Signal transduction by the α6β4 integrin: distinct β4 subunit sites mediate recruitment of Shc/Grb2 and association with the cytoskeleton of hemidesmosomes

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We have examined the mechanism of signal transduction by the hemidesmosomal integrin α6β4, a laminin receptor involved in morphogenesis and tumor progression. Immunoprecipitation and immune complex kinase assays indicated that antibody- or laminin-induced ligation of α6β4 causes tyrosine phosphorylation of the β4 subunit in intact cells and that this event is mediated by a protein kinase(s) physically associated with the integrin. Co-immunoprecipitation and GST fusion protein binding experiments showed that the adaptor protein Shc forms a complex with the tyrosine-phosphorylated β4 subunit. Shc is then phosphorylated on tyrosine residues and recruits the adaptor Grb2, thereby potentially linking α6β4 to the ras pathway. The β4 subunit was found to be phosphorylated at multiple tyrosine residues in vivo, including a tyrosine-based activation motif (TAM) resembling those found in T and B cell receptors. Phenylalanine substitutions at the β4 TAM disrupted association of α6β4 with hemidesmosomes, but did not interfere with tyrosine phosphorylation of Shc and recruitment of Grb2. These results indicate that signal transduction by the α6β4 integrin is mediated by an associated tyrosine kinase and that phosphorylation of distinct sites in the β4 tail mediates assembly of the hemidesmosomal cytoskeleton and recruitment of Shc/Grb2.

Keywords: hemidesmosomes/integrins/Shc/signaling/tyrosine phosphorylation

Introduction

Basement membranes provide cells with positional cues which can affect their proliferation and differentiation (Adams and Watt, 1993). It is now clear that cell–matrix interactions are in large part mediated by integrins (Ruoslathi, 1991; Hynes, 1992) and that ligation of integrins results in intracellular signaling (Juliano and Haskill, 1993; Giancotti and Mainiero, 1994). Many of the influences of basement membranes on cellular behavior can be recapitulated in vitro by laminins or blocked with anti-laminin antibodies (Adams and Watt, 1993). It is therefore important to elucidate the mechanisms by which binding of laminins to integrins results in the activation of signal transduction pathways.

Laminins are a growing family of obligatory components of basement membranes expressed in a tissue- and development-specific manner (Engvall, 1993). At least six cell surface receptors, including various β1 integrins and the α6β4 integrin, have been implicated in binding to laminins and in many cases their binding specificities appear to overlap (Mercurio, 1990; Hynes, 1992). Cell adhesion to laminins, however, results in different patterns of gene expression depending on cell type and perhaps developmental stage (Di Persio et al., 1991; Roskelley et al., 1994), suggesting that specific signals may result from the engagement of distinct laminin binding integrins in different cells.

Focal adhesion kinase (FAK) (Shaller et al., 1992) has been implicated in signaling from β1 and β3 integrins (Guan and Shalloway, 1992; Hanks et al., 1992; Lipfert et al., 1992). There is evidence suggesting that FAK can link integrins to the ras signaling pathway (Slaaepfer et al., 1994), as well as induce intracellular changes which are potentially important for assembly of the actin cytoskeleton, such as phosphorylation of paxillin and tensin (Burridge et al., 1992; Bockholt and Burridge, 1993) and activation of Rho (McNamee et al., 1992; Chong et al., 1994). However, the mechanisms by which β1 and β3 integrins activate FAK have remained elusive so far. In particular, since ligation of the platelet integrin αmβ3 causes a cascade of tyrosine phosphorylation events prior to activation of FAK (Huang et al., 1993) and since the latter event requires an additional co-stimulus provided by an agonist receptor (Shattil et al., 1994), it is possible that FAK does not lie immediately downstream of the integrins. Thus although these results establish the role of integrins in signaling, they do not clarify how laminin-derived signals are transduced at the plasma membrane and how specificity of signaling is achieved.

The α6β4 integrin is a receptor for various laminins and binds with the highest relative affinity to laminins 4 and 5 (Spinardi et al., 1995). The highest levels of expression of α6β4 are observed in the basal cell layer of stratified epithelia (Kajiji et al., 1989), at the ends of endothelial sprouts during angiogenesis (Enenstein and Kramer, 1994), in Schwann cells at the onset of myelination (Einheber et al., 1993) and in CD4+ CD8+ pre-T lymphocytes entering the thymus (Wadsworth et al., 1992), suggesting the involvement of α6β4 in various morphogenetic events. In addition, increased levels of α6β4 are expressed in squamous, but not basal, carcinomas in humans (Kimmel and Carey, 1986; Savoia et al., 1993) and suprabasal expression of α6β4 is associated with malignant progression during mouse skin carcinogenesis (Tenneman et al., 1993). Elucidation of the signal transduction mechanism of the α6β4 integrin may, therefore, help us to understand

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the differential effects induced by basement membranes in different normal cell types, as well as the significance of \( \alpha_6 \beta_4 \) up-regulation in cancer cells.

The cytoplasmic domain of \( \beta_4 \) may provide the \( \alpha_6 \beta_4 \) integrin with unique cytoskeletal and signaling interactions. The \( \beta_4 \) tail is very large (~1000 amino acids) and bears no homology with the short cytoplasmic domains of other known \( \beta \) subunits, including the \( \beta_1 \) and \( \beta_3 \) integrins, which are known to activate FAK. It contains, toward its C-terminus, two pairs of type III fibronectin (Fn)-like modules interrupted by a 142 amino acid long sequence (Connecting Segment) (Hogervorst et al., 1990; Suzuki and Naitoh, 1990). Furthermore, in contrast to the \( \beta_1 \) and \( \beta_2 \) integrins, which localize to focal adhesions, the \( \alpha_6 \beta_4 \) integrin is found concentrated in hemidesmosomes (Carter et al., 1990; Stepp et al., 1990). Recent results demonstrate that \( \alpha_6 \beta_4 \) plays a necessary role in the assembly of hemidesmosomes (Spinardi et al., 1995). Upon binding to extracellular ligand, \( \alpha_6 \beta_4 \) associates with cytoskeletal elements of hemidesmosomes, thereby linking the basement membrane to the keratin filament system. This \( \alpha_6 \beta_4 \) function requires a specific region of the unique \( \beta_4 \) cytoplasmic domain, comprising the first pair of type III Fn-like repeats and the Connecting Segment (Spinardi et al., 1993). Collectively, the unique structure, subcellular localization and cytoskeletal interactions of \( \alpha_6 \beta_4 \) suggest that it may transduce intracellular signals by mechanisms distinct from those used by other integrins.

We here provide evidence that signal transduction by the \( \alpha_6 \beta_4 \) integrin is mediated by an associated tyrosine kinase capable of phosphorylating the \( \beta_4 \) subunit. Mutations at a tyrosine activation motif (TAM) in the \( \beta_4 \) tail prevented the incorporation of \( \alpha_6 \beta_4 \) into hemidesmosomes, but not the binding of Shc and Grb2, indicating that these two functions are mediated by phosphorylation of distinct integrin motifs.

**Results**

**Ligation of the \( \alpha_6 \beta_4 \) integrin induces tyrosine phosphorylation of the \( \beta_4 \) subunit**

To examine the role of tyrosine phosphorylation in signal transduction by the \( \alpha_6 \beta_4 \) integrin we asked if ligation of the extracellular portion of the integrin resulted in tyrosine phosphorylation of its component \( \alpha \) or \( \beta \) subunits. To obtain selective ligation of \( \alpha_6 \beta_4 \) in the absence of any concomitant stimulation caused by growth factors or cell shape changes, human epidermoid carcinoma A431 cells were serum starved, detached and then incubated in suspension with polystyrene beads coated with the anti-\( \beta_4 \) monoclonal antibody 3E1 or the control anti-MHC monoclonal antibody W6.32. Tyrosine phosphorylation of \( \alpha_6 \beta_4 \) was monitored over time by immunoprecipitation with the 3E1 antibody followed by immunoblotting with anti-phosphotyrosine (P-Tyr) antibodies. As shown in Figure 1, the \( \beta_4 \) subunit was transiently phosphorylated on tyrosine in cells phosphorylated with anti-\( \beta_4 \) beads, but was not significantly phosphorylated in cells treated with control beads. In addition, no tyrosine phosphorylation of \( \beta_4 \) was observed in cells incubated with soluble 3E1 antibodies (not shown). These observations indicate that antibody-mediated cross-linking of \( \alpha_6 \beta_4 \) results in activation of a tyrosine kinase capable of phosphorylating the \( \beta_4 \) subunit.

To test whether the \( \alpha_6 \beta_4 \) integrin is associated with cytoplasmic protein kinase(s), immune complex kinase assays were performed. The A431 cells, which express several \( \beta_1 \) integrins, as well as \( \alpha_6 \beta_4 \), were immunoprecipitated with the monoclonal antibodies 3E1 and AIIB2, directed against the \( \beta_4 \) and the \( \beta_1 \) integrin subunits respectively. The immunoprecipitated samples were subjected to kinase assay and analyzed by SDS–PAGE. As shown in Figure 2A, incubation of the anti-\( \beta_4 \) immunoprecipitate with \( [\gamma-32P]ATP \) resulted in significant phosphorylation of a 200 kDa protein corresponding to \( \beta_4 \), as well as lower level phosphorylation of an additional 140 kDa protein. Occasionally, additional proteins with apparent molecular masses of 50–70 kDa also underwent specific phosphorylation in the in vitro reaction. In contrast, despite the presence of a tyrosine phosphorylation consensus site in the cytoplasmic domain of the \( \beta_1 \) subunit (Tamkun et al., 1986), incubation of the anti-\( \beta_4 \) immunoprecipitate with \( [\gamma-32P]ATP \) did not yield any specific phosphorylation product under these experimental conditions. Similar results were obtained with LoVo human colon carcinoma and 804G rat bladder carcinoma cells, which both express endogenous \( \alpha_6 \beta_4 \). In addition, analysis of 804G cells expressing either a wild-type or a tail-less human \( \beta_4 \) subunit from cDNA indicated that while the full-length subunit was efficiently phosphorylated in the immune complex kinase assay, the truncated protein was not (Figure 2A). These results indicate that the \( \beta_4 \) subunit is phosphorylated in vitro by a protein kinase(s) associated

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with the integrin and that this event requires an intact β4 cytoplasmic domain.

Phosphoamino acid analysis indicated that the in vitro phosphorylated β4 subunit contained a significant amount of phosphotyrosine, in addition to phosphothreonine and phosphoserine (Figure 2B). The incorporation of phosphate on tyrosine, threonine and serine residues was reduced, but not suppressed, if the immunoprecipitate was washed repeatedly under stringent conditions prior to the reaction (see Materials and methods), suggesting that the phosphorylation of β4 was specific and was not caused by kinases contaminating the immunoprecipitate. Since the intracellular portion of αβ4 does not contain a protein kinase domain, these results suggest that the integrin is physically associated with protein kinase(s) capable of phosphorylating β4 on tyrosine, threonine and serine residues in vitro. Although it is likely that αβ4 is associated with two protein kinases with distinct amino acid selectivity, these results do not exclude the possibility of an association with a dual specificity kinase.

Figure 2. The αβ4 integrin is associated with a protein kinase(s) which can phosphorylate the β4 subunit in vitro. (A) Phosphorylation of β4 in an immune complex kinase assay. A431 cells were either directly extracted with Brij 96 buffer or treated with 10 μM vanadate for 10 min prior to extraction. Rat 804G cells expressing a recombinant wild-type (clone A) or tail-less human β4 subunit (clone B) were directly lysed with Brij 96 buffer. Equal amounts of total proteins were immunoprecipitated with control rabbit anti-mouse IgGs (C), anti-β4 monoclonal antibody 3E1 (3E1) or anti-β1 monoclonal antibody AIIIB2 (AIIIB2). The samples were subjected to an in vitro kinase assay and separated by SDS-PAGE. The arrow points to β4. (B) Phosphoamino acid analysis of in vitro labeled β4 from untreated A431 cells (–Van). (C) Phosphoamino acid analysis of in vitro labeled β4 from vanadate-treated A431 cells (+ Van). Identical amounts of radioactivity were loaded in (B) and (C).

Association of αβ4 with Shc and Grb2

Since tyrosine phosphorylation regulates the recruitment of SH2 domain molecules to activated cell surface receptors, we examined the possible involvement of SH2 domain proteins in signaling by αβ4. To test if the adaptor protein Shc formed a complex with tyrosine-phosphorylated αβ4, A431 cells were stimulated with anti-β4 or anti-MHC beads and the resulting extracts were either immunoprecipitated with anti-β4 antibodies and probed by immunoblotting with anti-Shc antibodies or immunoprecipitated with anti-Shc antibodies and probed with anti-β4 antibodies. The results showed that p52Shc is co-immunoprecipitated with αβ4 from cells incubated with anti-β4 beads, but not from those treated with anti-MHC beads (Figure 3A). Although the other two Shc isoforms, p46Shc and p66Shc, are expressed at levels comparable with that of p52Shc in A431 cells (Pellici, 1992) and are recognized by the antibodies used in this study, only a very modest amount of p46Shc and no p66Shc was detected in association with αβ4. In addition, in accordance with the observation that αβ4 does not contain tyrosine phosphorylation sites conforming to the consensus for binding to the p85 subunit of phosphatidylinositol-3-hydroxyl kinase or phospholipase C-γ (Songyang et al., 1993), we did not detect an association of these SH2 molecules with tyrosine-phosphorylated αβ4. Taken
Fig. 3. Association of αβ4 with Shc and Grb2. (A) Shc forms a complex with activated αβ4 in intact cells. A431 cells were stimulated as indicated in Figure 1. Equal amounts of total proteins were immunoprecipitated with rabbit anti-β4 peptide serum (top) or rabbit anti-Shc serum (bottom). The samples were probed by immunoblotting with anti-Shc monoclonal antibody (top) or anti-β4 monoclonal antibody 450-11A (bottom). (B) Binding of the Shc PID and SH2 domains to β4. Rat 804G cells expressing the human wild-type β4 subunit (clone A) were serum starved and treated with medium alone, 100 μM sodium orthovanadate plus 3 mM H2O2 or stimulated in suspension with anti-β4 beads for 10 min at 37°C. Denatured lysates were incubated with glutathione-agarose beads carrying the GST leader protein alone (GST) or GST–Shc PID domain (PID) or GST–Shc SH2 domain (SH2). Bound proteins were separated by SDS-PAGE and analyzed by immunoblotting with polyclonal anti-β4 antibodies.

Together, these results indicate that p52Sc forms a specific complex with the activated αβ4 integrin.

We next wondered if Shc could interact directly with the tyrosine-phosphorylated β4 subunit and whether the interaction was mediated by the SH2 domain or the Phosphotyrosine Interaction Domain (PID) of Shc. 804G cells expressing a recombinant wild-type β4 subunit were treated with sodium orthovanadate or incubated with anti-β4 beads to induce β4 phosphorylation. The extracts were denatured by heating in 1% SDS and incubated with agarose-immobilized GST fusion proteins encoding either the PID or the SH2 domain of Shc. Bound proteins were analyzed by immunoblotting with anti-β4 antibodies. As shown in Figure 3B, both the PID and the SH2 domain of Shc bound to the β4 subunit extracted from cells treated with vanadate or anti-β4 antibodies, but not to β4 from control, untreated cells. These results suggest that the tyrosine-phosphorylated β4 subunit can interact directly with both the PID and the SH2 domain of Shc.

To examine the effect of αβ4 ligation on tyrosine phosphorylation of Shc, A431 cells were incubated with anti-β4 or control beads and immunoprecipitated with anti-Shc antibodies. The samples were analyzed by immunoblotting with anti-P-Tyr antibodies. As shown in Figure 4 (upper panel), treatment of the cells with anti-β4, but not control, beads led to tyrosine phosphorylation of p52Sc. Two tyrosine-phosphorylated proteins were co-immunoprecipitated with Shc, a 195 kDa component which appeared to be constitutively associated with Shc and was not investigated further (lower arrow) and a 200 kDa molecule which was detected in association with Shc in cells treated with anti-β4, but not control, antibodies (upper arrow). Reprobing of the blot with the anti-β4 monoclonal antibody 450-11A revealed that this latter protein corresponded to the tyrosine-phosphorylated β4 subunit. These results indicate that upon forming a complex with activated αβ4, p52Sc becomes phosphorylated on tyrosine.

To examine the possibility that tyrosine-phosphorylated Shc associates with Grb2 upon ligation of αβ4, extracts derived from A431 cells treated with anti-β4 or control beads were subjected to immunoprecipitation with anti-Shc antibodies followed by immunoblotting with anti-Grb2 antibodies. The result indicated that Grb2 forms a complex with Shc in cells stimulated with anti-β4, but not control, beads (Figure 4, lower panel). Grb2 could also be detected in anti-β4 immunoprecipitates from stimulated cells, but in lower amounts than in the anti-Shc immunoprecipitates. Together with the observation that the β4 tail does not contain consensus Grb2 binding motifs (Songyang et al., 1993), these results suggest that the association of Grb2 with αβ4 is mediated by p52Sc and contingent upon its tyrosine phosphorylation. Collectively these findings indicate that the two adaptors Shc and Grb2 interact sequentially with αβ4, thereby potentially linking the integrin to the ras signaling pathway.
Cell adhesion to laminin 5 results in tyrosine phosphorylation of \( \beta_4 \) and p52\text{Shc}

We next asked whether the above-described intracellular events also occurred in response to engagement of \( \alpha_6 \beta_4 \) by extracellular matrix ligand. A431 cells were serum starved, detached with EDTA and either kept in suspension or plated on laminin 5 matrix-coated dishes for the indicated times. After extraction, the samples were immunoprecipitated with monoclonal antibodies to \( \beta_4 \) (top panel) or Shc (bottom panel). The samples were probed by immunoblotting with a mixture of the two anti-P-Tyr monoclonal antibodies 4G10 and PY20.

Phosphorylation of a tyrosine-based activation motif (TAM) in the \( \beta_4 \) cytoplasmic domain

To assess the biological significance of \( \beta_4 \) phosphorylation, we sought to examine the tyrosine phosphorylation sites in \( \beta_4 \). Preliminary studies using a combination of deletion mutagenesis and immunoblotting with anti-P-Tyr antibodies pointed to the presence of major tyrosine phosphorylation sites in the \( \beta_4 \) Connecting Segment (data not shown). Inspection of the amino acid sequence of the Connecting Segment revealed three potential tyrosine phosphorylation sites: Tyr1343, Tyr1422 and Tyr1440. We noted that the closely spaced Tyr1422 and Tyr1440 are embedded in very similar amino acid contexts. In particular, both residues are followed at position +3 by a leucine. Tandem tyrosine phosphorylation sites with a leucine at position +3 play a critical role in signal transduction by antigen receptors and are commonly referred to as TAMs or antigen recognition activation motifs (ARAMs) (Weiss and Littman, 1994). Figure 6 shows an alignment of the \( \beta_4 \) TAM with the other previously identified TAMs, which include those present in the T cell receptor (TCR), B cell receptor (BCR), Fce\( \epsilon \) and Fcy receptors and the bovine leukemia virus gp30 glycoprotein.

To determine if the \( \beta_4 \) TAM sequence is phosphorylated in vivo and examine the physiological significance of this event, we generated and then introduced into 804G cells \( \beta_4 \) CDNAs carrying either individual phenylalanine substitutions at Tyr1343, Tyr1422 and Tyr1440 or a combined replacement of Tyr1422 and Tyr1440. Fluorescence activated cell sorting (FACS) analysis indicated that the cDNA encoded mutant subunits Y1343F, Y1422F, Y1440F and Y1422F/Y1440F were expressed at the cell surface at levels comparable with that of wild-type recombinant \( \beta_4 \).

Wild-type \( \beta_4 \) and phenylalanine mutant subunits were examined by in vivo labeling and phosphopeptide mapping. Since antibody- or ligand-induced cross-linking of \( \alpha_6 \beta_4 \) did not produce the high level tyrosine phosphorylation of \( \beta_4 \) required for mapping, tyrosine phosphorylation of \( \beta_4 \) was obtained by exposing the cells to vanadate. Preliminary experiments of \(^{32}P\)orthophosphate labeling and phosphoamino acid analysis revealed that the wild-type \( \beta_4 \) subunit is constitutively phosphorylated on serine residues in vivo, but becomes phosphorylated on tyrosine residues upon vanadate treatment (Figure 7A and B). *Staphylococcus* V8 protease digestion of wild-type \( \beta_4 \) from vanadate-treated cells yielded five major phosphopeptides (S1–S3, Y5 and Y6) and a number of minor phosphopeptides (Y1–Y4) (Figure 7, top panel). Phosphoamino acid analysis of individual phosphopeptides indicated that the major phosphopeptides S1–S3 contain exclusively radioactive phosphoserine. This observation is consistent with their presence in phosphopeptide maps of \( \beta_4 \) isolated from unstimulated cells. In contrast, the two major phosphopeptides Y5 and Y6, as well as the minor phosphopeptides Y1–Y4, which were only detected in stimulated cells, were found to contain exclusively phosphothreonine. We concluded that \( \beta_4 \) is phosphorylated at multiple tyrosine residues in vivo.

We next examined the phosphopeptide maps of mutant proteins containing the wild-type or Y1343F, Y1422F, Y1440F or Y1422F/Y1440F substitutions.
subunits Y1422F and Y1440F. As shown in Figure 7C (middle panel), the replacement of Tyr1440 with phenylalanine caused the disappearance of peptides Y5 and Y6. The simultaneous disappearance of peptides Y5 and Y6 as a consequence of a single point mutation and their similar migration indicate that these peptides are closely related and that both contain Tyr1440. We also observed that the map derived from the Y1440F mutant subunit contained a number of novel peptides and that peptides Y1 and Y4 were more intensely radioactive than in wild-type β4. Presumably these events are a consequence of compensatory phosphorylation. The substitution of Tyr1422 with phenylalanine caused a reduction in the intensity of only a couple of phosphopeptides (Figure 7C, bottom panel). Also in this case we noticed compensatory phosphorylation (see, for example, peptide Y1). In contrast, the replacement of Tyr1343 with phenylalanine did not result in modification of any phosphopeptide (data not shown). We conclude that the β4 tail is phosphorylated in vivo at multiple tyrosine residues: the C-terminal element of the TAM corresponds to one of the major sites of phosphorylation, while its N-terminal element may correspond to a minor one.

Activation of Shc by αβ4 integrin is not affected by mutations at the β4 TAM

The role of the β4 TAM in activation of the Shc/Grb2 pathway was examined using recombinant β4 subunits carrying either a deletion of the Connecting Segment or phenylalanine substitutions in the β4 TAM. Rat 804G cells expressing human wild-type or mutant β4 subunits were incubated with beads coated with either the anti-human β4 antibody 3E1 or anti-MHC class II antibody W6,32. The samples were immunoprecipitated with anti-Shc antibodies and probed with either anti-P-Tyr or anti-Grb2 antibodies. As shown in Figure 8 (top panel), β4 subunits with a double mutation in the TAM, a single phenylalanine substitution outside the TAM but within the Connecting Segment or a complete deletion of the Connecting Segment mediated tyrosine phosphorylation of Shc as efficiently as wild-type β4. In all cases tyrosine phosphorylation of Shc resulted in recruitment of Grb2 (Figure 8, bottom panel).
Re-probing of the blot with the anti-human β₄ monoclonal antibody 450-9Δ indicated that all the mutant subunits had formed a specific complex with Shc upon stimulation with anti-β₄ beads (data not shown). These results indicate that the β₄ TAM sequence and the entire Connecting Segment are not required for linking α₆β₄ to Shc and Grb2.

**Phosphorylation of the β₄ TAM mediates association of the α₆β₄ integrin with the cytoskeleton**

Previous results indicated that association of the α₆β₄ integrin with the cytoskeleton and consequent assembly of hemidesmosomes require a specific segment of the β₄ tail (Spinardi et al., 1993, 1995). Since the β₄ TAM is part of this segment and selective inhibition of β₄ phosphorylation with the tyrosine kinase inhibitor herbimycin correlates with inhibition of hemidesmosome assembly (A.Pepe, F.Mainiero and F.G.Giancotti, unpublished results), we asked if phosphorylation of the β₄ TAM played a role in association of the integrin with the hemidesmosomal cytoskeleton. As the α₆β₄ integrin incorporated in hemidesmosomes is largely resistant to extraction in non-ionic detergents (Spinardi et al., 1993), we examined the Triton X-100 solubility of recombinant β₄ subunits carrying phenylalanine substitutions in the TAM. The result of this experiment indicated that the wild-type β₄ subunit and the control mutant subunit Y1343F, which carries a mutation outside the TAM, are associated predominantly with the Triton X-100-insoluble fraction. In contrast, the mutant subunit Y1422F was equally distributed in the detergent-soluble and -insoluble fractions and the mutant protein Y1440F was exclusively associated with the soluble fraction (Figure 9A). The mutant protein Y1422F/Y1440F was also recovered exclusively from the soluble fraction (data not shown). These results indicate that phosphorylation of the β₄ TAM is important for association of α₆β₄ with the detergent-insoluble cytoskeleton.

We next examined the subcellular localization of the phenylalanine mutant β₄ subunits by immunofluorescence. Immunostaining with the 3E1 monoclonal antibody showed that wild-type human β₄ is in part diffusely distributed on the plasma membrane and in part concentrated at the basal cell surface within punctate, 'Swiss-cheese-like' structures corresponding to hemidesmosomes (Figure 9B, panel a; Spinardi et al., 1993, 1995). In accordance with previous results, treatment with Triton X-100 prior to fixation eliminated the diffuse staining associated with the plasma membrane, but rendered more evident the 'Swiss-cheese-like' staining of hemidesmosomes (panel d). Cells expressing the control mutant subunit Y1343F, which carries a single phenylalanine substitution outside the connecting segment, displayed a staining pattern identical to that of control cells, indicating that this recombinant molecule is correctly targeted to hemidesmosomes (data not shown). In contrast, the staining pattern generated by the 3E1 antibody in cells expressing the mutant subunit Y1422F was mostly diffuse and associated with the plasma membrane (panel b). Although punctate staining could be detected in cells treated with Triton X-100 before fixation, this staining was much more scarce than that in control cells expressing wild-type β₄ and 'Swiss-cheese-like' structures were never observed (panel e). This indicates that association of the mutant subunit Y1422F with hemidesmosomes is impaired as compared with that of wild-type β₄. Finally, the 3E1 antibody generated only diffuse staining of the plasma membrane in cells expressing the mutant subunit Y1440F (panel c). Notably, virtually all staining was suppressed if the cells were treated with Triton X-100 prior to immunostaining (panel f). Identical results were obtained from an analysis of the subcellular localization of mutant subunit Y1422F/Y1440F (data not shown). Thus mutant β₄ subunits carrying either a single phenylalanine permutation at position 1440 or a double substitution at positions 1422 and 1440 can be detected at the cell surface, but not in hemidesmosomes. Taken together, these findings indicate that stable association of α₆β₄ with the cytoskeleton at hemidesmosomes requires phosphorylation of both elements of the β₄ TAM.

**Discussion**

Although observations made in the past two decades point to a pivotal role of the extracellular matrix in controlling gene expression (Adams and Watt, 1993), the question of how integrins transduce signals at the plasma membrane level has remained in large part unsolved, despite intensive investigation. In this study we have examined the mechanism of signal transduction by the α₆β₄ integrin. Our results indicate that ligand or antibody binding to α₆β₄ causes tyrosine phosphorylation of the β₄ subunit and suggest that this event is mediated by a protein tyrosine kinase associated with the integrin. The results of phosphopeptide mapping and mutagenesis experiments indicate that the β₄ cytoplasmic domain is phosphorylated at multiple sites: one site, which corresponds to a bidentate TAM similar to those found in several immune receptors, mediates association of α₆β₄ with the cytoskeleton of hemidesmosomes, while one or more distinct sites are involved in sequential recruitment of the adaptor molecules Shc and Grb2.

The mechanism of signaling by α₆β₄ suggested by our results incorporates elements of other receptor systems, such as the recruitment of Shc and Grb2, as well as unique features, such as association with the hemidesmosomal cytoskeleton. Like many cytokine and immune receptors (Kishimoto et al., 1994; Weiss and Littman, 1994), α₆β₄ lacks an intracellular catalytic domain and relies on its association with a cytoplasmic tyrosine kinase for signal transduction. As tyrosine phosphorylation of β₄ can be triggered by adhesion to a laminin 5 matrix, as well as by antibody-mediated cross-linking, but not by soluble antibodies to α₆β₄, it is likely that dimerization or oligomerization of the integrin is required either for activating the associated tyrosine kinase or for bringing it into close proximity to its target sequences in the β₄ tail. The identity of the tyrosine kinase associated with α₆β₄ remains to be determined, but the selective ability of src family kinases to induce β₄ phosphorylation in co-transfection experiments (A.Curatola and F.G.Giancotti, unpublished results), together with previous observations indicating that the T cell and B cell receptor TAMs are phosphorylated by src family kinases (Weiss and Littman, 1994), suggest that α₆β₄ may be associated with a src family member. The observation that the β₄ subunit can be phosphorylated on
Signal transduction by the $\alpha_5\beta_4$ integrin

Fig. 9. Phenylalanine replacements in the $\beta_4$ TAM interfere with incorporation of $\alpha_5\beta_4$ in hemidesmosomes. (A) Triton X-100 solubility of wild-type and mutant $\beta_4$ subunits. Triton X-100-soluble (Sol) and -insoluble (Ins) cell fractions were derived from rat 804G cells expressing the human wild-type $\beta_4$ subunit (Clone A) or the indicated phenylalanine substituted subunits (Y1343F, Y1422F and Y1440F). After immunoprecipitation with the 3E1 antibody, the samples were probed by immunoblotting with rabbit anti-$\beta_4$ serum. In this experiment a smaller number of cells was used to generate detergent-soluble and -insoluble fractions from 804G cells expressing the Y1440F mutant. (B) Localization of wild-type and mutant $\beta_4$ subunits to hemidesmosomes. Rat 804G cells expressing human wild-type $\beta_4$ (a and d), the mutant Y1422F (b and e) or the mutant Y1440F (c and f) were plated on coverslips, cultured for 48 h and then either fixed directly with cold methanol for 2 min (a, b and c) or treated with 0.2% Triton X-100 for 5 min prior to fixation (d, e and f). Immunofluorescent staining was performed using the 3E1 antibody followed by FITC-conjugated goat anti-mouse IgG. Identical results were obtained with three independent clonal cell lines of each type.

Serine and threonine residues in immune complex kinase assays indicates that $\alpha_5\beta_4$ may also be associated with other kinases, highlighting the complexity of $\alpha_5\beta_4$ function. We have observed that ligation of $\alpha_5\beta_4$ results in its association with the adaptor protein Shc. This molecule contains two distinct domains capable of interacting with tyrosine-phosphorylated sequences: an N-terminal PID (Kavanaugh and Williams, 1994; Bork and Margolis, 1995) and a C-terminal SH2 domain (Pellicci et al., 1992). The GST fusion protein binding experiments of this study suggest that both Shc domains can interact independently and directly with the tyrosine-phosphorylated $\beta_4$ subunit. Interestingly, the $\beta_4$ tail contains two tyrosine-based motifs potentially able to interact with the Shc SH2 domain (Songyang et al., 1994) and three N-X-X-Y motifs which could bind to the Shc PID (Kavanaugh et al., 1995). Although definition of the $\beta_4$ sequences involved in interaction with Shc requires further mutagenesis experiments, the present results suggest that the PID and SH2 domains of Shc may bind to $\beta_4$ by a cooperative mechanism similar to that described for their binding to the epidermal growth factor receptor (Batzer et al., 1995). As a consequence of its binding to $\alpha_5\beta_4$, Shc is phosphorylated on tyrosine, an event presumably mediated by the kinase associated with $\alpha_5\beta_4$, and then binds to Grb2. Several recent studies have indicated that Grb2 is stably associated...
with the \textit{ras} GTP exchanger mSOS (Schlessinger, 1994; Pawson, 1995). However, while in unactivated cells the complex is confined to the cytoplasm, in stimulated cells it is recruited to the activated receptors and therefore translocated to the plasma membrane, where it can activate \textit{ras}. Our results therefore describe a molecular mechanism potentially linking the \( \alpha_6\beta_4 \) integrin to the \textit{ras} signaling pathway. In the future it will be important to delineate the specific intracellular pathways activated by recruitment of Shc and Grb2 to \( \alpha_6\beta_4 \) and elucidate their effects on cell function.

Binding of laminin 5 to \( \alpha_6\beta_4 \) integrin plays an essential role in the organization of hemidesmosomes (Spinardi \textit{et al.}, 1995). The results of this study suggest that this function requires phosphorylation of the \( \beta_4 \) TAM. Mutations which prevented tyrosine phosphorylation of the \( \beta_4 \) TAM also suppressed association of \( \alpha_6\beta_4 \) with hemidesmosomes. Interestingly, the replacement of Tyr1440 had a more drastic effect on \( \alpha_6\beta_4 \) function than mutation of Tyr1422, indicating that phosphorylation of the C-terminal tyrosine may be sufficient for partial functioning of the \( \beta_4 \) TAM. It must be noted that tyrosine phosphorylation of \( \beta_4 \) occurs only transiently in response to ligation of \( \alpha_6\beta_4 \). In fact, virtually no tyrosine phosphorylated \( \beta_4 \) is detected in stably adherent cells, in which the majority of \( \alpha_6\beta_4 \) is in hemidesmosomes. Thus it is unlikely that the formation of hemidesmosomes depends on a stable interaction mediated by tyrosine-phosphorylated \( \beta_4 \) TAM. Instead, it is possible that the \( \beta_4 \) TAM is primarily involved in transducing a signal required for hemidesmosome assembly.

What is the nature of this signal? The TAM was originally identified as a common motif present in several immune receptors (Reth, 1989). In the TCR system, as a result of simultaneous binding of the TCR \( \alpha/\beta \) heterodimer and co-receptor CD4 to the peptide-bearing MHC molecule, \textit{lk} comes into close proximity to and phosphorylates the TAMs present in the multichain invariant CD3 complex. Phosphorylation of \( \zeta \) chain TAMs provides a template for binding of the tyrosine kinase ZAP70 involved in subsequent downstream signaling events (Weiss and Littman, 1994). It is possible that the mechanism by which phosphorylation of the \( \beta_4 \) TAM regulates cytoskeletal assembly also involves binding to an SH2
domain-containing protein. The tyrosine kinases ZAP70 and syk contain two tandem SH2 domains through which they bind to the phosphorylated TAMs of the T cell and B cell receptors respectively (Weiss and Littman, 1994). These molecules, however, are restricted to the immune system. In addition, the spacing between Tyr1422 and Tyr1440 in β4 is larger than the distance between the tyrosines in other TAMs. These observations raise the possibility that the β4 TAM has a distinct binding specificity. To prove this model it will be necessary to identify the protein kinase or adaptor interacting with the β4 TAM.

In sum, the results of this study suggest a model of signal transduction by αββ4 integrin that involves a number of sequential steps (Figure 10). We hypothesize that upon binding to a multivalent extracellular matrix ligand αββ4 dimerizes or oligomerizes on the plasma membrane, thereby activating an associated intracellular tyrosine kinase and/or juxtaposing it to its target sequences in the β4 tail. The phosphorylated β4 subunit then interacts with Shc and Grb2, as well as with molecules involved in assembly of hemidesmosomes. These two functions appear to be mediated by distinct motifs, because mutations in the β4 TAM selectively interfere with association of the integrin with the hemidesmosomal cytoskeleton.

The αββ4 signaling mechanism proposed here appears to be especially suited to allow fine tuning of distinct intracellular functions in response to diverse environmental cues. The level of phosphorylation of distinct receptor sites may diverge substantially depending on the nature of the extracellular ligand (Sloan-Lancaster et al., 1994). Thus it is possible that the β4 TAM and the distinct site involved in binding to Shc are differentially phosphorylated depending on the specific laminin isoform encountered by the cell or its oligomerization state. In addition, the level of phosphorylation of each site may vary with the cell type and its state of differentiation. This potential mechanism is attractive because it would allow a differential regulation of the ras pathway and assembly of hemidesmosomes depending on the matrix and cellular context. It is possible that the growth advantage of squamous carcinoma cells is at least in part related to overexpression of αββ4 in these cells (Kimmel and Carey, 1986; Savoia et al., 1993; Tennenbaum et al., 1993) and to its ability to link to the ras pathway. Squamous carcinoma cells, however, lack well-organized hemidesmosomes (Schenk, 1979), suggesting that the signals responsible for hemidesmosome assembly may be defective in these cells. Thus these cells may represent an extreme example of the divergent regulation of αββ4-mediated signals.

Finally, the signal transduction mechanism described in this paper provides a rational basis for the effects of αββ4 on morphogenesis and tumor progression. Although it is likely that the intracellular signals elicited by laminin binding to αββ4 are unique, future studies will undoubtedly reveal the extent of signaling overlap between various integrins. The recent observation that αββ4 associates with insulin receptor substrate 1 in insulin-stimulated cells (Vuori and Ruoslahti, 1994) suggests that an additional level of complexity in integrin signaling may result from interaction between growth factor- and adhesion-dependent pathways. In this context, the results of this study represent a first step toward understanding the mechanisms of signal transduction by integrins.

### Materials and methods

#### Antibodies

The monoclonal antibody 3E1, reacting with the extracellular portion of human β4, and the rabbit polyclonal antisemur to the C-terminal peptide of β4 have been described previously (Giancotti et al., 1992). The anti-β4 monoclonal antibody 450-9D and 450-11A have also been previously characterized (Kennel et al., 1990). The monoclonal antibody AIIIB2 binds to the extracellular portion of the human β1 subunit (Werb et al., 1989). The anti-MHC monoclonal antibody W6.32 reacts with human and cultured rat cells (Kahn-Perles et al., 1987). The rabbit polyclonal anti-P-Tyr serum 72 was produced according to published procedures (Kamps and Seftron, 1988). The monoclonal anti-P-Tyr antibody PY20 and the monoclonal anti-Shc antibody were from Transduction Laboratories (Lexington, KY). The polyclonal anti-Shc serum 410 was obtained by immunizing a rabbit with a GST fusion protein containing the SH2 domain of the protein (Battner et al., 1995). The monoclonal antibody EL-6 recognizes an epitope in the SH2 domain of Grb2.

#### Constructs and transfections

All recombinant expression constructs were assembled in the CMV promoter-based vector pRC-CMV (Invitrogen Corp., San Diego, CA). The plasmids encoding the wild-type and tail-less human β4 subunits have been previously described (Spinardi et al., 1993). To generate the construct pCMV-β4Δ1314–1486, which directs expression of a truncated β4 subunit lacking the Connecting Segment (ΔCS), we employed the polymerase chain reaction (PCR) to engineer a DNA fragment encoding β4 residues 1315–1486 flanked by NcoI (5'-end) and NotI (3'-end) sites. The 4.8 kb HindIII–XbaI fragment of β4 was subcloned into pSL1180 (Pharmacia, Piscataway, NJ), thus generating pSL1180–β4, and the 5.2 kb NotI–SacII fragment of this plasmid was ligated to the PCR-generated β4 fragment. The 4.3 kb BspEI–XbaI fragment of the resulting plasmid was finally ligated to the 6.3 kb XbaI–BspEI fragment of pCMV-β4. Phenylalanine substitutions were introduced into β4 using the Altered Site in vitro mutagenesis system (Promega, Madison, WI). Confirmation of each of the constructs was verified by sequencing. Rat bladder carcinoma 804G cells were transfected with the various expression constructs and pSV-neo as previously described (Giancotti et al., 1994). Clones expressing comparable levels of each recombinant β4 polypeptide were selected by FACS analysis. Immunoprecipitation of cells labeled metabolically with [35S]methionine was used to verify correct assembly of the recombinant β4 polypeptides with the endogenous α6 subunit (Spinardi et al., 1993).

GST fusion proteins encoding the murine Shc PID (residues 1–209) and SH2 domains were expressed and purified on glutathione–agarose beads as previously described (Blakie et al., 1994).

#### Biochemical methods

To obtain selective ligation of αββ4 in the absence of any co-stimulus, the cells were serum starved, detached with 10 mM ethylenediamine tetraacetate (EDTA) and then resuspended at 20×10^6/ml. Aliquots (200 μl) of this cell suspension were incubated at 37°C and either stimulated with 1.8×10^8 polystyrene sulfate latex beads (2.5 μm diameter; IDC, Portland, OR) coated with the 3E1 or the control W6.32 monoclonal antibody (400 μg/ml) for the indicated times or left untreated. To obtain engagement of αββ4 by a physiological ligand, the cells were serum starved, detached with EDTA and either kept in suspension or plated on laminin 5 matrix-coated dishes (Spinardi et al., 1995) for the indicated times. At the end of the incubation the cells were extracted for 30 min at 0°C with RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) or lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100) containing 1 mM sodium orthovanadate, 50 mM sodium pyrophosphate, 100 mM sodium fluoride, 0.01% aprotinin, 4 μg/ml pepstatin A, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA and 1 mM ethyleneglycol-bis(β-aminoethyl) ether)-N,N,N',N'-tetraacetate (EGTA) (all from Sigma, St Louis, MO).

To examine the detergent solubility of phenylalanine mutant β4 subunits, subconfluent monolayers of the various clones were extracted on ice with 50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton X-100 and the protease inhibitors for 5 min. The detergent-insoluble fraction was recovered and the insoluble cytoskeletons were washed and then extracted with RIPA buffer and protease inhibitors. Detergent-soluble and -insoluble fractions derived from the same sample were directly compared.
Immunoprecipitation and immunoblotting were performed as previously described (Giancotti and Ruoslahti, 1990; Giancotti et al., 1992). Nitrocellulose-bound antibodies were detected by chemiluminescence with ECL (Amersham Life Sciences, Little Chalfont, UK).

For binding studies, rat 804G cells expressing the human wild-type β2 subunit were serum-starved and treated with 100 μM sodium orthovanadate plus 3 mM H2O2 or stimulated in suspension with anti-β2 beads for 10 min at 37°C. After extraction in SDS buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% SDS) with protease inhibitors, the lysates were heated for 5 min at 80°C, sonicated and diluted with 9 vol. lysis buffer. Glutathione–agarose beads carrying the GST fusion proteins were incubated with the denatured lysates (10 μg fusion protein/1 mg total proteins) for 2 h at 4°C, washed and boiled in SDS–PAGE sample buffer. Samples were separated by SDS–PAGE and analyzed by immunoblotting with polyclonal anti-β2 antibodies.

For immune complex kinase assay, subconfluent cell monolayers were extracted with 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Brij 96 and phosphatase and protease inhibitors. After clarification, the extracts were immunoprecipitated as described above. The affinity beads were washed extensively with the extraction buffer without phosphatase inhibitors and then equilibrated in kinase buffer (10 mM Tris, pH 7.4, 10 mM MnCl2, 20 mM p-nitrophosphorylase). The kinase reaction was initiated by adding 50 μl kinase buffer containing 20 μCi [γ-32P]ATP (4500 Ci/mmol; ICN Biomedicals Inc., Irvine, CA) to the beads and continued at 30°C for 30 min. The reaction was stopped by boiling the samples for 5 min in SDS–PAGE sample buffer.

Phosphoamino acid analysis was performed as described by Boyle et al. (1991). 32P-Labeled β2 was eluted from fixed polyacrylamide gels and precipitated with 20% trichloroacetic acid. 32P-Labeled peptides were scraped off TLC plates, eluted in pyridine and lyophilized. Both types of sample were subjected to acid hydrolysis in 6 N HCl at 110°C for 1 h. Phosphoamino acids were separated by two-dimensional TLC electrophoresis in pH 1.9 buffer (2% formic acid, 7.9% 25 mM sodium orthovanadate and 3 mM H2O2) for 10 min at 37°C or left untreated. After immunoprecipitation with the 3E1 antibody, the samples were transferred to nitrocellulose. The nitrocellulose fragments containing β2 were soaked in 0.5% polyvinylpyrrolidone (PVP-360; Sigma), 100 mM acetic acid at 37°C for 30 min. Complete digestion was achieved by incubating the bands in 200 μl 50 mM phosphate buffer, pH 7.8, with 25 μg Streptomyces aureus V8 protease (Worthington Biochemical Corp., Freehold, NJ) for 48 h at 37°C. The samples were separated by two-dimensional TLC. Separation in the first dimension was achieved by electrophoresis in pH 1.9 buffer (1.5 kV, 50 min) and in the second by ascending chromatography in Phospho Chromatography buffer (37.5% n-butanol, 25% pyridine, 7.5% acetic acid).

Immunofluorescence Cells were either fixed directly with cold methanol for 2 min or treated with phosphate-buffered saline containing 0.2% Triton X-100 for 5 min on ice prior to fixation with methanol. Immunostaining with 3E1 antibody was performed as previously described (Spinardi et al., 1993, 1995). Secondary antibodies were species-specific. Samples were examined with a Zeiss Axiopt Fluorescent Microscope.

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