Tyrosine Phosphorylation of the β4 Integrin Cytoplasmic Domain Mediates Shc Signaling to Extracellular Signal-regulated Kinase and Antagonizes Formation of Hemidesmosomes

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Ligation of the α6β4 integrin induces tyrosine phosphorylation of the β4 cytoplasmic domain, followed by recruitment of the adaptor protein Shc and activation of mitogen-activated protein kinase cascades. We have used Far Western analysis and phosphopeptide competition assays to map the sites in the cytoplasmic domain of β4 that are required for interaction with Shc. Our results indicate that, upon phosphorylation, Tyr1440, or secondarily Tyr1422, interacts with the SH2 domain of Shc, whereas Tyr1526, or secondarily Tyr1462, interacts with its phosphotyrosine binding (PTB) domain. An inactivating mutation in the PTB domain of Shc, but not one in its SH2 domain, suppresses the activation of Shc by α6β4. In addition, mutation of β4 Tyr1526, which binds to the PTB domain of Shc, but not of Tyr1422 and Tyr1440, which interact with its SH2 domain, abolishes the activation of ERK by α6β4. Phenylalanine substitution of the β4 tyrosines able to interact with the SH2 or PTB domain of Shc does not affect incorporation of α6β4 in the hemidesmosomes of 804G cells. Exposure to the tyrosine phosphatase inhibitor orthovanadate increases tyrosine phosphorylation of β4 and disrupts the hemidesmosomes of 804G cells expressing recombinant wild type β4. This treatment, however, exerts a decreasing degree of inhibition on the hemidesmosomes of cells expressing versions of β4 containing phenylalanine substitutions at Tyr1422 and Tyr1440, at Tyr1526 and Tyr1462 or at all four tyrosine phosphorylation sites. These results suggest that β4 Tyr1526 interacts in a phosphorylation-dependent manner with the PTB domain of Shc. This event is required for subsequent tyrosine phosphorylation of Shc and signaling to ERK but not formation of hemidesmosomes.

Basement membranes regulate the survival, proliferation, and differentiation of cells through ligation of integrin receptors (1–4). The α6β4 integrin is a major receptor for the basement membrane component laminin-5 and is expressed in a variety of epithelial cells, in Schwann cells, in certain endothelial cells, and in CD4+ CD8+ T cells (5, 6). The cytoplasmic domain of β4, which is unusually long and dissimilar in amino acid sequence from the corresponding portions of other integrin β subunits, enables α6β4 to recruit the adaptor protein Shc as well as to promote the assembly of hemidesmosomes (5, 6).

Shc is an SH2/PTB1 domain adaptor protein that couples a variety of receptor and nonreceptor tyrosine kinases, cytokine receptors, immune receptors, and adhesion receptors to Ras signaling (7, 8). In most cases, Shc binds to upstream tyrosine phosphorylated molecules through its SH2 domain, PTB domain, or both. It is then phosphorylated on tyrosine and recruits the Grb2/SOS complex, which can subsequently activate Ras. By this mechanism, Shc participates in mediating the proliferative functions of many receptors. In addition, it regulates cell migration (9–11). Although many receptor tyrosine kinases must interact directly with Shc to induce its tyrosine phosphorylation and activation, the EGF receptor is capable of signaling through Shc even when prevented from binding to it (12, 13). In addition, whereas α6β4 can interact directly with Shc (14), a subset of β4 and α6 integrins recruit Shc indirectly through Src family kinases (15, 16).

Dominant negative studies suggest that Shc is required to couple the α6β4 integrin to Ras and thereby both the Raf-ERK and Rac-JNK signaling cascades. Through these pathways, α6β4 cooperates with growth factor receptors to promote immediate-early gene expression and progression through the G1 phase of the cell cycle (17). Mice carrying a targeted deletion of the cytoplasmic domain of β4 display proliferative defects in the skin and gastro-intestinal tract, suggesting that signaling pathways activated by the cytoplasmic domain of β4, probably through Shc, are required for optimal epithelial cell proliferation in vivo (18).

In addition to its signaling function, the cytoplasmic domain of β4 plays a crucial role in the assembly of hemidesmosomes (18, 19). The hemidesmosomes are adhesive junctions that mediate stable attachment of stratified and transitional epithelia to the basement membrane. They differ from focal adhe-

* This work was supported by National Institutes of Health Grants R01-CA58976 and P30-CA08748. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviations used are: SH2, Src homology 2; PTB, phosphotyrosine binding; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; mAb, monoclonal antibody; GST, glutathione S-transferase; HRP, horseradish peroxidase; FTTC, fluorescentes isothiocyanate; DMEM, Dulbecco’s modified Eagle’s medium; PBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; Fn, fibronectin; TAM, tyrosine-based activation motif.
sions because they are linked to the keratin instead of the actin cytoskeleton. In accordance with the role of hemidesmosomes in mediating strong adhesion to the basement membrane, defects in hemidesmosome integrity cause epidermal fragility and skin blistering (6, 20). The assembly of hemidesmosomes is likely to require interaction of the cytoplasmic domain of β4 with HDI/plectin and BPAG2 (21–23).

Recent studies have indicated that ligation of αbβ4 promotes phosphorylation of the β4 cytoplasmic domain through activation of an integrin-associated Src family kinase. Because Src kinases are known to activate Shc (24), it is possible that αbβ4 activates Shc through the integrin-associated Src kinase independently of direct binding of Shc to the cytoplasmic domain of β4. We have thus examined whether αbβ4-mediated Shc signaling requires direct binding of Shc to the β4 cytoplasmic domain. Our results indicate that the SH2 and PTB domain of Shc bind to separate phosphoryrosines in the cytoplasmic domain of β4. The interaction mediated by the PTB domain is essential for phosphorylation of the Shc binding sites in β4. This phosphorylation of the Shc binding sites in β4 antagonizes formation of hemidesmosomes.

MATERIALS AND METHODS

Immunohistochemical Reagents—The rabbit anti-phosphotyrosine antibody and the mAb against the extracellular domain of β4 (3E1) were characterized previously (17, 25). The rabbit polyclonal antibodies to GST were affinity purified on a GST-Sepharose column from sera of rabbits immunized with GST fusion proteins. The biotinylated anti-phosphotyrosine mAb 4G10 and protein A-agarose were purchased from Upstate Biotechnologies. The recombinant anti-phosphotyrosine antibody RC20 was from Transduction Laboratories. The anti-Myc tag mAb 9E10 was obtained from the Sloan-Kettering Hybridoma Facility and horseradish peroxidase-labeled anti-Myc tag mAb 9E10 from Roche Molecular Biochemicals. The mouse monoclonal antibody M2 to FLAG tag was from Kodak. The rabbit polyclonal antibodies to phospho-ERK were purchased from VEC Technologies and cultured on gelatin-coated dishes in DMEM with 10% FBS (Life Technologies, Inc.) in a total of 10 ml of DMEM for 5 h, according to manufacturer’s recommendations. Cells were allowed to recover for 24 h prior to serum starvation. Ras bladder carcinoma. Rat bladder carcinoma. Rat bladder carcinoma.

Fusion Proteins—GST fusion proteins were expressed in BL21-RES cells (Strategene) treated with 0.1 mM isosporyl-1-thio β-D-galactopyranoside for 3 h at 37 °C and purified on glutathione-agarose as described previously (28). Proteins were eluted by incubating glutathione-agarose beads three times for 5 min at room temperature with an equal volume of 100 mM Tris, pH 5.5, 120 mM sodium chloride, 0.1% Triton X-100, and freshly added 20 mM glutathione. Aliquots were stored at −20 °C until use.

Far Western Analysis—After overnight serum starvation, 293T cells were left untreated or treated for 5 min at 37 °C with 100 μM orthovanadate and 3 mM hydrogen peroxide, washed with ice-cold PBS, and lysed in lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10 mM EDTA) supplemented with 5 mM EDTA, 1% Triton X-100, 1 mg/ml G418. Aliquots containing 1 mg of total proteins were immunoprecipitated for 3 h with 5 μg of 3E1 plus 30 μl of packed protein-G agarose. Immunocomplexes were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with TBS, 0.1% Tween (TBST) containing 5% milk for 1 h at room temperature and incubated with 1 μg/ml GST Shc SH2 or PTB domain fusion proteins in TBST-2.5% milk, 1% dithiothreitol, 10 μg/ml aprotinin, leupeptin, and pepstatin A. After three washes with TBST, the membranes were incubated with 1 μg/ml anti-GST rabbit polyclonal antibody in TBST-2.5% milk, washed again, and then incubated with protein-A-HRP in TBST-5% milk. After five washes with TBST and two washes with TBST-0.2% Triton X-100, the membranes were rinsed with TBS and incubated with ECL for 1 min before exposure to film. To assess phosphorylation of β4, the membranes were stripped for 30 min at 50 °C in 62.5 mM Tris, pH 6.7, 2% SDS, and 100 mM β-mercaptoethanol, rinsed extensively with TBS, and then probed by immunoblotting with RC20 according to the manufacturer’s recommendations. To control for 4x levels of β4, membranes were reprobed, blocked with TBST, 5% milk and probed by immunoblotting with 1 μg/ml anti-β4 exo in TBS, 3% BSA followed by protein A-HRP.

Peptide Competition Assay—HaCat cells were serum starved and stimulated as described above. β4 was immunoprecipitated with the 3E1 mAb from lysates containing 2 mg of total proteins and transferred to nitrocellulose. Membranes were cut, and Far Western blotting was performed on gelatin-coated dishes in DMEM with 10% FBS (Life Technologies, Inc.) in a total of 10 ml of DMEM for 5 h, according to manufacturer’s recommendations. Cells were allowed to recover for 24 h prior to serum starvation. Ras bladder carcinoma. Rat bladder carcinoma. Rat bladder carcinoma.

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incubated with 20 μg/ml of biotin-conjugated 4G10 followed by streptavidin-HRP. To examine tyrosine phosphorylation of β4, HUVECs were transfected with mutant versions of β4, as described above, allowed to recover for 24 h, serum starved overnight, and treated with 1 μM sodium orthovanadate and 3 mM hydrogen peroxide. After extraction in lysis buffer, β4 was immunoprecipitated with 5 μg of 3E1 mAb and 30 μl of protein G-agarose, and probed by immunoblotting with RC20.

Mitogen-activated Protein Kinase Assay—Dishes were coated with 10 μg/ml rabbit anti-mouse IgGs for 2 h at room temperature, saturated with 0.5% heat-denatured BSA (fatty acid, globulin-free), and then incubated with 20 μg/ml 3E1 mAb overnight at 4 °C. Serum starved cells were detached with 2 mM EDTA, washed with DMEM, 0.2% BSA, and kept in suspension in DMEM, 0.2% BSA, and subdivided in 300–μl aliquots. Cells were incubated on ice for 40 min with 10 μg of anti-β4 mAb 3E1, washed with 700 μl of cold DMEM, resuspended in 200 μl of cold DMEM containing 5 μg of horse anti-mouse IgG, and incubated at 37 °C for 5 min. After one wash with cold PBS, the cells were pelleted and lysed in 800 μl of lysis buffer with phosphatase and protease inhibitors. Recombinant Shc proteins were immunoprecipitated with a rabbit anti-Myc polyclonal antibody, separated by SDS-PAGE, and transferred to nitrocellulose. The membranes were probed by immunoblotting with 1 μg/ml of biotin-conjugated 4G10 followed by streptavidin-HRP. To examine tyrosine phosphorylation of β4, HUVECs were transfected with mutant versions of β4, as described above, allowed to recover for 24 h, serum starved overnight, and treated with 1 μM sodium orthovanadate and 3 mM hydrogen peroxide. After extraction in lysis buffer, β4 was immunoprecipitated with 5 μg of 3E1 mAb and 30 μl of protein G-agarose, and probed by immunoblotting with RC20.

Immunofluorescence—804G cells were cultured on glass coverslips for 24 h in DMEM, 10% FBS and then overnight in DMEM, 1% FBS in the presence of the indicated concentrations of sodium orthovanadate. The cells were fixed with methanol at −20 °C for 20 min and stained with 10 μg/ml 3E1 mAb followed by 2 μg/ml FITC-conjugated anti-mouse IgGs. Samples were examined with a Zeiss fluorescent microscope.

RESULTS

Identification of the β4 Tyrosine Phosphorylation Sites Required for Interaction with the SH2 and the PTB Domain of Shc—GST pull-down assays with SDS-denatured extracts have shown that the isolated SH2 and PTB domain of Shc can interact directly with the tyrosine phosphorylated β4 subunit in vitro (17). To identify the sequences of β4 cytoplasmic domain mediating the interaction with the SH2 and the PTB domain of Shc, we transiently transfected 293T cells with the constructs encoding the wild type or deleted β4 subunits illustrated in Fig. 1. Antibody or laminin-5-mediated ligation of α5β1 promotes tyrosine phosphorylation of β4 in vivo, but this event is rapidly reversed, presumably by tyrosine phosphatases. By contrast, treatment of the cells with the tyrosine phosphatase inhibitor pervanadate causes high level and persistent tyrosine phosphorylation of β4. We thus used this protocol to increase tyrosine phosphorylation of β4 in mapping experiments.

After immunoprecipitation from cells treated with pervanadate, α5β1 was separated by SDS-PAGE and probed by Western blotting with GST fusion proteins containing the SH2 or PTB domain of Shc or by immunoblotting with anti-phosphotyrosine antibodies. As shown in Fig. 2A, the SH2 domain of Shc bound to wild type β4 and to β4 mutants containing the connecting segment (E and M) in a tyrosine phosphorylation-dependent manner. It did not, however, bind to mutants lacking the connecting segment (G and I), despite the fact that they were still phosphorylated on tyrosine. We consistently observed that the mutants E and M, which lack the membrane proximal region of the cytoplasmic domain, were phosphorylated to a higher stoichiometry than wild type β4. It is possible that the segment deleted in E and M is necessary for efficient association with a tyrosine phosphatase able to reverse β4 phosphorylation. Alternatively, the deletion may bring some of the β4 tyrosine phosphorylation sites in closer proximity to the tyrosine kinase that phosphorylates them. The mutants truncated after the second fibronectin (Fn) type III repeat (J and C) did not become phosphorylated on tyrosine. Because these mutants can still associate with Src kinases, it is likely that the major tyrosine phosphorylation sites in β4 reside downstream of the second Fn type III module. Taken together, these observations suggest that the SH2 domain of Shc binds to phosphorylated tyrosines located within the connecting segment of β4.

The connecting segment contains two potential tyrosine phosphorylation sites that conform to the consensus for binding to the SH2 domain of Shc (31). We thus mutated these two tyrosines (Tyr4122 and Tyr4140) to phenylalanine either separately or in combination. The resulting versions of β4 were able to bind their ability to bind the SH2 domain of Shc. Fig. 2A shows that the SH2 domain of Shc does not bind to β4 Y1422F/Y1440F and binds to a modest extent to β4 Y1440F. By contrast, the SH2 domain of Shc interacts effectively with β4 Y1422F. These results suggest that the SH2 domain of Shc binds primarily to phosphorylated Tyr4140 and secondarily to phosphorylated Tyr4122 in the connecting segment of β4.

The PTB domain of Shc binds to phosphorylated tyrosines in the context of NXXY motifs (24, 29). The cytoplasmic domain of

FIG. 1. Structure of integrin β4 constructs. The features of wild type (A) and mutant forms of human β4 used in this study are shown. TM, transmembrane domain; black box, type III Fn-like repeat; gray box, connecting segment; F, phenylalanine substitution. For brevity, some of the mutants are referred to in the text as indicated on the right.
\( \beta_4 \) contains three NXXY motifs: one in the region between the transmembrane domain and the other two downstream of the connecting segment, one in the third and the other in the fourth Fn type III repeat. As shown in Fig. 2B, the PTB domain of Shc bound to phosphorylated \( \beta_4 \) mutant E as effectively as to phosphorylated wild type \( \beta_4 \), but it did not interact with phosphorylated mutant M. This result suggests that the major binding site for the PTB domain of Shc in \( \beta_4 \) resides downstream of the connecting segment and possibly corresponds to the NXXY motif in the third or that in the fourth Fn type III repeat. Therefore, versions of \( \beta_4 \) containing phenylalanine substitutions of the tyrosines within each of these two NXXY sites (Tyr\(^{1526}\) and Tyr\(^{1642}\)) were analyzed by Far Western blotting with the Shc PTB domain (Fig. 2B). Phenylalanine substitution of either Tyr\(^{1526}\) or Tyr\(^{1642}\) decreased only partially binding of the PTB domain to \( \beta_4 \), with substitution of Tyr\(^{1526}\) resulting in a significantly greater reduction. Mutation of both sites in combination (Y1526F/Y1642F) completely prevented the binding of PTB domain (Fig. 2B). Phenylalanine substitution of either Tyr\(^{1422}\), Tyr\(^{1526}\), or Tyr\(^{1642}\) in \( \beta_4 \) decreased only partially binding of the PTB domain to \( \beta_4 \), with substitution of Tyr\(^{1526}\) resulting in a significantly greater reduction. Mutation of both sites in combination (Y1526F/Y1642F) completely prevented the binding of PTB domain to \( \beta_4 \). These results suggest that the PTB domain of Shc binds primarily to Tyr\(^{1526}\) and secondarily to Tyr\(^{1642}\) in \( \beta_4 \).

Phosphopeptide competition assays were performed to compare the relative affinities of the potential binding sites for the SH2 or PTB domain of Shc in \( \beta_4 \) (see Table I for peptide sequences). For these experiments, we used HaCaT keratinocytes, which express endogenous \( \alpha_6 \beta_4 \). Cells were either left untreated or treated with pervanadate. After immunoprecipitation, \( \alpha_6 \beta_4 \) was transferred to nitrocellulose and probed with GST fusion proteins containing the SH2 or PTB domain of Shc in the absence or presence of tyrosine phosphorylated synthetic peptides modeled after the sequences surrounding Tyr\(^{1422}\), Tyr\(^{1526}\), or Tyr\(^{1642}\) in \( \beta_4 \) (Table I). As positive controls, we used tyrosine phosphorylated peptides reproducing the high affinity binding sites for the SH2 and PTB domain of Shc in the

![Fig. 2. Far Western analysis indicates that \( \beta_4 \) Tyr\(^{1440} \) is the major binding site for the SH2 domain of Shc and Tyr\(^{1526} \) is the major binding site for its PTB domain.](http://www.jbc.org/)

**Table I**

| Peptide Sequence | Sequence |
|------------------|----------|
| \( \beta_4 \) Tyr(P)\(^{1422} \) | LTRD pY NSLTRSE |
| \( \beta_4 \) Tyr(P)\(^{1440} \) | LPRD pY STLTSVS |
| Platelet-derived growth factor receptor | DGHE pY IVYDFMQ |
| \( \beta_4 \) Tyr(P)\(^{1526} \) | DLLPNHS pY VFRV |
| \( \beta_4 \) Tyr(P)\(^{1642} \) | GLSENVP pY VFKY |
| MT Ag | SLLSNPT pY SVMR |
| \( \beta_4 \) Tyr(P)\(^{1526} \) | DLLPNHS Y VFRV |

As shown in Fig. 3A, the peptides \( \beta_4 \) pY\(^{1422} \) and \( \beta_4 \) pY\(^{1440} \) inhibited the binding of the SH2 domain of Shc to wild type \( \beta_4 \) to a similar extent, suggesting that Tyr\(^{1422} \) and Tyr\(^{1440} \) are both potential binding sites for the SH2 domain of Shc. It is likely that the SH2 domain of Shc binds preferentially to Tyr\(^{1440} \) (Fig. 2B) because this tyrosine is more efficiently phosphorylated than Tyr\(^{1422} \) in vivo (14, 17). Tyr\(^{1440} \) may thus be the main physiologic binding site for the SH2 domain of Shc in \( \beta_4 \). Neither \( \beta_4 \) Tyr(P)\(^{1526} \) nor a nonphosphorylated peptide (\( \beta_4 \) Tyr\(^{1526} \)) were able to inhibit the binding of the SH2 domain of Shc to \( \beta_4 \).

![Fig. 3B.](http://www.jbc.org/)

Fig. 3B shows that the phosphorylated peptide \( \beta_4 \) Tyr(P)\(^{1526} \), but not \( \beta_4 \) Tyr(P)\(^{1642} \), inhibited the binding of the PTB domain of Shc to wild type \( \beta_4 \). Unphosphorylated \( \beta_4 \) Tyr(P)\(^{1526} \) as well as phosphorylated \( \beta_4 \) Tyr(P)\(^{1440} \) had no effect. Together with those of mutational analysis (Fig. 2B), these results indicate that Tyr\(^{1526} \) is the primary binding site for the PTB domain of Shc in \( \beta_4 \).

**Table II**

| Sequences of peptides used in competition studies |
|--------------------------------------------------|
| Peptide Sequence | |
| \( \beta_4 \) Tyr(P)\(^{1422} \) | LTRD pY NSLTRSE |
| \( \beta_4 \) Tyr(P)\(^{1440} \) | LPRD pY STLTSVS |
| Platelet-derived growth factor receptor | DGHE pY IVYDFMQ |
| \( \beta_4 \) Tyr(P)\(^{1526} \) | DLLPNHS pY VFRV |
| \( \beta_4 \) Tyr(P)\(^{1642} \) | GLSENVP pY VFKY |
| MT Ag | SLLSNPT pY SVMR |
| \( \beta_4 \) Tyr(P)\(^{1526} \) | DLLPNHS Y VFRV |

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Phosphorylation of the \( \beta_4 \) Integrin Cytoplasmic Domain

\( \beta_4 \) cytoplasmic domain, we used versions of Shc carrying inactivating mutations in either the SH2 domain (R397K), PTB domain (F198V), or both domains. The SH2 domain mutation resides within the conserved FLVRES motif and prevents the interaction with phosphotyrosine, whereas the PTB domain mutation prevents interaction with the hydrophobic residue at position -5 and with the asparagine at position -3 in the \( \Psi X X X Y \) motif (32). As shown in Fig. 4A, each of these mutations prevented the binding of a GST fusion protein containing the corresponding domain of Shc to tyrosine phosphorylated \( \beta_4 \) in vitro (Fig. 4A).

We examined the ability of \( \alpha_6 \beta_4 \) to activate in vivo versions of Shc containing inactivating mutations in either or both the SH2 and PTB domains. HeLa cells were transfected with Myc-tagged wild type or mutant versions of Shc. HeLa cells were chosen because they are easily transfectable, express endogenous \( \alpha_6 \beta_4 \), and were used previously to study \( \alpha_6 \beta_4 \) signaling (17) as well as EGF receptor activation of the same Shc mutants (13). To specifically ligate \( \alpha_6 \beta_4 \), cells were incubated in suspension with the anti-\( \beta_4 \) mAb 3E1, followed by an anti-mouse secondary antibody. The recombinant Shc proteins were immunoprecipitated with an anti-Myc antibody and analyzed by immunoblotting with anti-phosphotyrosine antibodies. As shown in Fig. 4B, both wild type and SH2 mutant Shc (R397K) were efficiently phosphorylated on tyrosine in response to ligation of \( \alpha_6 \beta_4 \). By contrast, the PTB mutant (F198V) and double mutant (F198V/R397K) versions of Shc were not phosphorylated upon EGF receptor stimulation. All four versions of Shc were efficiently phosphorylated upon treatment of the cells with EGF (Fig. 4C), as shown previously (13). These results indicate that a functional PTB domain is necessary for \( \alpha_6 \beta_4 \)-mediated Shc signaling and imply that Shc has to bind to \( \beta_4 \).

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**Fig. 3.** Phosphopeptides modeled after \( \beta_4 \) sequences comprising Tyr\(^{1422} \) and Tyr\(^{1440} \) inhibit binding of \( \beta_4 \) to the SH2 domain of Shc, whereas a phosphopeptide including Tyr\(^{1428} \) prevents interaction with the PTB domain. HaCat cells were either left untreated (−) or treated with pervanadate (+). After immunoprecipitation, \( \alpha_6 \beta_4 \) was transferred to nitrocellulose. Pieces of membrane containing individual bands of phosphorylated \( \beta_4 \), were incubated with GST-Shc-SH2 (A) or GST-Shc-PTB (B) in the absence or presence of 100 \( \mu M \) of the indicated phosphorylated and nonphosphorylated peptides (top panels). Membranes were stripped and reprobed with anti-phosphotyrosine antibodies (middle panels) and then stripped again and reprobed with antibodies against the extracellular domain of \( \beta_4 \) (bottom panels).

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**Fig. 4.** \( \alpha_6 \beta_4 \)-mediated phosphorylation of Shc requires an intact PTB, but not SH2, domain. A, mutations that inactivate the SH2 or PTB domain of Shc abolish binding of the corresponding domain to \( \beta_4 \) in vitro. HaCat cells were either left untreated (−) or treated with pervanadate (+). After immunoprecipitation, \( \alpha_6 \beta_4 \) was transferred to nitrocellulose. Pieces of membrane containing bands of phosphorylated \( \beta_4 \) were probed with wild type (WT) or mutant (R397K) GST-Shc-SH2 domain or with wild type or mutant (F198V) GST-Shc-PTB domain (top panels). Membranes were then stripped and reprobed with anti-phosphotyrosine antibodies (middle panels) and then stripped again and reprobed with antibodies against the extracellular domain of \( \beta_4 \) (bottom panels). B, an inactivating mutation in the PTB domain of Shc, but not one in its SH2 domain, prevents phosphorylation of Shc upon ligation of \( \alpha_6 \beta_4 \). HeLa cells were transiently transfected with Myc-tagged versions of wild type Shc or versions of Shc carrying mutations in the SH2 domain (R397K), the PTB domain (F198V), or both (F198V/R397K). Cells were incubated in suspension with the anti-\( \beta_4 \) mAb 3E1 followed by anti-mouse IgGs. Control cells (lanes C) were stimulated with secondary anti-mouse IgGs alone. After immunoprecipitation, the Myc-recombinant proteins were probed with anti-phosphotyrosine antibodies (top panel). The membranes were then stripped and reprobed with anti-Myc antibodies (bottom panel). C, as a control, adherent cells were either left untreated (−) or were treated with 250 ng/ml EGF for 5 min (+). After immunoprecipitation, the Myc-recombinant proteins were probed with anti-phosphotyrosine antibodies (top panel). The membranes were then stripped and reprobed with anti-Myc antibodies (bottom panel). EGF receptor-mediated phosphorylation of Shc is not prevented by mutation of the SH2 domain of Shc, its PTB domain, or both, as reported previously.
through this domain to become phosphorylated on tyrosine by the αβ₄-associated kinase.

Signaling by αβ₄ Requires Interaction with the PTB, but Not SH2, Domain of Shc—In HeLa cells, αβ₄ signaling to ERK proceeds through Shc (17). We thus examined whether the binding of Shc to β₄ was required also for activation of ERK. To test this hypothesis, we chose to use primary HUVECs because they do not express αβ₄ and can therefore be transfected with various mutant versions of β₄. These cells offer a better model for examining αβ₄ signaling than fibroblastic cells because αβ₄ is expressed in certain endothelial cells in vivo (5, 6). In addition, because ERK is only constitutively activated in established cell lines, nonimmortalized cells such as the HUVECs enable a more accurate assessment of the activation of ERK.

To verify that αβ₄ activated ERK in a Shc-dependent manner in HUVECs, these cells were transfected with constructs encoding αβ₄ alone or in combination with increasing doses of a FLAG-tagged version of dominant negative Shc carrying phenylalanine permutations at both potential Grb2 binding sites. After serum starvation, the cells were detached and replated onto dishes coated with the anti-β₄ mAb 3E1. As shown in Fig. 5A, dominant negative Shc inhibited αβ₄-induced activation of ERK in a dose-dependent manner, suggesting that αβ₄ signaling to ERK proceeds through Shc also in HUVECs.

We next analyzed the relative efficiency of phosphorylation of the β₄ tyrosines involved in binding to the SH2 and PTB domain of Shc. Previous studies using phosphopeptide mapping had identified Tyr₁⁴⁴⁹ as a major tyrosine phosphorylation site in β₄ and revealed that β₄ is phosphorylated at several additional tyrosines in vivo (14, 17). HUVECs were transfected

**FIG. 5.** aβ₄-mediated activation of ERK requires phosphorylation of β₄ Tyr₁⁴⁴⁹, which mediates interaction with the PTB domain of Shc. A, dominant negative Shc inhibits activation of ERK by αβ₄ in HUVECs. HUVECs were transiently transfected with wild type α and β₄ in combination with increasing amounts of vector encoding FLAG-tagged dominant negative (Dn) Shc (7.5, 15, and 30 μg). Cells were detached and replated for 60 min on dishes coated with the anti-β₄ mAb 3E1. Because dishes were post-coated with BSA, only the cells expressing αβ₄ (approximately 30%) attached. Total proteins from cells in suspension or plated for the indicated times on dishes coated with the 3E1 mAb were probed by immunoblotting with antibodies against Shc and then stripped and reprobed with antibodies against the extracellular domain of Shc. Previous studies using phosphopeptide mapping had identified Tyr₁⁴⁴⁹ as a major tyrosine phosphorylation site in β₄ and revealed that β₄ is phosphorylated at several additional tyrosines in vivo (14, 17). HUVECs were transfected with αβ₄ and either wild type (A) or the indicated mutant versions of β₄ (Y1422F, Y1440F, Y1526F, Y1642F, and 4F). Cells were either left untreated (−) or treated with pervanadate (+) and lysed. After immunoprecipitation, αβ₄ was separated by SDS-PAGE and probed with antibodies against phosphorylated ERK (top panel) or the FLAG epitope (middle panel). Blots were stripped and reprobed with antibodies against total ERK (bottom panel) as control. B, phenylalanine substitution of Tyr₁⁴⁴⁹ and Tyr₁⁵₂⁶ inhibits vanadate-induced phosphorylation of β₄. HUVECs were transiently transfected with αβ₄ and either wild type (A) or the indicated mutant versions of β₄ (Y1422F, Y1440F, Y1526F, Y1642F, and 4F). Cells were either left untreated (−) or treated with pervanadate (+) and lysed. After immunoprecipitation, αβ₄ was separated by SDS-PAGE and probed by immunoblotting with antibodies against phosphotyrosine (top panel) and then stripped and reprobed with antibodies against the extracellular domain of β₄ (bottom panel). C, a version of β₄ containing phenylalanine substitutions at the PTB domain binding sites (Y1526F/Y1642F) does not activate ERK as efficiently as wild type β₄ or a version with phenylalanine substitutions at the SH2 domain binding sites (Y1422F/Y1440F). HUVECs were transfected with αβ₄ together with wild type (A) or the indicated mutant forms of β₄ (Y1526F/Y1642F and Y1422F/Y1440F). Total proteins from cells in suspension or plated for the indicated times on dishes coated with the 3E1 mAb were probed by immunoblotting with antibodies against β₄ (top panel) or phosphorylated ERK (middle panel). The blot was stripped and re-probed with an antibody to total Erk2 (bottom panel) as control. D, phenylalanine substitution of the primary PTB domain binding site in β₄ prevents Erk activation. HUVECs transfected with αβ₄ (Y1526F, Y1642F, Y1526F/Y1642F, and L) were plated on 3E1-coated dishes for 60 min. Total proteins were probed by immunoblots with antibodies against β₄ (top panel) or phosphorylated ERK (middle panel). The blot was then stripped and reprobed with an antibody recognizing total Erk2 (bottom panel).
with mutant forms of $\beta_4$ carrying phenylalanine substitutions at each one or all four tyrosines (Tyr1422, Tyr1440, Tyr1526, and Tyr1642) capable of binding to Shc and stimulated with pervanadate (Fig. 5F). The phosphorylation of $\beta_4$ was analyzed by immunoblotting with antibodies against phosphorytosine. Phenylalanine substitution of either Tyr1440 or Tyr1526 resulted in a significant decrease in total phosphorylation of $\beta_4$, whereas phenylalanine substitutions at Tyr1422 or Tyr1642 had a negligible effect. Mutation of all four tyrosines to phenylalanine (4F) resulted in an almost complete block in tyrosine phosphorylation. With the caveat that the anti-phosphotyrosine antibodies may not interact with equal affinity with all phosphorylated tyrosines in $\beta_4$, these results suggest that Tyr1440 and Tyr1526 are the major phosphorylation sites in $\beta_4$.

To examine whether the interaction of Shc with $\beta_4$ was required for signaling to ERK, the HUVECs were transfected with constructs encoding o6 in combination with versions of $\beta_4$ unable to bind to either the SH2 domain (Y1422F/Y1440F) or the PTB domain (Y1526F/Y1642F) of Shc. Cells were then replated onto 3E1-coated dishes and analyzed by immunoblotting with anti-phospho-ERK antibodies. As shown in Fig. 5C, ligation of $\beta_4$ Y1422F/Y1440F caused activation of ERK, although slightly less efficiently than ligation of wild type $\beta_4$, whereas ligation of $\beta_4$ Y1526F/Y1642F did not induce activation of ERK. Immunoblotting with a polyclonal antibody against the extracellular domain of $\beta_4$ (Fig. 5C, top panel) and fluorescence-activated cell sorting analysis (data not shown) confirmed that the different versions of $\beta_4$ were expressed at comparable levels. These results provide evidence that $\alpha\beta_4$ signaling to ERK requires interaction of the PTB domain of Shc with $\beta_4$. This finding is consistent with the observation that $\alpha\beta_4$ signaling to ERK proceeds through Shc (Fig. 5B) and the activation of Shc by $\alpha\beta_4$ requires a functional PTB domain (Fig. 4B).

Finally, we examined the relative importance of the two potential PTB binding sites in $\beta_4$ for activation of ERK. As shown in Fig. 5D, ligation of $\beta_4$ Y1526F did not cause activation of ERK, whereas ligation of $\beta_4$ Y1642F induced activation of ERK, although slightly less efficiently than wild type $\beta_4$. These results indicate that $\beta_4$ Tyr1526, the primary binding site for the PTB domain of Shc, is required for activation of ERK.

**Mutation of the Major Tyrosine Phosphorylation Sites Does Not Prevent Incorporation of the Canonical Form A of $\beta_4$ into Hemidesmosome-like Adhesions**—The cytoplasmic domain of $\beta_4$, and in particular the segment comprising the first two Fn type III modules and the connecting segment, is required for formation of hemidesmosomes (18, 19). Our initial studies had suggested that Tyr1422 and Tyr1440 (which resemble a tyrosine-based activation motif or TAM) were necessary for incorporation of recombinant $\beta_4$ into the hemidesmosome-like adhesions formed by 804G cells in culture (14). Subsequent studies have, however, indicated that residues C-terminal to residue 1355 in the $\beta_4$ cytoplasmic domain are not required for the formation of hemidesmosome-like adhesions by cultured cells (23, 33). To resolve this discrepancy, we have performed a number of additional studies. Among them, an analysis of the entire coding sequence of the constructs used in our previous study revealed that the $\beta_4$ mutant Y1422F/Y1440F (called the TAM mutant or YZ in (14)) originated from a version of $\beta_4$ that contained an in-frame deletion of the sequences encoding amino acids 880–886 (DHTIVDT). Because this sequence is located in the membrane proximal portion of the cytoplasmic domain of $\beta_4$, which previous studies had indicated to be dispensable for incorporation in hemidesmosome-like adhesion structures (19, 23), we had not covered it during our initial sequence reanalysis. The nature and origin of the variant cDNA lacking amino acids 880–886 remains to be examined. As shown below, this deletion (termed the Gap) in combination with the Y1422F/Y1440F mutation prevents incorporation of $\beta_4$ into hemidesmosomes in 804G cells, in agreement with our original result.

The 804G cells are a rat bladder carcinoma cell line that expresses endogenous $\alpha\beta_4$ and forms hemidesmosome-like adhesions in culture. Immunofluorescence staining of hemidesmosomal components reveals that the hemidesmosome-like adhesions of these cells are arranged in a “Swiss cheese” pattern (34). We transfected 804G cells with constructs encoding human versions of either the canonical (A) or various variant forms of $\beta_4$ (Y1422F/Y1440F, Y1526F/Y1642F, 4F, Gap or Gap-Y1422F/Y1440F). Pools of cells stably expressing these recombinant forms of $\beta_4$ (see “Materials and Methods”) were stained with the 3E1 mAb that recognizes human but not rat $\beta_4$. As shown in Fig. 6A, the $\beta_4$ variants Y1422F/Y1440F, Y1526F/Y1642F, 4F, and Gap localized to hemidesmosomes as efficiently as wild type $\beta_4$ (A), whereas the $\beta_4$ mutant Gap-Y1422F/Y1440F displayed a significantly decreased ability to localize to hemidesmosomes. Thus, mutation of the potential $\beta_4$ TAM impairs localization of $\beta_4$ to hemidesmosomes only in the context of the Gap version of $\beta_4$. These results indicate that the integrity of Tyr1422 and Tyr1440 is not required for incorporation of $\beta_4$ in hemidesmosome-like adhesions, as shown previously by others (23). However, the synergy between the TAM mutation and the Gap suggests that Tyr1422 and Tyr1440 may play a role in assembly of hemidesmosomes. It is possible that a full assessment of this role requires examination of the assembly of bona fide hemidesmosomes in skin organ culture systems or in vivo.

**Phosphorylation of the $\beta_4$ Tyrosines Involved in the Recruitment of Shc Antagonizes Assembly of Hemidesmosomes**—Hemidesmosomes are structures that provide stable adhesion of epithelial cells to the underlying basement membranes and are therefore disassembled during cell migration (34, 35). Because phenylalanine substitution of the tyrosines that bind to Shc does not prevent incorporation of the canonical form of $\beta_4$ in hemidesmosomes, it is very unlikely that the phosphorylation of these sites participates in the assembly of hemidesmosomes (Ref. 23 and Fig. 6A).

We have previously shown that treatment with EGF induces tyrosine phosphorylation of $\beta_4$ and disassembly of hemidesmosomes in keratinocytes, although we did not establish a cause-effect relationship (36). To examine whether tyrosine phosphorylation of $\beta_4$ causes disassembly of hemidesmosomes, we used 804G cell lines expressing wild type $\beta_4$ (A) or the $\beta_4$ mutants Y1422F/Y1440F, Y1526F/Y1642F, or 4F. Because these cells express very low levels of EGF receptor, they were treated with either 20 or 100 $\mu$M orthovanadate for 15 h to increase tyrosine phosphorylation of $\beta_4$. When orthovanadate is added in the absence of hydrogen peroxide, it behaves as a competitive inhibitor of tyrosine phosphatases, whereas in the presence of hydrogen peroxide it is converted to the irreversible inhibitor pervanadate (37). We used orthovanadate for these experiments because prolonged exposure to pervanadate causes cell toxicity. Treatment with 20 $\mu$M orthovanadate induced tyrosine phosphorylation of wild type $\beta_4$ but not of the mutant 4F in 804G cells (data not shown). As shown in Fig. 6B, 20 $\mu$M orthovanadate caused disruption of hemidesmosomes in 804G cells expressing wild type $\beta_4$ (A) but not in those expressing any of the mutant versions of $\beta_4$ (Y1422F/Y1440F, Y1526F/Y1642F, or 4F). In the presence of 100 $\mu$M orthovanadate, most 804G cells expressing wild type $\beta_4$ (A) rounded up and almost completely detached (>90% detached), whereas cells expressing the mutant versions of $\beta_4$ were protected from disruption of hemidesmosomes to varying degrees. Those expressing the $\beta_4$
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mutant 4F were the most protected (−20% detached, some remaining hemidesmosomes), whereas those expressing the \(\beta_4\) mutant Y1526F/Y1642F were protected to a lesser degree (−30% detached), and those expressing the \(\beta_4\) mutant Y1422F/Y1440F were the least protected (−60% detached). Therefore, phosphorylation of these tyrosines can lead to a reduction of hemidesmosomes and may be a physiologic mechanism for regulating hemidesmosome turnover. Treatment with 100 ng/ml EGF for 15 h resulted in a modest and equivalent disruption of hemidesmosomes in all four transfectants (data not shown), suggesting that the partial disassembly of hemidesmosomes caused by EGF in 804G cells may also involve an additional mechanism, as suggested previously (38).

**DISCUSSION**

Ligation of the \(\alpha_\text{d}\beta_4\) integrin induces tyrosine phosphorylation of the cytoplasmic domain of \(\beta_4\), recruitment of Shc, and activation of Ras-dependent mitogen-activated protein kinase cascades (14, 17). Biochemical and genetic evidence implies that \(\alpha_\text{d}\beta_4\)-dependent signaling promotes proliferation of both keratinocytes and intestinal epithelial cells (17, 18). To assess the specific role of Shc in these processes, it is necessary to identify the mechanism by which Shc binds to \(\beta_4\) and determine whether this binding is required for activation of downstream signaling pathways. The results of this study provide evidence that the PTB domain of Shc interacts in a phosphorylation-dependent manner with Tyr\(^{1526}\) in \(\beta_4\), whereas the SH2 domain binds to Tyr\(^{1440}\). Both phenylalanine substitution of Tyr\(^{1526}\) in \(\beta_4\) and inactivation of the PTB domain in Shc suppress \(\alpha_\text{d}\beta_4\)-mediated phosphorylation of Shc and signaling to ERK. By contrast, mutation of both the primary and secondary binding site for the Shc SH2 domain in \(\beta_4\) or inactivation of the SH2 domain itself exert only a minor effect on Shc signaling to ERK. These observations suggest that the binding of the PTB domain of Shc to Tyr\(^{1526}\) in \(\beta_4\) is crucial for subsequent phosphorylation of Shc by the \(\alpha_\text{d}\beta_4\)-associated kinase and thus activation of Ras-dependent pathways. It remains, however, possible and indeed likely that the interaction mediated by the SH2 domain of Shc contributes to some extent to the stability of the association of Shc with \(\beta_4\) in vitro.

The primary \(\beta_4\) binding site for the PTB domain of Shc, Tyr\(^{1526}\), is located in the third Fn type III module. Interestingly, all known type III Fn repeats contain a tyrosine at this position. Fn type III repeats are found not only in extracellular matrix proteins (fibronectin and tenascin) and cell surface receptors (for growth hormone, prolactin, and insulin) but also in cytoplasmic components (twitchin, titin, and the \(\beta_4\) cytoplasmic domain) (39). To our knowledge, this study is the first to identify a physiologically relevant phosphorylation site in a Fn type III module. Because several of these repeats have been crystallized, including the N-terminal pair in \(\beta_4\), it is possible to predict the conformation of \(\beta_4\) Tyr\(^{1526}\) and surrounding amino acids. The residues N-terminal to tyrosine 1526 that are also important for interaction with the PTB domain of Shc, namely the asparagine and leucine at the −3 and −5 positions, are predicted to be in a loop between the E and F strands of the \(\beta\)-sandwich. Based on its location in a loop between \(\beta\) strands, this sequence motif is likely to be exposed to solvent in the intact molecule. This position would facilitate its phosphorylation and subsequent interaction with the PTB domain of Shc. In addition, there is evidence suggesting that the C-terminal portion of the \(\beta_4\) tail binds to a more proximal segment comprising the second Fn type III repeat and the connecting segment in vitro (23). If this intramolecular interaction occurs also in vivo, it may bring the third Fn type III repeat closer to the plasma membrane and thus facilitate the interaction of the Src family kinase with Tyr\(^{1526}\) in \(\beta_4\) and thereby the recruitment of...
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Shc. This mechanism would also enable Shc to position Grb2/SOS in closer proximity to its target Ras.

The SH2 domain of Shc interacts in a phosphorylation-dependent manner primarily with Tyr$^{1440}$ and secondarily with Tyr$^{1422}$ in $\beta_4$. In agreement with the observation that these two sites are homologous to one another and both conform well to the consensus for binding to the SH2 domain of Shc (XXL), our results show that phosphopeptides encompassing both sites inhibit to a similar extent the interaction of the SH2 domain of Shc with $\beta_4$. It is likely that Tyr$^{1440}$ is more important in both

in vitro and in vivo experiments because it is more efficiently phosphorylated (see also Refs. 14 and 17). Based on the observation that phenylalanine substitutions at Tyr$^{1422}$ and Tyr$^{1440}$ prevent incorporation of a form of $\beta_4$ lacking amino acids 880–886 in the hemidesmosome-like adhesions of 804G cells, we had made the hypothesis that phosphorylation of both tyrosines would interfere with the association of $\beta_4$ with this model and thus destabilize hemidesmosomes.

The simplest hypothesis is that these tyrosines of $\beta_4$ interact with a component, such as BPAG2 (21, 23) that confers stability to hemidesmosomes. Phosphorylation of these tyrosines would interfere with the association of $\beta_4$ with this component and thus disrupt hemidesmosomes. This model is attractive also because it potentially explains why phenylalanine substitution of these tyrosines reduces association of BPAG2 with hemidesmosomes (21, 23), and we have shown here that it prevents incorporation of a form of $\beta_4$ lacking amino acids 880–886 in the hemidesmosome-like adhesions of 804G cells. Further experiments will be required to test this model.

Our results indicate that tyrosine phosphorylation of $\beta_4$ induces Shc signaling but antagonizes formation of hemidesmosomes, suggesting that these two processes may be mutually exclusive. It has been reported that processing of the $\alpha_{5}\beta_4$ ligand laminin-5 inhibits assembly of hemidesmosomes and promotes cell migration (40–42). It will be interesting to determine whether this process also affects $\alpha_{5}\beta_4$ signaling to Shc. If so, differential processing of laminin-5 may help direct whether $\alpha_{5}\beta_4$ ligation leads to tyrosine phosphorylation and Shc signaling or hemidesmosome formation.

The finding that the region of the $\beta_4$ cytoplasmic domain required for Shc signaling is distinct from that required for hemidesmosome formation may enable further studies on the role of $\alpha_{5}\beta_4$ signaling. For example, analysis of knock-in mice expressing a version of $\beta_4$ unable to signal through Shc but still able to promote assembly of hemidesmosomes will enable a more comprehensive understanding of the physiologic role of $\alpha_{5}\beta_4$ signaling.
Tyrosine Phosphorylation of the β4 Integrin Cytoplasmic Domain Mediates Shc Signaling to Extracellular Signal-regulated Kinase and Antagonizes Formation of Hemidesmosomes

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J. Biol. Chem. 2001, 276:1494-1502.
doi: 10.1074/jbc.M008663200 originally published online October 23, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M008663200

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