 mM KCl, 200 mM sucrose, 10 mM EGTA, 2 mM MgCl₂, 1 mM PMSF, and 10 mM PIPES at pH 6.8 for 1 min, discarding the supernatant (soluble fraction) and adding 200 μ l of sample buffer (two times) to the remaining fraction (insoluble fraction I _{$\beta 4$}).

The samples were sonicated and boiled, and 15- μ l aliquots were resolved by SDS-PAGE (6 or 8%) and immunoblotted with the anti-pS₁₄₂₄ Ab first, developed using chemiluminescence, and then stripped and restained using a $\beta 4$ polyclonal antibody. Densitometric studies were performed with NIH Image software (<http://rsb.info.nih.gov/ij/>), and the fractions were calculated as follows: Soluble fraction = T _{$\beta 4$} - I _{$\beta 4$} ; Insoluble fraction = I _{$\beta 4$} .

Figure 1. Phosphopeptide analysis of the $\beta 4$ integrin and $\beta 4$ mutant S_{1424D}. (A) Radiolabeled HaCat cells were treated with EGF (100 ng/ml 15 min) and $\alpha 6\beta 4$ was immunoprecipitated and resolved by electrophoresis. The band corresponding to $\beta 4$ was excised and processed for peptide mapping. The phosphopeptide pp_f (arrow) was further examined by (B) Edman degradation, showing that pp_f has a phosphoserine on the fourth position; (C) phosphoamino acid analysis, showing that pp_f contains phosphoserine only; and (D) digestion with chymotrypsin, showing that pp_f can be cut with this enzyme and therefore indicating the presence of Y, W, or F. Two candidate peptides were identified (DYNS₁₄₂₄LTR and SQVS₁₀₂₅YR), and the corresponding serine was mutated within the wt $\beta 4$ cDNA using site-directed mutagenesis. The mutant cDNAs were transfected into Cos-7 cells and analyzed using peptide mapping. (E) Peptide map for DYND₁₄₂₄LTR

RESULTS

Identification of a Novel Phosphorylation Site on the $\beta 4$ Integrin Subunit: S₁₄₂₄

Using a peptide-mapping strategy, we previously identified three major sites of phosphorylation in the $\beta 4$ integrin: S₁₃₅₆, S₁₃₆₀, and S₁₃₆₄ (Rabinovitz *et al.*, 2004). To identify additional sites of phosphorylation by peptide mapping, HaCat cells were metabolically labeled with ³²PO₄ and stimulated with EGF, and detergent extracts were immunoprecipitated using a $\beta 4$ Ab. $\beta 4$ was then resolved by electrophoresis in one dimension, and the corresponding band was excised, digested with trypsin and then resolved using TLE in one dimension and TLC in the second dimension. This analysis yielded ~15 discernable phosphopeptides (Figure 1A), consistent with previous results (Rabinovitz *et al.*, 2004) and shows that most of the radioactivity (~50%) is contained in three phosphopeptides: pp₀, pp₁, and pp₂, that were previously shown to contain S₁₃₅₆, S₁₃₆₀, and S₁₃₆₄. A fourth major phosphopeptide pp_f (Figure 1A, arrow) was selected for identification by recovering the peptide from the TLC plate and performing the following assays: manual Edman degradation (Figure 1B), phosphoamino acid analysis (Figure 1C), and diagnostic secondary digest with chymotrypsin (Figure 1D). Phosphoamino acid analysis revealed that pp_f contained only phosphoserine (Figure 1C). It was apparent from Edman degradation that the pp_f was phosphorylated on the fourth position, because phosphate (³²PO₄) is shed during the fourth cycle by the cleaved amino acid (Figure 1B). Additional information on the phosphopeptides was obtained by comparing their digestion patterns with specific enzymes. Digestion with chymotrypsin (Figure 1D) showed that pp_f can be cut with this enzyme, therefore indicating the presence of a Y-X, F-X, or W-X bond. The PepSort4 program (www.genestream.org) was used to identify putative sequences for pp_f based on its mobility in TLE/TLC. This analysis revealed two phosphopeptides that have consistent mobility, are phosphorylated on the fourth serine and contain a Y or F as suggested by digestion assays: DYNS₁₄₂₄LTR and SQVS₁₀₂₅YR. To assess the validity of the identified sequences, site-specific mutagenesis and subsequent biochemical analyses were performed. We mutated the fourth serine of each of the candidate peptides. The $\beta 4$ _{S1025D} and $\beta 4$ _{S1424D} constructs were transiently transfected into Cos-7 cells, and 2 d later the cells were metabolically labeled with ³²PO₄, stimulated with EGF, and processed for peptide map-

Figure 2. Phosphorylation of S₁₄₂₄ mobilizes $\alpha 6\beta 4$ from HDs. (A–D) Double immunofluorescence analysis of 804G rat bladder carcinoma cells transfected with human $\beta 4$ cDNA. An anti-plectin Ab was used to detect any HD (A and C), and an anti-human $\beta 4$ mAb (B and D) was used to detect expression and incorporation of the wild-type (A and B) or mutant S $\rightarrow$ D₁₄₂₄ (C and D) $\beta 4$ cDNAs into HDs. Notice the characteristic pattern of HDs as “leopard spots” in the positive transfectant example of the wild-type construct (A and B; cell area is delineated) and the failure of the positive transfectant of the S $\rightarrow$ D₁₄₂₄ construct to get incorporated into HDs (C and D). Arrows point to the positive transfectants. (E) Quantitation of the effect of $\beta 4$ S $\rightarrow$ D mutations on HD dynamics. 804G transfected with the indicated S $\rightarrow$ D single or multiple mutation constructs were grown for 48 h and processed for indirect immunofluorescence using an anti-human $\beta 4$ mAb for analysis. The graph describes the percentage of positive transfectants showing the presence of the human mutant $\beta 4$ in HD plaques in relation to the wild-type human $\beta 4$ (wt, 100%). Data shown are means $\pm$ SE of three independent experiments in which at least 50 cells were analyzed. *p < 0.05 versus wild type, **p < 0.05 quadruple versus single or triple mutation.

ping (Figure 1E). The analysis showed that the construct $\beta 4_{S1424D}$ eliminated completely and specifically the signal of pp_f, whereas other phosphopeptides were preserved. In contrast transfection with $\beta 4_{S1025D}$ yielded results similar to the HaCat peptide map, failing to eliminate pp_f or any other phosphopeptide (results not shown). These results identify the phosphorylated residue in phosphopeptide pp_f as S₁₄₂₄.

Phosphorylation of S₁₄₂₄ Promotes Mobilization of $\beta 4$ from HD

To assess whether S₁₄₂₄ phosphorylation can induce the mobilization of $\beta 4$ from HDs, we transfected a phosphorylation-mimicking mutant of S₁₄₂₄ (SIID₁₄₂₄) or the wild-type $\beta 4$ into 804G rat bladder carcinoma cells. These cells produce type I HDs that are easily detected as a leopard-spot pattern where $\beta 4$, plectin, and BPAGs colocalize (Riddelle *et al.*, 1992; Figure 2, A and B). 804G HDs can be disassembled using EGF or OKA. We selected this cell line because our $\beta 4$ -derived constructs are from human origin and can be discerned from the endogenous $\beta 4$ of rat origin by immunofluorescence analysis using an anti-human $\beta 4$ Ab. Figure 2 exemplifies positive transfectants showing the presence of the expressed human construct in HD plaques (wild-type $\beta 4$; Figure 2, A and B) or the failure to do so ($\beta 4$ S $\rightarrow$ D₁₄₂₄; Figure 2, C and D). A quantitative analysis was performed by counting the percentage of human $\beta 4$ -pos-

itive transfectants showing HD plaques. Mutation of S₁₄₂₄ into aspartate (S $\rightarrow$ D₁₄₂₄) decreased the number of transfectants showing incorporation of the mutant $\beta 4$ into HDs by 40% (Figure 2E). Because previous works have suggested that the mobilization of $\beta 4$ is a cooperative effect of several phosphorylation sites (Rabinovitz *et al.*, 2004; Wilhelmsen *et al.*, 2007), we evaluated whether S₁₄₂₄ phosphorylation would collaborate with the previously described triple $\beta 4$ mutant (S $\rightarrow$ D₁₃₅₆D₁₃₆₀D₁₃₆₄). As shown in Figure 2E, D₁₃₅₆D₁₃₆₀D₁₃₆₄ was more potent than D₁₄₂₄ in reducing incorporation of the mutant into HDs (60% reduction), but its effect was significantly enhanced by the combination of the four mutations: D₁₃₅₆D₁₃₆₀D₁₃₆₄D₁₄₂₄ (95% reduction). These data suggest that S₁₄₂₄ phosphorylation by itself has a moderate impact in the mobilization of $\beta 4$ from HDs and further collaborates with other phosphorylation sites to complete HD disassembly.

Phosphorylation of S₁₄₂₄ Participates in the Disassembly of HDs Induced by EGF

To further characterize S₁₄₂₄ phosphorylation, we produced a phospho-specific antibody raised against a $\beta 4$ -derived peptide containing phosphorylated S₁₄₂₄. The antibody was affinity-purified by sequential columns containing the phosphorylated and unphosphorylated form of the S₁₄₂₄-containing peptide as ligands. To assess the specificity and sensi-

Figure 3. Epidermal growth factor and OKA induce the phosphorylation of S₁₄₂₄ and S→A₁₄₂₄ mutation increases resistance to EGF-induced mobilization of α6β4 from HDs. (A) Specificity of the anti-pS₁₄₂₄ Ab. Cos-7 cells were transfected with α6 integrin and wild-type or S→A₁₄₂₄ mutated β4 constructs. The transfectants were stimulated with EGF and lysed. β4 immunoprecipitates were separated using PAGE and electro-transferred onto nitrocellulose membranes. The membrane was blotted with the anti-pS₁₄₂₄ Ab, visualized using chemiluminescence, and then stripped and reprobed with a β4 polyclonal Ab. (B) HaCat keratinocytes were grown on plates, serum-starved overnight, and then treated or not with EGF (100 ng/ml) or OKA (1 μM) for 30 min. Lysates were processed for Western analysis (left). Membranes were blotted using anti-pS₁₄₂₄ Ab and then stripped and reprobed using a β4 polyclonal Ab. Right, densitometric analysis showing the mean ± SE of three independent experiments. (C) HaCat keratinocytes were grown on plates, serum-starved overnight and stimulated with EGF (100 ng/ml) for different times (min) as indicated. Western analysis was performed as described above (left). Right, densitometric analysis showing the mean ± SE of three independent experiments. (D) Resistance to EGF-induced mobilization of β4 from HD by nonphosphorylatable S→A₁₄₂₄ mutation of β4: 804G cells transfected with β4 wild-type or β4 S→A₁₄₂₄ constructs were incubated or not with EGF (25 ng/ml) for 2 h and processed for indirect immunofluorescence using an anti-human β4 mAb for analysis. The graph describes the percentage of positive transfectants showing the presence of the human β4 (S→A₁₄₂₄) mutant in HD plaques in relation to the wild-type human β4 (wt, 100%). Data shown are means ± SE of five independent experiments in which at least 50 cells were analyzed. *p < 0.05 versus wild type.

tivity of the phospho-specific Ab (anti-pS₁₄₂₄ Ab), we blotted β4 immunoprecipitates obtained from Cos-7 cells transfected with wild-type β4 or the β4 mutant S→A₁₄₂₄ and stimulated with EGF. Results in Figure 3A show that the S→A mutation of S₁₄₂₄ efficiently prevents reaction with the phospho-specific Ab, corroborating the specificity of the Ab.

We further characterized the phosphorylation of S₁₄₂₄ induced by EGF or the serine-phosphatase inhibitor okadaic acid (OKA, Figure 3B). Surprisingly, the phosphorylation of S₁₄₂₄ was relatively high in nonstimulated cells and moderately increased by EGF (2×) or more robustly with OKA (3×) at 30 min. We further assessed the kinetics of S₁₄₂₄ phosphorylation by EGF at different times (Figure 3C). EGF peaked the phosphorylation levels of S₁₄₂₄ at early times.

To evaluate the role of S₁₄₂₄ in the disassembly of HD induced by EGF, we transfected the mutant S→A₁₄₂₄ or wild-type β4 into 804G cells and evaluated their resistance to mobilization from HDs upon stimulation with EGF. We first confirmed that 804G cells respond to EGF and can phosphorylate their own β4 (Figure S1), as well as express similar levels of the transfected human β4 constructs (Figure S2). As shown in Figure 3D, the relative number of cells that incorporated the β4 mutant (S→A₁₄₂₄) into HDs in the absence of EGF was increased by 30% relative to the wild-type

control. In the presence of EGF the wild-type β4 was substantially mobilized from HDs (62%), whereas the β4 mutant (S→A₁₄₂₄) was resistant to mobilization. These results suggest that pS₁₄₂₄ is important in the mobilization of β4 produced by EGF, whereas the nonphosphorylated S₁₄₂₄ may stabilize β4 in HDs. The high level of S₁₄₂₄ constitutive phosphorylation suggests an additional function.

HaCat Keratinocytes Contain High Levels of S₁₄₂₄ Constitutive Phosphorylation That Can Be Further Increased in Carcinoma Cells

Previous studies have shown that the β4 integrin contains high levels of constitutive phosphorylation, the identity or function of which has not been defined (Mainiero *et al.*, 1996; Rabinovitz *et al.*, 1999). Our initial evaluation of S₁₄₂₄ suggested that this site may be highly phosphorylated in non-stimulated cells, and therefore we addressed the function of constitutively phosphorylated β4. To confirm that our antibody was not reacting against unphosphorylated β4 giving the impression of high levels of constitutive phosphorylation in HaCat keratinocytes, we dephosphorylated α6β4 immunocomplexes obtained from HaCat keratinocytes that were stimulated or not with the serine-phosphatase inhibitor calyculin A using alkaline phosphatase and then per-

Figure 4. High levels of constitutive pS₁₄₂₄ are observed in resting keratinocytes, and further increased in carcinoma cells. (A) Reactivity of anti-pS₁₄₂₄ Ab with dephosphorylated $\beta 4$. Phosphorylated $\alpha 6\beta 4$ was obtained from HaCat cells treated or not with phosphatase inhibitor calyculin A (50 ng/ml, 30 min) by immunoprecipitation. Immunocomplexes were or not dephosphorylated using alkaline phosphatase, resolved for Western blot analysis using as primaries first the anti-pS₁₄₂₄ Ab, and then, after stripping the membrane, a $\beta 4$ Ab. (B) Immunodepletion of S₁₄₂₄-phosphorylated $\beta 4$. A HaCat cell lysate containing S₁₄₂₄-phosphorylated $\beta 4$ was sequentially depleted in several rounds of immunoprecipitation using the anti-pS₁₄₂₄ Ab (lanes 1–4). The remaining fraction of $\beta 4$ was recovered by concentrating the lysate and running one fifth of the concentrate (lane 5). (C) Levels of pS₁₄₂₄ in different cell lines. Cell lysates obtained from HaCat keratinocytes, A431, or SCC25 squamous carcinoma cell lines were analyzed by Western blot using the anti-pS₁₄₂₄ Ab and then stripped and reprobed with $\beta 4$ Ab. The number under the lanes corresponds to densitometric ratio pS₁₄₂₄: $\beta 4$.

formed a Western analysis using the anti-pS₁₄₂₄ Ab. As shown in Figure 4A, the phosphorylation signal was completely eliminated by dephosphorylation of $\beta 4$, validating the observed constitutive phosphorylation of S₁₄₂₄.

To assess the prevalence of constitutively phosphorylated S₁₄₂₄ in resting cells, we used an immunodepletion strategy. A lysate obtained from HaCat cells was sequentially immunoprecipitated three times with the anti-pS₁₄₂₄ Ab and the nonprecipitated fraction of $\alpha 6\beta 4$ was recovered by concentrating the remaining lysate volume (Figure 4B). A densitometric analysis of these lanes reveals that 10% of the $\beta 4$ population is phosphorylated on S₁₄₂₄ in resting conditions.

To assess the levels of constitutively phosphorylated S₁₄₂₄ in normal and carcinoma cells in vitro, we performed a comparative Western analysis of HaCat keratinocytes and the squamous cell carcinoma lines A431 and SCC25, all of which express the $\beta 4$ integrin. The content of $\beta 4$ varied among the different cell lines but all showed a substantial level of pS₁₄₂₄ (Figure 4C). The pS₁₄₂₄: $\beta 4$ densitometric ratio was increased in squamous carcinoma cells as much as threefold. These data suggest that constitutive phosphorylation of S₁₄₂₄ $\beta 4$ in vitro can be significantly increased in carcinoma cells.

Constitutively Phosphorylated S₁₄₂₄ Functions at the Trailing Edge of Migrating Cells

We hypothesized that constitutively phosphorylated S₁₄₂₄ may reflect localized disassembly of HDs as a result of some asymmetric cellular process, such as migration, which can be observed in nonstimulated keratinocytes. We therefore determined the localization of pS₁₄₂₄ in HaCat keratinocytes using indirect immunofluorescence. $\alpha 6\beta 4$ in HaCat keratinocytes can be found in characteristic HD plaques and in

actin-rich protrusions (Figure 5, C and D) in both single and clustered cells, although in the latter HD plaques are more numerous. A variety of interesting distribution patterns was observed for pS₁₄₂₄. A frequent pattern was the enrichment of pS₁₄₂₄ at the trailing edge or areas of retraction and a reduced signal at the front of a cell, where lamellipodia were protruding. Figure 5 shows an example of a single cell and a group of two cells. The single cell shows a characteristic polarized orientation with a fan-shaped lamellae and a trailing edge with retraction fibers. In the cell group, the bottom cell shows lamellipodia, and the top cell exhibits characteristic retraction fibers, suggesting a trailing edge or retraction of the upper cell. The $\beta 4$ integrin (Figure 5, C and D) is distributed in the retraction fibers in the two examples as well as in the characteristic HD plaques distributed all around the cells (thick arrows). In contrast, pS₁₄₂₄ is enriched at the trailing edge of the single (Figure 5E) or the top cell (Figure 5F) particularly in retraction fibers (thin arrows), and in the case of the top cell, in some of the neighboring HD plaques near the retraction fibers. Staining gradually decreases toward the front of the cell, suggesting that HDs are phosphorylated and disassembled as the cell retracts an area rich in HDs. Colocalization of $\beta 4$ and pS₁₄₂₄ is shown in Figure 5G and H ($\beta 4$: green; pS₁₄₂₄: red; colocalization: yellow). In general, lamellipodia showed little pS₁₄₂₄ signal, as can be observed in Figure 5E and the lower cell of Figure 5F (arrowhead). To corroborate that in cell groups S₁₄₂₄ is enriched at the trailing edges, we performed videomicroscopy analysis to track the historical trajectory of a cell group before fixation and staining for immunofluorescence analysis. Figure 6A and Video S1 show a cluster of cells migrating in a coordinated manner in a clockwise direction (arrows) that were filmed for 15 min before fixation. Figure 6, B–D, illustrate the indirect immunofluorescence analysis of the filmed cells (total $\beta 4$: green; pS₁₄₂₄: red; colocalization: yellow) showing that S₁₄₂₄ at the trailing edge is phosphorylated in several of the migrating cells, and in most of the cells it shows a gradient of phosphorylation pointing opposite to the direction of the migration. These data suggest that phosphorylation of S₁₄₂₄ is induced at the trailing edge as the cell moves forward.

To assess whether the observed phosphorylated S₁₄₂₄ at the trailing edge has already disassembled, we treated the cells with a Triton X-100 buffer before fixation, because previously it has been shown that HD-associated $\beta 4$ is resistant to the detergent buffer (Rabinovitz *et al.*, 1999; Carter *et al.*, 1990). Most of the enriched $\beta 4$ and pS₁₄₂₄ signal in the retraction fibers disappears when cells are treated with the detergent buffer before fixation (Figure 7A, $\beta 4$; B, pS₁₄₂₄; and D, colocalization), suggesting that phosphorylated $\beta 4$ in this region has already detached from the HD. However, pS₁₄₂₄ could still be found within the HD plaques close to the margin of the cell or distributed within a localized region of the total HD area (Figure 7, arrows), suggesting that phosphorylation of S₁₄₂₄ may not be sufficient to disassemble the HD. As can be seen in this Figure 7, A–D, it was common to see increased pS₁₄₂₄ signal in only a few cells within a larger group, suggesting a local factor regulation. To assess more accurately the fraction of S₁₄₂₄-phosphorylated $\beta 4$ that is still associated with HDs, we determined percentage of $\beta 4$ that is present in the detergent-insoluble compartment. As can be observed in Figure 7E, 20.7% of the pS₁₄₂₄ signal is observed in the detergent insoluble compartment, indicating that a considerable amount of phosphorylated $\beta 4$ can still be associated to HDs.

Figure 5. pS_{1424} is enriched at the trailing edge and sites of retraction. HaCat keratinocytes plated on coverslips (A and B: phase contrast) were fixed and processed for double-stain indirect immunofluorescence using a $\beta 4$ mAb (C and D) and the anti- pS_{1424} Ab (E and F) and analyzed for coimmunolocalization (G and H: green, $\beta 4$; red, anti- pS_{1424} Ab: yellow, colocalization). Examples of single cells (A, C, E, and G) or cell clusters (B, D, F, and H). Notice the retraction fibers (arrows) and lamellipodia (arrowhead) suggesting movement in the direction of the lamellipodia and the characteristic pattern of HD plaques (thick arrows). Bars, 10 μm .

Constitutively S_{1424} -phosphorylated $\beta 4$ Is Present in HD Type II But Not Type I

We reasoned that the presence of pS_{1424} in HD plaques at the rear of the cell or areas undergoing retraction might be related to the differential ability of pS_{1424} to modulate interactions with other HD components. Keratinocytes express two types of HDs: type I, in which $\beta 4$ is associated with plectin and the BPAGs, and type II, in which $\beta 4$ is associated only with plectin (Koster *et al.*, 2003). Type I and type II probably represent two dynamic stages of HD maturation or disassembly (Koster *et al.*, 2003). We addressed this question by evaluating the degree of colocalization of pS_{1424} with plectin and the BPAGs using double immunostaining analysis. As shown in Figure 8, A–C, (plectin: green; pS_{1424} : red; colocalization: yellow) pS_{1424} was partially colocalized with plectin (arrow), usually at the margins of HD plaques closer

to the trailing edge (arrowhead: retraction fibers). In contrast, HD plaques containing BPAG1 and 2 conspicuously excluded pS_{1424} (Figure 8, D–F and G–I respectively). These data suggest that S_{1424} phosphorylation may be found in HD type II but not type I and that this phosphorylation may prevent the interaction of $\beta 4$ with BPAGs but not with plectin.

Constitutive Phosphorylation of S_{1424} Is Attachment Dependent

Cell attachment has been shown to modulate the stability and dynamics of $\beta 4$ (Geuijen and Sonnenberg, 2002). To determine whether S_{1424} constitutive phosphorylation depends on cell attachment, we compared levels of S_{1424} phosphorylation between cells kept in suspension, attached for long periods of time (20 h) or attached to laminin 5–enriched

Figure 6. Video-microscopy analysis of pS_{1424} distribution in migrating cells. HaCat cells were filmed by videomicroscopy for 15 min before fixation. The cells were processed for double-stain indirect immunofluorescence using the anti- pS_{1424} Ab and a $\beta 4$ mAb as primary Abs and Cy3- or Cy2-conjugated Abs as secondary. The cells were analyzed using (A) phase-contrast and (B–D) fluorescence microscopy. (B) Merged image of C and D: $\beta 4$, green; pS_{1424} , red; colocalization, yellow. (C) $\beta 4$, green. (D) pS_{1424} , red. Bar, 10 μm .

(LM5) or collagen I-coated dishes for short periods of time (min). As can be observed in Figure 9, cells in suspension drop their high level of constitutive phosphorylation and do

not start to recover until after 2.5 h of attachment in either LM5 or collagen I. Phosphorylation levels are completely recovered after 6 h on either substrate (data not shown).

Figure 7. Distribution of HD-associated pS_{1424} in HaCat keratinocytes. HaCat keratinocytes plated on coverslips were permeabilized with a Triton X-100 containing buffer before fixation to remove unassociated $\beta 4$ and double-stained for indirect immunofluorescence analysis using a $\beta 4$ mAb (A) and the anti- pS_{1424} Ab (B). Phase-contrast image of the cells (C). The cells were analyzed for coimmunolocalization (D: green, $\beta 4$; red, anti- pS_{1424} Ab; yellow, colocalization). Notice that the detergent treatment removes the strong pS_{1424} signal previously observed in retraction fibers and cell margins. Note that pS_{1424} signal is restricted to the HD plaques from one cell of the group. Bars, 10 μm . E. Detergent fractionation of S_{1424} -phosphorylated $\beta 4$. HaCat cells were plated on tissue culture dishes overnight and extracted or not with Triton X-100 buffer before lysing the remaining cells in sample buffer to obtain the total whole cell (WC) or insoluble (I) fractions of $\beta 4$. Lysate aliquots were analyzed by Western blot using the anti- pS_{1424} Ab. Densitometric measurements were used to calculate the insoluble pS_{1424} fraction as a percentage of the total pS_{1424} of the whole cell lysate as described in *Materials and Methods*.

E	WC	I	% insoluble:
pS_{1424}			20.7

Figure 8. pS₁₄₂₄ distributes to HD type II but not type I. HaCat cells were plated on coverslips and fixed with methanol. The cells were processed for double-stain immunofluorescence analysis to detect colocalization of pS₁₄₂₄ and plectin (HD type I and II) or BPAG1 and 2 (HD type I only). (A–C) Cells were stained with anti-plectin (A) and anti-pS₁₄₂₄ (B). (C) Merged image indicating colocalization area (yellow); arrowhead indicates retraction fibers and arrow points to a limited colocalization area close the cell's edge. (D–F) Cells were stained with anti-BPAG1 (D) and anti-pS₁₄₂₄ (E). Merged image (F): no colocalization was detected. (G–I) Cells were stained with anti-BPAG2 (G) and anti-pS₁₄₂₄ (H). (I) Merged image: no colocalization was detected. Bar, 10 μ m.

These data suggest that long-term attachment is clearly necessary for S₁₄₂₄ phosphorylation and that short-term binding to LM5 or collagen is not sufficient to produce high levels of phosphorylation.

The Phosphorylation of S₁₄₂₄ Is Mediated by PKC

Previous studies have shown that serine phosphorylation of S₁₃₅₆, S₁₃₆₀, and S₁₃₆₄ induced by EGF is mediated by PKC and PKA (Rabinovitz *et al.*, 2004; Wilhelmsen *et al.*, 2007). We assessed the role of conventional PKCs, PKA and MAPK in the phosphorylation of S₁₄₂₄ induced by EGF using inhibitors of these kinases. As shown in Figure 10A, Go6976, an inhibitor of conventional PKCs (Qatsha *et al.*, 1993) was able

to substantially inhibit the phosphorylation of S₁₄₂₄. In contrast PKA and MEK inhibitors H89 and PD697856 were unable to modify the response, suggesting that a conventional PKC is specifically involved in the phosphorylation of S₁₄₂₄. Moreover, the high constitutive levels of phosphorylated S₁₄₂₄ and those induced by OKA were reduced as well (Figure 10A), suggesting that these pathways share common downstream effectors. Our previous data have shown that PKC α can phosphorylate directly S₁₃₅₆, S₁₃₆₀, and S₁₃₆₄. We therefore examined the ability of this kinase to phosphorylate S₁₄₂₄ using an *in vitro* kinase assay. α 6 β 4 immunocomplexes obtained from HaCat cells were dephosphorylated using alkaline phosphatase and then exposed or not to recombinant PKC α . As shown in Figure 10B, PKC α efficiently phosphorylated S₁₄₂₄. Altogether these data suggest that a conventional PKC mediates the phosphorylation of S₁₄₂₄ in resting or EGF stimulated cells by directly phosphorylating β 4.

Figure 9. Constitutive phosphorylation of S₁₄₂₄ depends on long-term cell attachment. HaCat cells in suspension were plated on LM5-enriched or collagen I-coated plates for the indicated times (min). Cells were lysed and analyzed using Western blot and anti-pS₁₄₂₄ Ab. The membranes were stripped after visualization and reprobbed using a β 4 Ab.

DISCUSSION

During wound healing and carcinoma invasion HDs are disassembled in response to chemotactic factors such as EGF, allowing the cells to migrate. The mechanisms of regulation of HD disassembly are incompletely understood. Phosphorylation of the main components of the HD by chemotactic factors plays an important role in this process

Figure 10. Phosphorylation of S_{1424} is mediated by conventional PKC. (A) HaCat cells were plated on tissue culture dishes and serum-starved. The cells were then preincubated or not with inhibitors of conventional PKCs (Go6976), MEK (PD98059), or PKA (H89) for 30 min and then treated or not with EGF or OKA for an additional 30 min. The cells were lysed, and the samples resolved for a Western blot analysis using the anti- pS_{1424} Ab. The membrane was stripped after visualization and reprobed using a $\beta 4$ Ab. (B) In vitro phosphorylation of S_{1424} by $PKC\alpha$. Immunocomplexes of the $\beta 4$ integrin were obtained from HaCat keratinocytes using a $\beta 4$ Ab and agarose beads. The immunocomplexes were dephosphorylated with alkaline phosphatase, and aliquots were exposed to $PKC\alpha$ or kinase buffer only (control) as described in *Materials and Methods*. The eluted samples were resolved for a Western blot using anti- pS_{1424} Ab. After visualization using chemiluminescence, the membrane was stripped and reprobed with a $\beta 4$ Ab.

(Mainiero *et al.*, 1996; Rabinovitz *et al.*, 1999, 2004; Wilhelmsen *et al.*, 2007). The integrin $\alpha 6\beta 4$ is a downstream target of several chemotactic factors and contains about 15 serine and tyrosine phosphorylation sites, some of which have been shown to be important in promoting HD disassembly (Dans *et al.*, 2001; Rabinovitz *et al.*, 2004; Wilhelmsen *et al.*, 2007). An important observation in these studies has been that no individual phosphorylation site by itself can achieve the complete disassembly of the HD, and each needs the cooperation of additional sites to produce the effect. This generalization may be a reflection of the multiple interactions between the $\beta 4$ integrin and other hemidesmosomal components that need to be disrupted to disaggregate the structure. Therefore we have continued in our efforts to identify additional sites of phosphorylation. We have previously identified three phosphorylation sites on the connecting segment of the $\beta 4$ integrin, S_{1356} , S_{1360} , and S_{1364} , and we now identify a fourth phosphorylation site in the same region, S_{1424} , that appears to be functionally different. The identification of this site was supported by different independent tests such as peptide mapping, phosphoamino acid analysis, Edman degradation, TLE/TLC mobility, restricted digestion, mutational analysis, and phospho-specific antibodies. Although S_{1356} , S_{1360} , and S_{1364} are not significantly phosphorylated in resting keratinocytes until the cells are stimulated with EGF or PMA triggering HD disassembly (Rabinovitz *et al.*, 2004; Wilhelmsen *et al.*, 2007), S_{1424} shows a high level of constitutive phosphorylation in nonstimulated keratinocytes ($\sim 10\%$ of the total population of $\beta 4$). HaCat keratinocytes can still substantially increase the phosphorylation levels of S_{1424} after growth factor stimulation ($2\times$) or OKA treatment ($3\times$). Our data indicate that disabling this site by point mutation increases the incorporation of $\beta 4$ into HDs and resists HD disassembly induced by EGF, as demonstrated in 804G cells transfected with $\beta 4$ $S \rightarrow A_{1424}$ construct. Consistent with the idea that phosphorylation of S_{1424} mobilizes $\beta 4$ from HDs, the $\beta 4$ mutant $\beta 4$ $S \rightarrow D_{1424}$, which mimics constitutive phosphorylation, reduces the presence of $\beta 4$ in HDs. Our results also show that S_{1424} collaborates with S_{1356} , S_{1360} , and S_{1364} phosphorylation sites in HD

disassembly as the constructs containing the four $S \rightarrow D$ mutations are more efficient in producing disassembly than each group by itself.

The relatively high levels of constitutive S_{1424} phosphorylation in nonstimulated cells may indicate additional forms of regulation and function for the $\beta 4$ integrin using this site. Possible scenarios for S_{1424} function are processes that do not require the presence of chemoattractants such as HD turnover (Geuijen and Sonnenberg, 2002) or HD disassembly in response to local factors such as tension, which can promote tail retraction or repositioning of anchorage sites (Ridley *et al.*, 2003). Previous studies support the idea of a rapid turnover of $\beta 4$ in the HD, showing that even though the HD is a stable structure its equilibrium is highly dynamic, with turnover rates for the $\beta 4$ integrin of up to 50% in 5–30 min, depending on its interactions with the ECM (Geuijen and Sonnenberg, 2002; Tsuruta *et al.*, 2003). S_{1424} is a good candidate to be involved in turnover although its heterogeneous distribution within the $\beta 4$ population would imply that HD turnover varies according to the position of the HD within the cell. Rather, the distribution pattern of S_{1424} suggests a role in the HDs near the trailing edge and other sites of retraction. We observed that pS_{1424} is highly concentrated in retraction fibers and trailing edge and gradually reduced toward the front of the cell. This was confirmed by videomicroscopy analysis. The importance of tail retraction as a critical step in cell migration and polarization is well documented (Ridley *et al.*, 2003), and S_{1424} could have an indirect role in these processes by releasing HD anchors. Several regulatory molecules such as calpain, (Huttenlocher *et al.*, 1997; Wells *et al.*, 2005) Rho (Pertz *et al.*, 2006), and FAK (Iwanicki *et al.*, 2008), are thought to participate in the retraction process, and it will be interesting to evaluate their impact on $\beta 4$ function. Most of the S_{1424} -phosphorylated $\beta 4$ in retraction fibers and the trailing edge is loosely associated with HDs because a detergent buffer removes most of the pS_{1424} signal from these areas, suggesting that S_{1424} phosphorylation favors HD disassembly and that the disassembled S_{1424} -phosphorylated $\beta 4$ is being retrieved as the cell moves. However, a significant amount of phosphorylated $\beta 4$ is still associated with HDs as part of the detergent-insoluble fraction (20.7% of the S_{1424} -phosphorylated $\beta 4$). Importantly, these HDs show colocalization of pS_{1424} with plectin but completely exclude the BPAGs, suggesting that S_{1424} may initiate the disassembly by triggering the separation of the BPAGs and therefore transforming HD type I into HD type II, ($\beta 4$ + plectin). Because pS_{1424} may still be found in HD type II, additional events may be required to complete the disassembly, such as phosphorylation of additional sites on $\beta 4$ or plectin, or changes in conformation induced by other factors, such as tension. Keratinocytes contain both types of HDs within the same cell (Koster *et al.*, 2003), probably as part of a dynamic multistep process of gradual assembly/disassembly. It is worth mentioning that HDs type II are not incompatible with migration. Intestinal cells that are normally migrating along the villi present abundant HD type II but not type I (Beaulieu, 1997). and Cos-7 cells made to generate type II HDs do not curtail their migration (Rabinovitz *et al.*, 2004), suggesting that the BPAGs may play an important role in hindering migration. It is interesting that S_{1424} is immediately adjacent to a domain that is critical for BPAG1 binding (residue 1436–1457) and therefore may be modulating the interaction with this HD component (Koster *et al.*, 2003). In contrast, the observed colocalization of pS_{1424} with plectin suggests that it may play a secondary role in the regulation of the interaction of these two proteins, although it is worth mentioning that S_{1424} is within a region (1383–

1437) that has been shown to be important in the interaction of $\beta 4$ with a secondary site of plectin (Koster *et al.*, 2004). Altogether these data suggest that S_{1424} phosphorylation is induced by a retracting tail, which in turn promotes the gradual disassembly of HDs, relieving the anchoring, and allowing migration and retrieval of HD components.

Our data indicate that long-term attachment is required for keratinocytes to show S_{1424} phosphorylation. Cells in suspension quickly lose their high levels of pS_{1424} and it takes several hours of attachment before those levels are recovered. Moreover, cell spreading is also not sufficient to explain the delay in phosphorylation because the cells are spread after 40 min on LM5 or collagen (results not published). One possible scenario to explain why S_{1424} phosphorylation is dependent on long-term attachment is that it requires completion of HD assembly before it can be phosphorylated. Supporting this idea we found that HDs start to appear after 1 h of plating but need several hours for completion (results not published). It can also be inferred that pS_{1424} is not necessary for the assembly process. The fact that the mutation $S \rightarrow A_{1424}$ increases incorporation of $\beta 4$ into HDs further supports that phosphorylation is not necessary for HD formation. Similar S_{1424} phosphorylation kinetics were noted when cells were plated on LM5 and collagen I. This may be due to the rapid deposition of endogenous LM5 by keratinocytes (deHart *et al.*, 2003), which would allow cells plated on collagen I to assemble HDs. Consistent with this idea we observed that HDs are formed on LM5 or collagen I at a similar rate (results not published).

In regards to the dynamics of S_{1424} phosphorylation in the disassembly phase, it may be argued that phosphorylation is important during a short period of time during and after the disassembly process because most of the unassembled $\beta 4$, i.e., that in the detergent-soluble fraction, is not phosphorylated (~90%), suggesting that phosphatases may dephosphorylate $\beta 4$ quickly after disassembly is accomplished. If unassembled $\beta 4$ is not phosphorylated, what prevents it from incorporating into the HD? One possibility is that the reincorporation of $\beta 4$ into the HD may be limited by the availability of the other HD components. Supporting this idea is the massive mobilization of plectin in Cos-7 cells when $\alpha 6\beta 4$ integrin is transfected (Rabinovitz *et al.*, 2004). Initially plectin can be detected ubiquitously within the Cos-7 cells that lack $\alpha 6\beta 4$, but when $\alpha 6\beta 4$ is expressed, most of the plectin is quickly sequestered into plaques organized by the integrin on the basal surface while overexpressed $\alpha 6\beta 4$ may still be diffusely found outside the plaques, suggesting that plectin may be a limiting factor.

A conventional PKC has been implicated in the phosphorylation of S_{1356} , S_{1360} , and S_{1364} induced by EGF (Rabinovitz *et al.*, 2004), and recently PKA has been found to be involved specifically in the phosphorylation of S_{1364} (Wilhelmsen *et al.*, 2007). Go6976, a conventional PKC inhibitor, was previously shown to reduce $\beta 4$ phosphorylation, increase HD stability and resistance to growth factor-induced disassembly (Rabinovitz *et al.*, 1999, 2004). Our data suggest that either constitutive or induced phosphorylation of S_{1424} is regulated as well by a conventional PKC, as suggested by the inhibition of S_{1424} by Go6976, but not by H89 (PKA inhibitor), or PD98059 (MEK inhibitor). Moreover, the conventional PKC α can directly phosphorylate S_{1424} *in vitro*. These results suggest that PKC may be a common downstream effector of both growth factors and local cellular processes such as cell retraction. The preferential phosphorylation of S_{1424} at the rear of the cell raises the question whether PKC is increased at the trailing edge. We were not able to find enrichment of PKC α in this region (data not

shown), although activation of the kinase is a strong possibility because this region experiences tension during cell migration that can open stretch-activated calcium channels and PKC may be one of the downstream targets (Cullen, 2003; Ridley *et al.*, 2003). Calcium oscillations have been linked to an increase in PKC activity (Cullen, 2003) and could be a potential mechanism to explain the local phosphorylation of $\beta 4$ on S_{1424} . We are currently evaluating this hypothesis. A question that remains to be answered is why activated PKC in nonstimulated cells phosphorylates S_{1424} but not the other residues S_{1356} , S_{1360} , and S_{1364} in the connecting segment, which are not far away (~65 amino acids) and have been shown to be PKC substrates (Rabinovitz *et al.*, 2004). One possible scenario is that S_{1356} , S_{1360} , and S_{1364} may be masked by a certain $\beta 4$ conformation or by the presence of plectin that would prevent kinase access, implying that an additional event triggered by EGF would be required to unmask the serines. Clearly further studies are needed to address the dynamics of phosphorylation of the serines in the connecting segment, a region that seems to be critical in the regulation of $\beta 4$ interactions.

Changes in the dynamics of HDs have important implications in physiological and pathological processes such as wound healing and carcinoma invasion that depend on cell migration. We observed an increase in the levels of S_{1424} phosphorylation in two squamous carcinoma cell lines, suggesting the possibility that an increase in the phosphorylation of S_{1424} may be an important factor determining tumor progression in these types of carcinoma where HDs are reduced or absent but where $\beta 4$ expression remains elevated and redistributed around the membrane (Van Waes and Carey, 1992; Van Waes *et al.*, 1995; Herold-Mende *et al.*, 2001). Future studies examining the degree of $\beta 4$ phosphorylation in this type of carcinoma would be warranted.

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REFERENCES

- Beaulieu, J. F. (1997). Extracellular matrix components and integrins in relationship to human intestinal epithelial cell differentiation. *Prog. Histochem. Cytochem.* 31, 1–78.
- Boyle, W. J., van der Geer, P., and Hunter, T. (1991). Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. *Methods Enzymol.* 201, 110–149.
- Carter, W. G., Kaur, P., Gil, S. G., Gahr, P. J., and Wayner, E. A. (1990). Distinct functions for integrins $\alpha 3 \beta 1$ in focal adhesions and $\alpha 6 \beta 4$ in bullous pemphigoid antigen in a new stable anchoring contact (SAC) of keratinocytes: relation to hemidesmosomes. *J. Cell Biol.* 111, 3141–3154.
- Cormack, B. (2003). Directed mutagenesis using the polymerase chain reaction. In: *Current Protocols in Molecular Biology*, New York: John Wiley & Sons, Unit 8.5 (online).
- Cullen, P. J. (2003). Calcium signalling: the ups and downs of protein kinase C. *Curr. Biol.* 13, R699–R701.
- Dans, M., Gagnoux-Palacios, L., Blaikie, P., Klein, S., Mariotti, A., and Giancotti, F. G. (2001). Tyrosine phosphorylation of the $\beta 4$ integrin cytoplasmic domain mediates Shc signaling to extracellular signal-regulated kinase and antagonizes formation of hemidesmosomes. *J. Biol. Chem.* 276, 1494–1502.
- deHart, G. W., Healy, K. E., and Jones, J. C. (2003). The role of $\alpha 3 \beta 1$ integrin in determining the supramolecular organization of laminin-5 in the extracellular matrix of keratinocytes. *Exp. Cell Res.* 283, 67–79.
- Falcioni, R., Sacchi, A., Resau, J., and Kennel, S. J. (1988). Monoclonal antibody to human carcinoma-associated protein complex: quantitation in normal and tumor tissue. *Cancer Res.* 48, 816–821.

- Geuijen, C. A., and Sonnenberg, A. (2002). Dynamics of the $\alpha 6 \beta 4$ integrin in keratinocytes. *Mol. Biol. Cell* 13, 3845–3858.
- Gipson, I. K., Spurr-Michaud, S., Tisdale, A., Elwell, J., and Stepp, M. A. (1993). Redistribution of the hemidesmosome components $\alpha 6 \beta 4$ integrin and bullous pemphigoid antigens during epithelial wound healing. *Exp. Cell Res.* 207, 86–98.
- Green, K. J., and Jones, J. C. R. (1996). Desmosomes and hemidesmosomes - structure and function of molecular components [Review]. *FASEB J.* 10, 871–881.
- Herold-Mende, C., Kartenbeck, J., Tomakidi, P., and Bosch, F. X. (2001). Metastatic growth of squamous cell carcinomas is correlated with upregulation and redistribution of hemidesmosomal components. *Cell Tissue Res.* 306, 399–408.
- Hieda, Y., Nishizawa, Y., Uematsu, J., and Owaribe, K. (1992). Identification of a new hemidesmosomal protein, HD1, a major, high molecular mass component of isolated hemidesmosomes. *J. Cell Biol.* 116, 1497–1506.
- Huttenlocher, A., Palecek, S. P., Lu, Q., Zhang, W., Mellgren, R. L., Lauffenburger, D. A., Ginsberg, M. H., and Horwitz, A. F. (1997). Regulation of cell migration by the calcium-dependent protease calpain. *J. Biol. Chem.* 272, 32719–32722.
- Hynes, R. O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 69, 11–25.
- Iwanicki, M. P., Vomastek, T., Tilghman, R. W., Martin, K. H., Banerjee, J., Wedegaertner, P. B., and Parsons, J. T. (2008). FAK, PDZ-RhoGEF and ROCKII cooperate to regulate adhesion movement and trailing-edge retraction in fibroblasts. *J. Cell Sci.* 121, 895–905.
- Koster, J., Geerts, D., Favre, B., Borradori, L., and Sonnenberg, A. (2003). Analysis of the interactions between BP180, BP230, plectin and the integrin $\alpha 6 \beta 4$ important for hemidesmosome assembly. *J. Cell Sci.* 116, 387–399.
- Koster, J., van Wilpe, S., Kuikman, I., Litjens, S. H., and Sonnenberg, A. (2004). Role of binding of plectin to the integrin $\beta 4$ subunit in the assembly of hemidesmosomes. *Mol. Biol. Cell* 15, 1211–1223.
- Litjens, S. H., de Pereda, J. M., and Sonnenberg, A. (2006). Current insights into the formation and breakdown of hemidesmosomes. *Trends Cell Biol.* 16, 376–383.
- Litjens, S. H., Koster, J., Kuikman, I., van Wilpe, S., de Pereda, J. M., and Sonnenberg, A. (2003). Specificity of binding of the plectin actin-binding domain to $\beta 4$ integrin. *Mol. Biol. Cell* 14, 4039–4050.
- Mainiero, F., Pepe, A., Yeon, M., Ren, Y., and Giancotti, F. G. (1996). The intracellular functions of $\alpha 6 \beta 4$ integrin are regulated by EGF. *J. Cell Biol.* 134, 241–253.
- Mariotti, A., Kedeshian, P. A., Dans, M., Curatola, A. M., Gagnoux-Palacios, L., and Giancotti, F. G. (2001). EGF-R signaling through Fyn kinase disrupts the function of integrin $\alpha 6 \beta 4$ at hemidesmosomes: role in epithelial cell migration and carcinoma invasion. *J. Cell Biol.* 155, 447–458.
- Mercurio, A. M. (1995). Laminin receptors: achieving specificity through cooperation. *Trends Cell Biol.* 5, 419–423.
- Nievers, M. G., Schaapveld, R. Q., and Sonnenberg, A. (1999). Biology and function of hemidesmosomes. *Matrix Biol.* 18, 5–17.
- Pertz, O., Hodgson, L., Klemke, R. L., and Hahn, K. M. (2006). Spatiotemporal dynamics of RhoA activity in migrating cells. *Nature* 440, 1069–1072.
- Qatsha, K. A., Rudolph, C., Marme, D., Schachtele, C., and May, W. S. (1993). Go 6976, a selective inhibitor of protein kinase C, is a potent antagonist of human immunodeficiency virus 1 induction from latent/low-level-producing reservoir cells in vitro. *Proc. Natl. Acad. Sci. USA* 90, 4674–4678.
- Rabinovitz, I., and Mercurio, A. M. (1997). The integrin $\alpha 6 \beta 4$ functions in carcinoma cell migration on laminin-1 by mediating the formation and stabilization of actin-containing motility structures. *J. Cell Biol.* 139, 1873–1884.
- Rabinovitz, I., Toker, A., and Mercurio, A. M. (1999). Protein kinase C-dependent mobilization of the $\alpha 6 \beta 4$ integrin from hemidesmosomes and its association with actin-rich cell protrusions drive the chemotactic migration of carcinoma cells. *J. Cell Biol.* 146, 1147–1160.
- Rabinovitz, I., Tsomo, L., and Mercurio, A. M. (2004). Protein kinase C- α phosphorylation of specific serines in the connecting segment of the $\beta 4$ integrin regulates the dynamics of type II hemidesmosomes. *Mol. Cell Biol.* 24, 4351–4360.
- Riddelle, K. S., Hopkinson, S. B., and Jones, J. C. (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.
- Ridley, A. J., Schwartz, M. A., Burridge, K., Firtel, R. A., Ginsberg, M. H., Borisy, G., Parsons, J. T., and Horwitz, A. R. (2003). Cell migration: integrating signals from front to back. *Science* 302, 1704–1709.
- Santoro, M. M., Gaudino, G., and Marchisio, P. C. (2003). The MSP receptor regulates $\alpha 6 \beta 4$ and $\alpha 3 \beta 1$ integrins via 14-3-3 proteins in keratinocyte migration. *Dev Cell* 5, 257–271.
- Sefton, B. M. (1997). Phosphoamino acid analysis. In: *Current Protocols in Molecular Biology*, New York: John Wiley & Sons, 18.13.11–18.
- Tsuruta, D., Hopkinson, S. B., and Jones, J. C. (2003). Hemidesmosome protein dynamics in live epithelial cells. *Cell Motil. Cytoskeleton* 54, 122–134.
- Uematsu, J., Nishizawa, Y., Sonnenberg, A., and Owaribe, K. (1994). Demonstration of type II hemidesmosomes in a mammary gland epithelial cell line, BMGE-H. *J. Biochem.* 115, 469–476.
- Van Waes, C., and Carey, T. E. (1992). Overexpression of the A9 antigen/ $\alpha 6 \beta 4$ integrin in head and neck cancer. *Otolaryngol. Clin. N. Am.* 25, 1117–1139.
- Van Waes, C., Surh, D. M., Chen, Z., Kirby, M., Rhim, J. S., Brager, R., Sessions, R. B., Poore, J., Wolf, G. T., and Carey, T. E. (1995). Increase in suprabasilar integrin adhesion molecule expression in human epidermal neoplasms accompanies increased proliferation occurring with immortalization and tumor progression. *Cancer Res.* 55, 5434–5444.
- Wells, A., Huttenlocher, A., and Lauffenburger, D. A. (2005). Calpain proteases in cell adhesion and motility. *Int. Rev. Cytol.* 245, 1–16.
- Wilhelmsen, K., Litjens, S. H., Kuikman, I., Margadant, C., van Rheeën, J., and Sonnenberg, A. (2007). Serine phosphorylation of the integrin $\beta 4$ subunit is necessary for epidermal growth factor receptor induced hemidesmosome disruption. *Mol. Biol. Cell* 18, 3512–3522.