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The coupling of $\alpha_6\beta_4$ integrin to Ras–MAP kinase pathways mediated by Shc controls keratinocyte proliferation

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The signaling pathways linking integrins to nuclear events are incompletely understood. We have examined intracellular signaling by the $\alpha_6\beta_4$ integrin, a laminin receptor expressed in basal keratinocytes and other cells. Ligation of $\alpha_6\beta_4$ in primary human keratinocytes caused tyrosine phosphorylation of Shc, recruitment of Grb2, activation of Ras and stimulation of the MAP kinases Erk and Jnk. In contrast, ligation of the laminin- and collagen-binding integrins $\alpha_3\beta_1$ and $\alpha_2\beta_1$ did not cause these events. While the stimulation of Erk by $\alpha_6\beta_4$ was suppressed by dominant-negative Shc, Ras and RhoA, the activation of Jnk was inhibited by dominant-negative Ras and Rac1 and by the phosphoinositide 3-kinase inhibitor Wortmannin. Adhesion mediated by $\alpha_6\beta_4$ induced transcription from the Fos serum response element and promoted cell cycle progression in response to mitogens. In contrast, $\alpha_3\beta_1$- and $\alpha_2\beta_1$-dependent adhesion did not induce these events. These findings suggest that the coupling of $\alpha_6\beta_4$ integrin to the control of cell cycle progression mediated by Shc regulates the proliferation of basal keratinocytes and possibly other cells which are in contact with the basement membrane *in vivo*.

Keywords: integrins/keratinocytes/MAP kinase/Ras/Shc

Introduction

In addition to promoting cell adhesion and contributing to the organization of tissues and organs, basement membranes exert complex and often divergent effects on the survival, proliferation and differentiation of epithelial cells (Adams and Watt, 1993; Lin and Bissel, 1993). The influences of basement membranes on epithelial cells are likely to be mediated by the ability of laminin-binding integrins to induce intracellular signaling (Giancotti and Mainiero, 1994; Clark and Brugge, 1995; Schwartz *et al.*, 1995), but the mechanisms involved are incompletely understood.

To elucidate the effects of laminins on epithelial cells, we have focused on the $\alpha_6\beta_4$ integrin. Cell adhesion assays with $\alpha_6\beta_4$-transfected K562 cells and radioligand binding studies with purified recombinant $\alpha_6\beta_4$ have indicated that this integrin is a receptor for various laminin isoforms and binds with the highest apparent affinity to laminins 5 and 4 (Niessen *et al.*, 1994; Spinardi *et al.*, 1995). In accordance with its role as a basement membrane receptor, $\alpha_6\beta_4$ is expressed in epithelial cells (Kajiji *et al.*, 1989), Schwann cells (Sonnenberg *et al.*, 1990; Einheber *et al.*, 1993) and a subset of endothelial cells (Kennel *et al.*, 1992; Klein *et al.*, 1993) and thymocytes (Wadsworth *et al.*, 1992).

The $\alpha_6\beta_4$ integrin has a distinctive structure and subcellular localization. The large cytoplasmic domain of the β_4 subunit, which is characterized by two pairs of type III fibronectin (Fn)-like domains separated by a 142 amino acid sequence (connecting segment), does not contain any region of homology with the cytoplasmic domains of other known integrin β subunits (Hogervorst *et al.*, 1990; Suzuki and Naitoh, 1990). Furthermore, while β_1 and α_v integrins are concentrated in focal adhesions and linked to the actin filament system, the $\alpha_6\beta_4$ integrin is found in hemidesmosomes both *in vivo* and in cultured cells (Carter *et al.*, 1990a; Stepp *et al.*, 1990). These observations suggest that the unique cytoplasmic domain of β_4 interacts with cytoskeletal elements of hemidesmosomes, thereby linking $\alpha_6\beta_4$ to the keratin filament system.

Our previous studies have indicated that the association of $\alpha_6\beta_4$ with the hemidesmosomal cytoskeleton is mediated by the cytoplasmic domain of β_4 and specifically by a region which includes the first pair of type III Fn-like repeats and the connecting segment (Spinardi *et al.*, 1993). In accordance with the hypothesis that $\alpha_6\beta_4$ plays a crucial role in the assembly of hemidesmosomes and their linkage to the keratin filament system, we have observed that the introduction of a truncated tail-less β_4 subunit into cells possessing endogenous $\alpha_6\beta_4$ integrins and hemidesmosomes results in a dominant-negative effect on hemidesmosome assembly (Spinardi *et al.*, 1995). Since the tail-less integrin binds efficiently to extracellular ligand, its dominant-negative effect is likely to result from its ability to co-cluster with the endogenous wild-type receptor and block a signal necessary for hemidesmosome assembly. In accordance with this hypothesis, recent studies have revealed that $\alpha_6\beta_4$ is associated with an intracellular tyrosine kinase. Mutagenesis experiments have provided evidence that the phosphorylation of a tyrosine activation motif (TAM) located in the connecting segment controls the association of $\alpha_6\beta_4$ with the hemidesmosomal

cytoskeleton, presumably via the recruitment of a signaling molecule containing two tandem Src homology 2 (SH2) domains (Mainiero *et al.*, 1995).

In the epidermis and other stratified epithelia, the expression of $\alpha_6\beta_4$ is restricted to the basal cell layer which contains cells endowed with proliferative capacity (Kajiji *et al.*, 1989). It is known that keratinocytes exit the cell cycle and begin their differentiation program when they detach from the basement membrane to migrate to the upper epidermal layers (Hall and Watt, 1989). In fact, this process can be replicated *in vitro* by depriving cultured keratinocytes of anchorage to their endogenously produced extracellular matrix (Green, 1977), which is particularly rich in laminin 5 (Carter *et al.*, 1991; Rousselle *et al.*, 1991). Furthermore, squamous carcinoma cells endowed with high proliferative potential often express elevated levels of $\alpha_6\beta_4$ (Kimmel and Carey, 1986; Wolf *et al.*, 1990). Finally, the basal keratinocytes of β_4 knock-out mice display signs of degeneration even in areas of epidermis where no significant detachment from the basement membrane is observed (Dowling *et al.*, 1996). These observations suggest that $\alpha_6\beta_4$ may provide epithelial cells with a signal important for cell survival and cell cycle progression.

What is the mechanism by which the $\alpha_6\beta_4$ integrin transduces biochemical signals capable of affecting cell proliferation? Immunoprecipitation and GST fusion protein binding experiments have indicated that ligation of $\alpha_6\beta_4$ results in the association of the adaptor protein Shc with tyrosine-phosphorylated β_4. Shc is then phosphorylated on tyrosine residues, presumably by the integrin-associated kinase, and combines with the other adaptor protein Grb2 (Mainiero *et al.*, 1995). These observations raise two key questions. First, what are the biochemical consequences of the recruitment of Shc and Grb2 to $\alpha_6\beta_4$? Second, what is the biological significance of $\alpha_6\beta_4$ signaling in epithelial cells?

In this study, we provide evidence that the $\alpha_6\beta_4$ integrin stimulates the Ras–Erk and Rac–Jnk mitogen-activated protein kinase (MAP kinase) signaling pathways via Shc and thereby controls immediate-early gene expression and keratinocyte proliferation in response to laminin.

Results

Ligation of $\alpha_6\beta_4$ causes activation of the Ras–Erk signaling pathway

To examine the intracellular signaling pathways activated by the $\alpha_6\beta_4$ integrin in a physiologically relevant cellular context, we elected to use primary human keratinocytes. These cells express high levels of $\alpha_6\beta_4$, $\alpha_2\beta_1$ and $\alpha_3\beta_1$ and adhere to laminin 5 through $\alpha_6\beta_4$ and $\alpha_3\beta_1$ (Xia *et al.*, 1996) and to collagen I through $\alpha_2\beta_1$ (Carter *et al.*, 1990b). Incubation of suspended keratinocytes with polystyrene beads coated with the anti-β_4 monoclonal antibody (Mab) 3E1 as well as adhesion to laminin 5-coated dishes caused tyrosine phosphorylation of the 52 and 46 kDa mol. wt isoforms of Shc (the 66 kDa isoform of Shc is expressed at very low levels in keratinocytes) and thereby recruitment of Grb2 (Figure 1A). No significant tyrosine phosphorylation of Shc and recruitment of Grb2 was observed in keratinocytes treated with beads coated with the anti-$\alpha_3\beta_1$ Mab P1B5 (not shown) or the control anti-MHC Mab W6.32 and in keratinocytes adhering to collagen I or poly-L-lysine-coated dishes (Figure 1A). The inability of collagen I to induce tyrosine phosphorylation of Shc and recruitment of Grb2 was not a consequence of insufficient adhesion because the keratinocytes spread equally well on laminin 5 and collagen I under our experimental conditions. Furthermore, we observed that antibody-mediated cross-linking of $\alpha_2\beta_1$ does not induce tyrosine phosphorylation of Shc and recruitment of Grb2 (not shown). These findings, which are consistent with previous results (Mainiero *et al.*, 1995; Wary *et al.*, 1996), indicate that ligation of $\alpha_6\beta_4$, but not $\alpha_2\beta_1$ and $\alpha_3\beta_1$, can promote signaling mediated by Shc in primary keratinocytes.

We next examined the role of cytoplasmic domain of β_4 in the recruitment and tyrosine phosphorylation of Shc. Rat bladder 804G cells expressing either a recombinant full-length human β_4 subunit (clone A) or a tail-less version (clone L) were cross-linked in suspension with the anti-β_4 Mab 3E1 or the control anti-MHC Mab W6.32. Immunoprecipitation with the 3E1 Mab followed by immunoblotting with anti-Shc antibodies revealed that the recruitment of all three isoforms of Shc by $\alpha_6\beta_4$ requires the cytoplasmic domain of the β_4 subunit (Figure 1B). To examine if recruitment to $\alpha_6\beta_4$ was a prerequisite for tyrosine phosphorylation of Shc, clone A and clone L were either cross-linked in suspension with the 3E1 or the W6.32 Mab or plated onto dishes coated with the same antibodies. Immunoprecipitation with anti-Shc antibodies followed by immunoblotting with anti-P-Tyr antibodies indicated that ligation of wild-type $\alpha_6\beta_4$ induces tyrosine phosphorylation of the 52 kDa isoform of Shc (the major isoform recruited to activated $\alpha_6\beta_4$ in these cells). In contrast, ligation of tail-less $\alpha_6\beta_4$ did not cause efficient tyrosine phosphorylation of Shc (Figure 1C). These results are consistent with the hypothesis that the recruitment of Shc to activated $\alpha_6\beta_4$ is mediated by tyrosine phosphorylation of the cytoplasmic domain of β_4. They also suggest that this event is necessary for subsequent tyrosine phosphorylation of the adaptor protein. Since in a number of experiments tail-less $\alpha_6\beta_4$ was able to induce a modest level of tyrosine phosphorylation of Shc, it is possible that an additional indirect mechanism contributes to the activation of Shc by $\alpha_6\beta_4$.

Since Grb2 is stably associated with the Ras-GTP exchange factor mSOS, the recruitment of Grb2 to the plasma membrane mediated by Shc is likely to bring mSOS in close proximity to its target Ras (Schlessinger, 1994). Ras-GTP loading experiments were therefore performed to examine if ligation of $\alpha_6\beta_4$ resulted in activation of Ras. After growth factor starvation and *in vivo* labeling with [^{32}P]orthophosphate, primary human keratinocytes were detached and either incubated in suspension with anti-β_4 or anti-MHC beads or replated on dishes coated with laminin 5 or anti-β_4 Mab. As a control, adherent keratinocytes were either left untreated or were stimulated with epidermal growth factor (EGF). As shown in Figure 2, chromatographic analysis of nucleotides bound to Ras indicated that adhesion to laminin 5- or anti-β_4 Mab-coated dishes results in an accumulation of GTP on Ras comparable with that caused by EGF. In suspended cells, however, antibody-mediated ligation of $\alpha_6\beta_4$ did not cause activation of Ras (Figure 2). The results of this experiment indicate that $\alpha_6\beta_4$-mediated cell adhesion causes activation

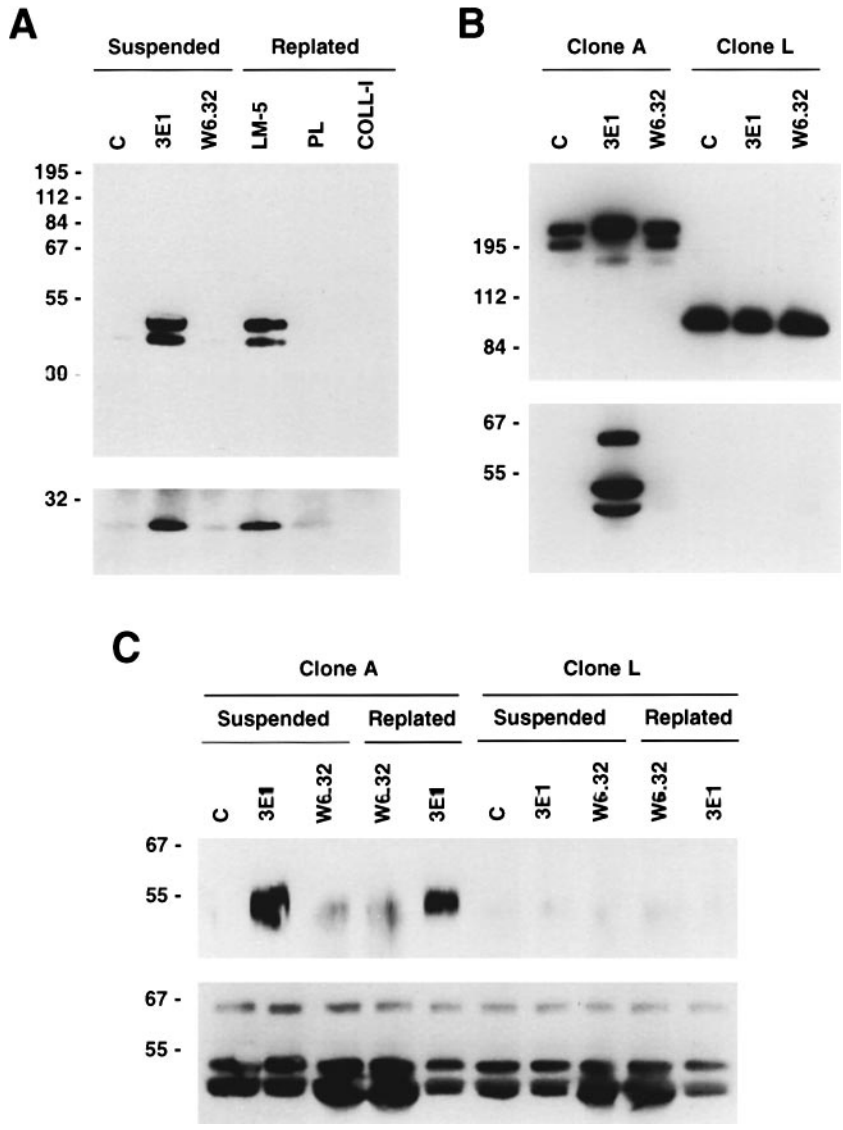

Fig. 1. Ligation of the $\alpha_6\beta_4$ integrin induces recruitment of Shc, tyrosine phoshorylation of Shc and association of Shc with Grb2. (**A**) Tyrosine phoshorylation of Shc and association of Shc with Grb2 in response to $\alpha_6\beta_4$ ligation. After growth factor starvation, primary human keratinocytes were detached and either incubated in suspension with medium alone (C), anti-β_4 beads (3E1) or anti-MHC beads (W6.32) for 20 min or plated on dishes coated with laminin 5 (LM-5), poly-L-lysine (PL) or collagen I (COLL-I) for 30 min. Equal amounts of total proteins were immunoprecipitated with anti-Shc Mab. The samples were probed by immunoblotting with HRP-conjugated anti-P-Tyr Mab RC20 (top panel) or polyclonal anti-Grb2 antibodies (bottom panel). (**B**) The recruitment of Shc to activated $\alpha_6\beta_4$ is mediated by the cytoplasmic domain of β_4. Clone A and clone L cells were serum starved and incubated in suspension with medium alone (C), with anti-β_4 beads (3E1) or anti-MHC beads (W6.32) for 20 min. Equal amounts of total proteins were immunoprecipitated with 3E1 Mab. The samples were probed by immunoblotting with polyclonal antibodies to the ectodomain of β_4 (top panel) and to Shc (bottom panel). (**C**) The cytoplasmic domain of β_4 is important for the activation of Shc in response to $\alpha_6\beta_4$ ligation. Clone A and clone L cells were serum starved and either incubated in suspension with medium alone (C), anti-β_4 beads (3E1) or anti-MHC beads (W6.32) for 20 min or plated on dishes coated with anti-β_4 (3E1) or anti-MHC (W6.32) Mab for 30 min. Equal amounts of total proteins were immunoprecipitated with anti-Shc Mab. The samples were probed by immunoblotting with HRP-conjugated anti-P-Tyr Mab RC20 (top panel) or polyclonal anti-Shc antibodies (bottom panel).

of Ras. They also suggest that, in contrast to the sequential recruitment of Shc and Grb2 which can occur in suspended cells treated with anti-β_4 beads, optimal activation of Ras by $\alpha_6\beta_4$ requires adhesion and/or spreading on a substratum coated with $\alpha_6\beta_4$ ligands.

We next examined if ligation of $\alpha_6\beta_4$ resulted in activation of the MAP kinase Erk. Growth factor-starved keratinocytes were detached and either kept in suspension or plated on dishes coated with the anti-β_4 Mab 3E1, the anti-MHC Mab W6.32, laminin 5, collagen I or poly-L-lysine. As a control, adherent keratinocytes were treated with EGF. As shown in Figure 3A, immunoprecipitation and *in vitro* kinase assays indicated that adhesion to laminin 5- or anti-β_4 Mab-coated dishes causes activation of Erk to a level similar to that induced by EGF. In contrast, adhesion to collagen I, poly-L-lysine or anti-MHC Mab did not result in significant activation of Erk (Figure 3A). Adhesion to anti-α_3 Mab-coated dishes also did not cause Erk activation (not shown). To examine the kinetics of Erk activation by $\alpha_6\beta_4$, keratinocytes were plated on laminin 5 for various times and subjected to Erk immunoprecipitation and kinase assay. As shown in Figure 3B, we detected a significant activation of Erk as early as 5 min after plating the keratinocytes on laminin

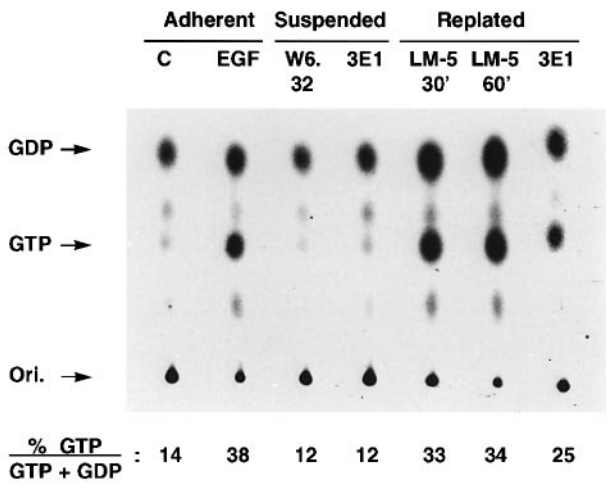

Fig. 2. Activation of Ras by the $\alpha_6\beta_4$ integrin. Primary human keratinocytes were growth factor starved and labeled *in vivo* with [^{32}P]orthophosphate. After detachment, they were then either incubated in suspension with anti-MHC (W6.32) or anti-β_4 (3E1) Mab-coated beads for 20 min or plated on dishes coated with laminin 5 (LM-5) for the indicated times or on dishes coated with anti-β_4 Mab (3E1) for 30 min. As a control, adherent cells were either left untreated or stimulated with 200 ng/ml EGF for 5 min. After immunoprecipitation of Ras, bound nucleotides were eluted and separated by TLC. Numbers indicate the molar ratio of GTP over total nucleotides.

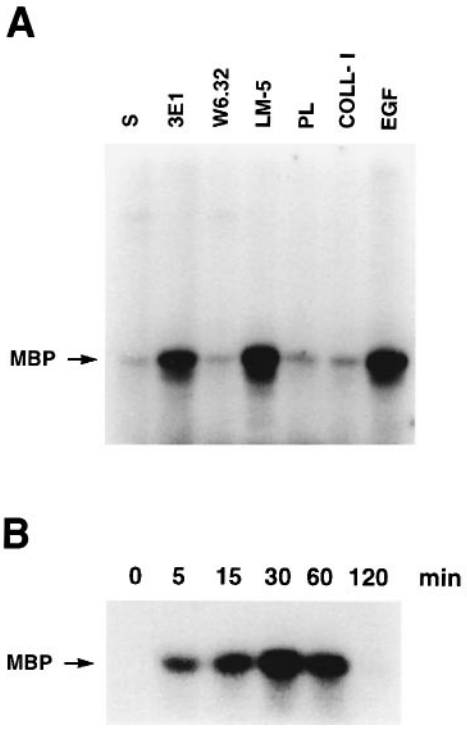

Fig. 3. Activation of Erk kinase by $\alpha_6\beta_4$. (**A**) Specificity of Erk activation by $\alpha_6\beta_4$. Primary human keratinocytes were growth factor starved, detached and either kept in suspension (S) or plated on dishes coated with anti-β_4 Mab (3E1), anti-MHC Mab (W6.32), laminin 5 (LM-5), collagen I (COLL-I) or poly-L-lysine (PL) for 30 min. As a control, adherent cells were stimulated with 200 ng/ml EGF for 5 min (EGF). (**B**) Kinetics of Erk activation by $\alpha_6\beta_4$. Primary human keratinocytes were growth factor starved, detached and either kept in suspension or plated on dishes coated with laminin 5 for the indicated times. Anti-Erk immunoprecipitates were subjected to *in vitro* kinase assay using MBP as a substrate.

5. The level of Erk activity peaked at 30 min of adhesion and declined thereafter. These results indicate that ligation of $\alpha_6\beta_4$ causes a significant and relatively persistent activation of the MAP kinase Erk and are in agreement with the observation that ligation of the laminin- and collagen-binding integrins $\alpha_3\beta_1$ and $\alpha_2\beta_1$ does not induce this event (Wary *et al.*, 1996). Since adhesion mediated by $\alpha_3\beta_1$ and $\alpha_2\beta_1$ is known to cause activation of focal adhesion kinase (FAK), these findings are also consistent with the notion that activation of FAK is not sufficient for stimulation of Erk in response to integrin ligation (Wary *et al.*, 1996).

The activation of Erk mediated by $\alpha_6\beta_4$ requires Shc, Ras and Rho

The mechanism of Erk activation in response to $\alpha_6\beta_4$ ligation was examined by testing the effect of various dominant-interfering mutant proteins. Since transient transfection of primary keratinocytes is very inefficient, we elected to use HeLa cells which express levels of $\alpha_6\beta_4$, $\alpha_3\beta_1$ and $\alpha_2\beta_1$ comparable with those of primary keratinocytes. HeLa cells were transfected with a hemagglutinin (HA)-tagged Erk2 vector in combination with different concentrations of cDNAs encoding dominant-negative Shc (317F), Ras (N17), RhoA (N19), CDC42 (N17) and Rac1 (N17). As shown in Figure 4, immunoprecipitation of HA-Erk2 followed by *in vitro* kinase assay indicated that the activation of Erk in response to $\alpha_6\beta_4$ ligation is suppressed by dominant-negative Shc (Figure 4A), Ras and RhoA (Figure 4B), but not by dominant-negative Cdc42 and Rac1 (Figure 4C). Although at the highest concentration tested dominant-negative Shc, Ras and RhoA completely inhibited the activation of Erk by $\alpha_6\beta_4$, at the intermediate and lowest concentration tested the inhibitory activity of the three dominant-negative mutants differed, perhaps as a consequence of the different relative abundance of their target protein or their different mechanism of action. These results indicate that Shc couples the $\alpha_6\beta_4$ integrin to the Ras–Erk signaling pathway and suggest that full activation of Erk in response to $\alpha_6\beta_4$ ligation requires the activity of both Ras and Rho.

Ligation of $\alpha_6\beta_4$ causes activation of the Rac–Jnk signaling pathway

We next examined if ligation of $\alpha_6\beta_4$ stimulated the MAP kinase Jnk. Growth factor-starved primary keratinocytes were detached and either kept in suspension or plated on dishes coated with anti-β_4 Mab, anti-MHC Mab, laminin 5, collagen I or poly-L-lysine. As a control, adherent keratinocytes were exposed to UV light. Jnk was precipitated by using a GST–Jun fusion protein and its activity examined by *in vitro* kinase assay. As shown in Figure 5, the binding of Mab 3E1 or laminin 5 to $\alpha_6\beta_4$ caused an activation of Jnk comparable with that induced by UV stimulation. In contrast, adhesion to dishes coated with collagen I, poly-L-lysine, the control Mab W6.32 (Figure 5) or the anti-$\alpha_3\beta_1$ Mab P1B5 (not shown) did not result in significant activation of Jnk. Time-course experiments indicated that the kinetics of activation of Jnk in response to laminin 5 were similar to those observed for Erk (not shown). These results indicate that ligation of $\alpha_6\beta_4$ causes activation of Jnk, and suggest that ligation of the collagen-binding integrin $\alpha_2\beta_1$ does not induce this event.

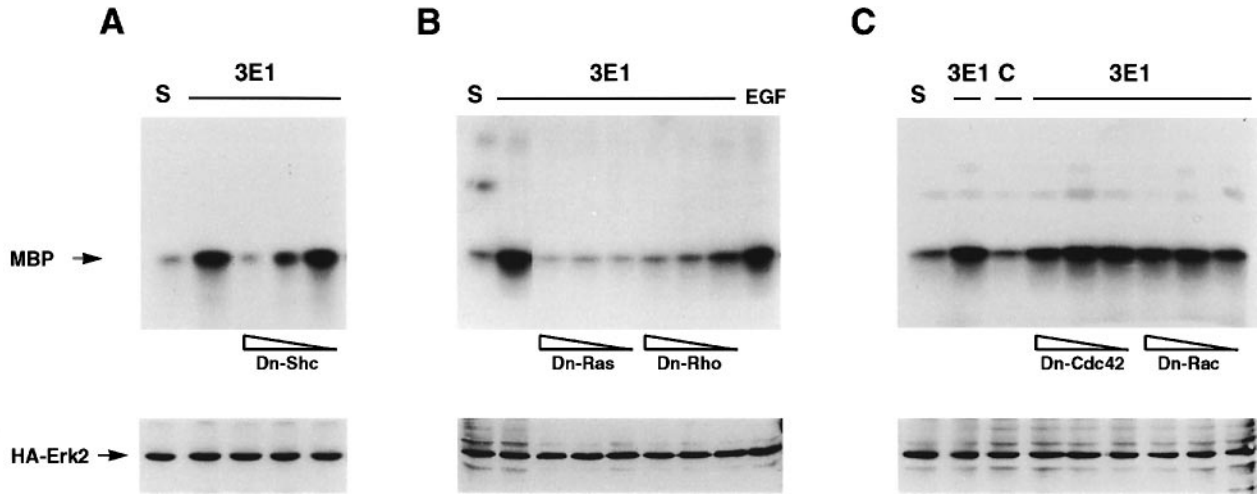

Fig. 4. The activation of Erk caused by $\alpha_6\beta_4$ ligation is inhibited by dominant-negative Shc, Ras and Rho. (**A**) HeLa cells were transiently transfected with 3 μg of HA-tagged Erk2 plasmid alone or in combination with 10, 5 and 2.5 μg of vector encoding dominant-negative Shc (Dn-Shc). The cells were then either kept in suspension (S) or plated on dishes coated with anti-β_4 Mab (3E1) for 30 min. (**B**) HeLa cells were transiently transfected with 1 μg of HA-tagged Erk2 plasmid alone or in combination with 1, 0.5 and 0.25 μg of vectors encoding dominant-negative Ras (Dn-Ras) or RhoA (Dn-Rho). The cells were then either kept in suspension (S) or plated on dishes coated with anti-β_4 Mab (3E1) for 30 min. As a control, adherent cells were treated with 200 ng/ml EGF for 5 min. (**C**) HeLa cells were transiently transfected with 1 μg of HA-tagged Erk2 plasmid alone or in combination with 1, 0.5 and 0.25 μg of vectors encoding dominant-negative Cdc42 (Dn-Cdc42) or Rac (Dn-Rac). The cells were then either kept in suspension (S) or plated on dishes coated with anti-β_4 Mab (3E1) or anti-MHC Mab (C) for 30 min. Anti-HA immunoprecipitates were subjected to *in vitro* kinase assay using myelin basic protein (MBP) as a substrate (top panels). Transfection efficiences were verified by immunoblotting aliquots of total proteins with anti-HA antibodies (bottom panels).

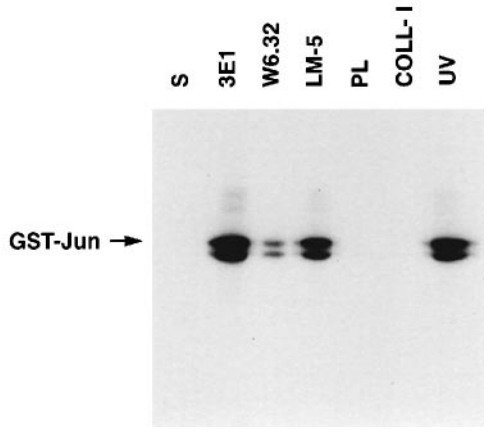

Fig. 5. Activation of Jnk kinase by $\alpha_6\beta_4$. Primary human keratinocytes were growth factor starved, detached and either kept in suspension (S) or plated on dishes coated with anti-β_4 Mab (3E1), anti-MHC Mab (W6.32), laminin 5 (LM-5), poly-L-lysine (PL) or collagen I (COLL-I) for 20 min. As a control, adherent cells were exposed to 40 J/m^2 of UV radiation (UV) and then kept in culture for 20 min. Jnk kinase was precipitated using glutathione beads coated with GST–Jun fusion protein and subjected to *in vitro* kinase assay. The position of phosphorylated GST–Jun is indicated. The lower band is a degradation product of GST–Jun.

The activation of Jnk mediated by $\alpha_6\beta_4$ requires Ras, PI-3K and Rac

The mechanism of Jnk activation in response to $\alpha_6\beta_4$ ligation was examined by testing the effect of various dominant-interfering mutant proteins and the phosphoinositide 3-kinase (PI-3K) inhibitor Wortmannin. HeLa cells were transfected with a Flag-tagged Jnk1 vector in combination with different concentrations of cDNAs encoding dominant-negative Ras (N17), Rac1 (N17), Cdc42 (N17) and RhoA (N19). As shown in Figure 6A, precipitation of Flag-Jnk1 followed by *in vitro* kinase assay indicated that the activation of Jnk in response to $\alpha_6\beta_4$ ligation is suppressed by dominant-negative Ras and Rac1, but not by dominant-negative RhoA, and very modestly by dominant-negative Cdc42. These results indicate that the activation of Jnk by $\alpha_6\beta_4$ requires the activity of both Ras and Rac. Although it has been suggested that Raf can activate Jnk by acting on the MAP kinase kinase kinase MEKK1 (Lange-Carter and Johnson, 1994), recent results indicate that the predominant mechanism by which Ras activates Jnk involves Rac, and not Raf (Kosravi-Far *et al.*, 1995; Minden *et al.*, 1995; Qiu *et al.*, 1995; Joneson *et al.*, 1996). The requirement for Rac in our system is consistent with this hypothesis. Since PI-3K is a downstream target effector of Ras (Rodriguez-Viciana *et al.*, 1994) and has been implicated in the activation of Rac (Nobes *et al.*, 1995; Klippel *et al.*, 1996), we tested if inhibition of PI-3K interfered with the activation of Jnk by $\alpha_6\beta_4$. As shown in Figure 6B, the activation of Jnk in response to ligation of $\alpha_6\beta_4$ was completely suppressed by as little as 50 nM Wortmannin, a concentration at which the inhibitor has very little effect on signaling molecules other than PI-3-K (Wymann *et al.*, 1996). Taken together, the results of these experiments suggest that $\alpha_6\beta_4$ activates the Rac–Jnk signaling pathway via Ras and PI-3K.

Induction of Fos SRE-dependent transcription by $\alpha_6\beta_4$

We next examined if the coupling of $\alpha_6\beta_4$ to Shc played a role in the control of immediate-early gene expression. Since Erk regulates transcription from the Fos serum response element (SRE) by phosphorylating the ternary complex factors Elk-1 and SAP-1, and Rho family proteins cooperate with this Erk function by acting on the serum response factor (Treisman, 1995), we examined the effect of $\alpha_6\beta_4$ ligation on the Fos SRE. HeLa cells were

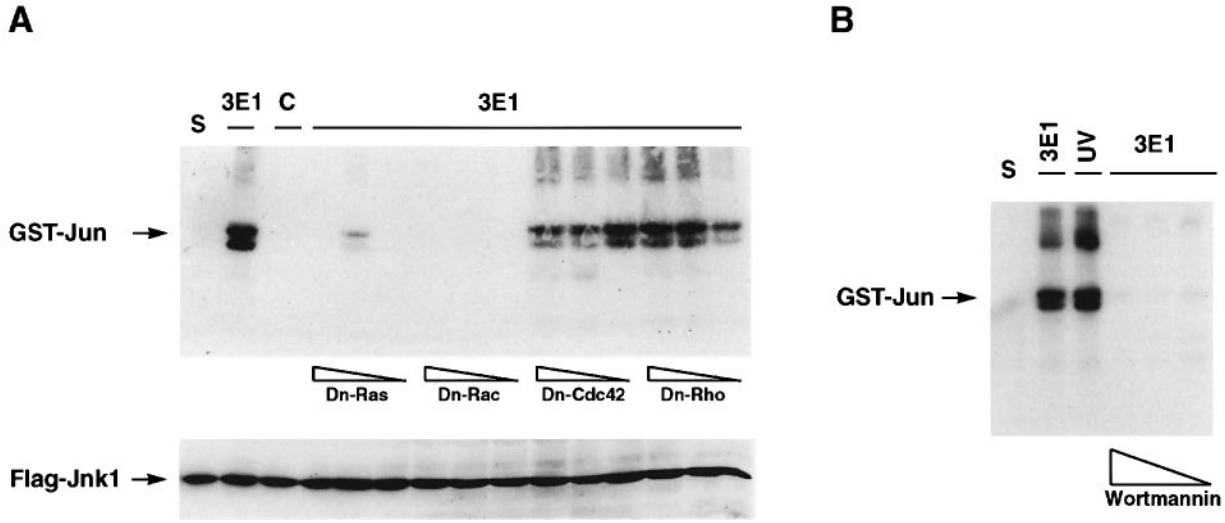

Fig. 6. The activation of Jnk kinase caused by $\alpha_6\beta_4$ ligation is inhibited by dominant-negative versions of Ras and Rac and by the PI-3K inhibitor Wortmannin. (**A**) HeLa cells were transiently transfected with 1 μg of Flag-tagged Jnk1 plasmid alone or in combination with 1, 0.5 and 0.25 μg of vectors encoding dominant-negative Ras (Dn-Ras), Rac (Dn-Rac), Cdc42 (Dn-Cdc42) and RhoA (Dn-Rho). The cells were then either kept in suspension or plated on dishes coated with anti-β_4 Mab (3E1) or anti-MHC Mab (C) for 20 min. Flag-Jnk was immunoprecipitated with the anti-Flag Mab M2 and subjected to *in vitro* kinase assay with GST–Jun as a substrate (top panel). Transfection efficiences were verified by immunoblotting aliquots of total proteins with Mab M2 (bottom panel). (**B**) HeLa cells were transiently transfected with 1 μg of Flag-tagged Jnk1 plasmid. After detachment, the cells were either kept in suspension or plated on dishes coated with anti-β_4 Mab (3E1) in the absence or presence of 200, 100 or 50 nM Wortmannin for 20 min. As a control, adherent cells were exposed to 40 J/m^2 of UV radiation for 20 min. Flag-Jnk was immunoprecipitated with Mab M2 and subjected to *in vitro* kinase assay with GST–Jun as a substrate. The position of phosphorylated GST–Jun is indicated. The lower band is a degradation product of GST–Jun.

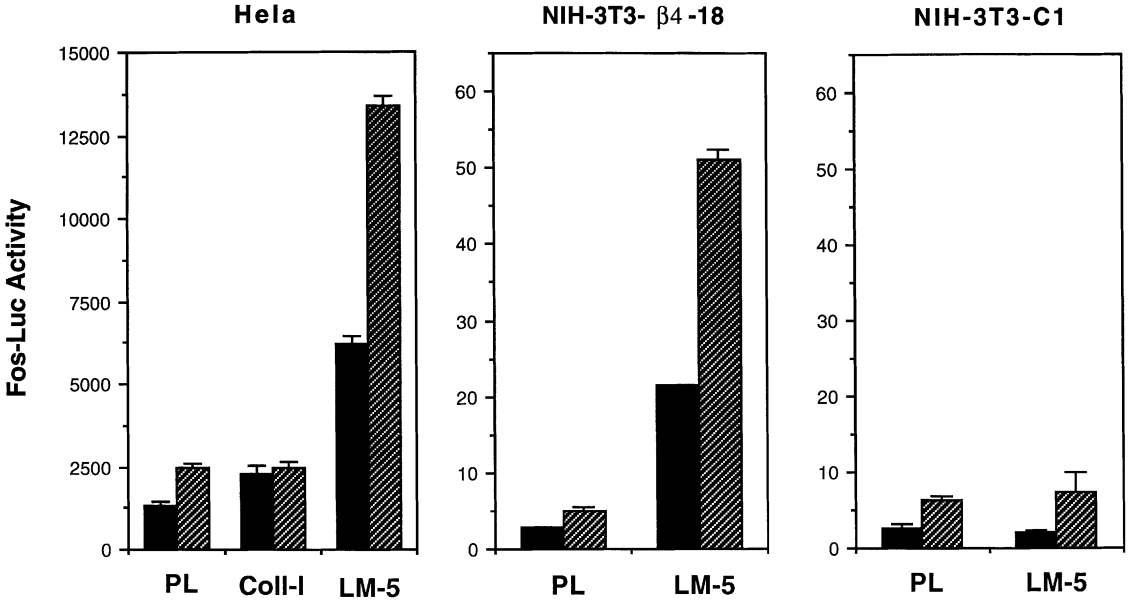

Fig. 7. Adhesion mediated by $\alpha_6\beta_4$ promotes transcription from the Fos SRE. HeLa cells, β_4-expressing NIH 3T3-β_4-18 cells and control NIH 3T3-C1 cells were transiently transfected with Fos-SRE–Luc plasmid. After growth factor starvation, the cells were detached and plated onto dishes coated with 10 μg/ml poly-L-lysine (PL), laminin 5 (LM-5) or collagen I (Coll-I) for 30 min. The cells were then either left untreated (solid bars) or exposed to mitogens for 10 min (shaded bars). Cell lysates were subjected to luciferase assay. Values are expressed in arbitrary units. The diagram shows the mean value and standard deviation from triplicate samples.

transiently transfected with the Fos-SRE–Luc vector, which contains the Fos SRE promoter element linked to the luciferase reporter gene. Upon plating on dishes coated with poly-L-lysine, collagen I or laminin 5, the cells were either left untreated or exposed to EGF. They were then subjected to luciferase assay. As shown in Figure 7, while adhesion to laminin 5 in the absence of EGF caused elevation of Fos SRE-dependent transcription, adhesion to poly-L-lysine or collagen I under the same conditions did not induce this activity. This suggests that ligation of $\alpha_6\beta_4$, but not $\alpha_2\beta_1$, is sufficient to promote transcription from the Fos SRE in the absence of mitogens. Treatment with EGF induced a significant elevation of Fos SRE activity in HeLa cells adhering to laminin 5, but caused a remarkably modest effect in cells attaching to poly-L-lysine or collagen I. This result suggests that ligation of $\alpha_6\beta_4$ is required for optimal induction of Fos SRE-dependent transcription in response to EGF.

To examine if laminin 5 is able to induce transcription from the Fos SRE in normal untransformed cells and to demonstrate the role of $\alpha_6\beta_4$ in this process, we transfected NIH 3T3 fibroblasts with a retroviral vector encoding human β_4 and isolated stable cell lines. Immunoprecipitation and fluorescence-activated cell sorting (FACS) analysis indicated that the recombinant β_4 subunit associated with endogenous α_6 and was regularly exported to the cell surface. Clones 8 and 18 displayed the highest levels of recombinant β_4 on the cell surface and were examined further. FACS analysis indicated that the level of expression of recombinant β_4 in these two clones approximated 40% of that of endogenous β_4 in primary keratinocytes. Since the NIH 3T3 cells do not express $\alpha_6\beta_4$ and adhere to laminin 5 through $\alpha_3\beta_1$, we examined the function of recombinant $\alpha_6\beta_4$ by comparing the kinetics by which the β_4 transfectants and control cells adhered to laminin 5-coated dishes. The results showed that the β_4 transfectants adhered to laminin 5 with faster kinetics than the controls. However, both types of cells became equally spread by 30 min of plating (not shown).

To analyze Fos SRE-dependent transcription in reponse to laminin 5, control and β_4-transfected NIH 3T3 cells were transiently transfected with the Fos-SRE–Luc plasmid. Upon plating on dishes coated with poly-L-lysine or laminin 5, the cells were either left untreated or exposed to basic fibroblast growth factor (bFGF), insulin and platelet-derived growth factor (PDGF). The results of luciferase assays indicated that adhesion to laminin 5 causes elevation of Fos SRE-dependent transcription in the β_4 transfectants even in the absence of mitogens and this induction is potentiated by mitogen treatment (Figure 7). The ability of laminin 5 to induce Fos SRE-dependent transcription was dependent on $\alpha_6\beta_4$ expression, because adhesion to laminin 5 did not cause this effect in control cells even after mitogen treatment (Figure 7). These results indicate that ligation of $\alpha_6\beta_4$, but not $\alpha_3\beta_1$, is sufficient to induce Fos SRE-dependent transcription, and confirm that $\alpha_6\beta_4$ cooperates with mitogens to cause optimal induction of this activity.

Adhesion mediated by $\alpha_6\beta_4$ promotes cell cycle progression

To examine if $\alpha_6\beta_4$ signaling played a role in cell cycle progression, primary keratinocytes were growth factor starved and then plated in the presence of EGF on plastic wells coated with laminin 5, collagen I or poly-L-lysine. Entry into the S phase was examined by 5′-bromo-2′-deoxyuridine (BrdU) incorporation and anti-BrdU staining. As shown in Figure 8, a significant fraction of keratinocytes plated on laminin 5 entered into the S phase during the 22 h of the assay. In contrast, only a modest percentage of cells plated on collagen I or poly-L-lysine entered into S during the assay. In the absence of mitogens, a similarly small percentage of cells plated on collagen I or poly-L-lysine entered into S phase (not shown). This fraction may consist of unsynchronized cells, which have already passed the G_1–S boundary at the time of plating. In addition, because the keratinocytes acquired and maintained a well-spread morphology on collagen I, their inability to enter into S on this substratum is not the result of insufficient spreading. The results of these experiments indicate that physical attachment and spreading on the extracellular matrix is not sufficient for progression of keratinocytes through G_1 in response to EGF, and suggest that this process requires ligation of a specific integrin, such as $\alpha_6\beta_4$.

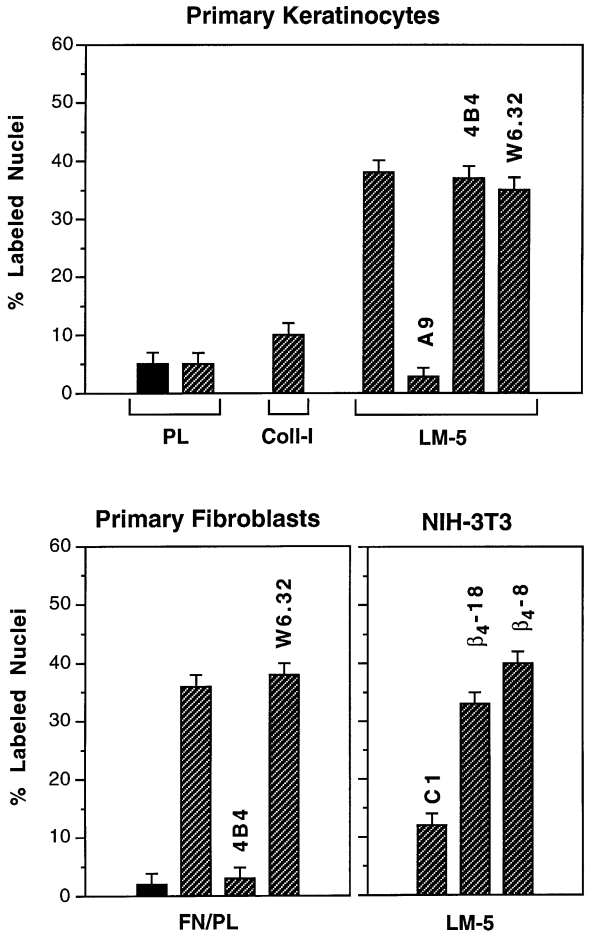

Fig. 8. Adhesion mediated by $\alpha_6\beta_4$ promotes cell cycle progression. Primary human keratinocytes, primary human dermal fibroblasts, control NIH 3T3-C1 cells and β_4-expressing NIH 3T3-18 and -8 cells were growth factor starved and plated on wells coated with 10 μg/ml poly-L-lysine (PL), collagen I (Coll-I) or laminin 5 (LM-5), or a mixture of 10 μg/ml fibronectin and 10 μg/ml poly-L-lysine (FN/PL). The cells were then incubated for 22 h in defined medium containing 10 μM BrdU and mitogens (shaded bars). To estimate the percentage of unsynchronized primary cells, the cells were also incubated for 22 h in defined medium containing 10 μM BrdU without mitogens (solid bars). When indicated, 10 μg/ml anti-β_1 Mab 4B4, 1:20 anti-β_4 Mab A9 ascites or 10 μg/ml control anti-MHC Mab W6.32 were included in the medium. After immunostaining with anti-BrdU Mab and alkaline phosphatase-conjugated secondary antibodies, the percentage of labeled nuclei was determined by scoring at least 500 cells from five different microscopic fields. The diagram shows the mean value and standard deviation from triplicate samples.

To examine the relative roles of $\alpha_6\beta_4$ and $\alpha_3\beta_1$ in keratinocyte proliferation, we tested the effect of inhibitory anti-β_4 and anti-β_1 antibodies. Growth factor-starved keratinocytes were plated on laminin 5 and exposed to EGF in the presence of the inhibitory anti-β_1 Mab 4B4, the inhibitory anti-β_4 Mab A9 or the control anti-MHC Mab W6.32. As shown in Figure 8, exposure to the anti-β_4 Mab completely suppressed keratinocyte entry into S. In contrast, treatment with the anti-β_1 or anti-MHC Mab did not inhibit keratinocyte proliferation on laminin 5. To control the efficacy of the anti-β_1 Mab 4B4, G_0 syncronized

primary human fibroblasts were plated on a mixed substrate consisting of poly-L-lysine and fibronectin and exposed to mitogens in the presence of the 4B4 or W6.32 Mab. In accordance with the recent observation that a class of β_1 integrins, which include the $\alpha_5\beta_1$ fibronectin receptor, is linked to the Ras–Erk pathway and the control of cell cycle progression by Shc (Wary *et al.*, 1996), plating of the primary fibroblasts on fibronectin/poly-L-lysine promoted cell cycle progression, and exposure to anti-β_1 Mab 4B4 blocked this process without inducing detachment (Figure 8). These results suggest that the ability of laminin 5 to promote keratinocyte cell cycle progression is mediated by $\alpha_6\beta_4$, and not by $\alpha_3\beta_1$.

We next examined the ability of control and β_4-expressing NIH 3T3 cells to progress through G_1 on laminin 5. While only a modest percentage of control cells progressed through G_1 when plated on laminin 5 for 22 h, a significant fraction of β_4 expressors entered into S under the same conditions (Figure 8), suggesting that ligation of $\alpha_6\beta_4$ is sufficient to promote progression through G_1 in response to mitogens. Taken together, the results of these assays indicate that ligation of $\alpha_6\beta_4$ is required and sufficient to promote keratinocyte proliferation in response to laminin 5.

Discussion

Although the notion that cell adhesion to the extracellular matrix regulates gene expression is supported by considerable experimental evidence, the signaling pathways linking integrins to nuclear events are not well known. In particular, the mechanisms by which integrin-dependent signals regulate cell cycle progression in normal epithelial cells are not fully understood. The results of recent studies have defined the membrane-proximal events induced by ligation of the $\alpha_6\beta_4$ integrin, a laminin receptor involved in various morphogenetic processes (Giancotti, 1996). Upon binding to extracellular ligand, $\alpha_6\beta_4$ becomes phosphorylated on tyrosine residues by the action of an integrin-associated kinase and thereby combines sequentially with the adaptor proteins Shc and Grb2 (Mainiero *et al.*, 1995). The results of the present study provide clear evidence that these receptor-proximal events result in the activation of Ras and of two distinct MAP kinase signaling pathways which regulate immediate-early gene expression. In contrast, other integrins, such as the $\alpha_2\beta_1$ and $\alpha_3\beta_1$ collagen and laminin receptors, do not induce these events. Since $\alpha_6\beta_4$-mediated adhesion promotes keratinocyte progression through G_1 in response to growth factor treatment, while $\alpha_2\beta_1$- and $\alpha_3\beta_1$-mediated adhesion does not, we propose that the linkage of $\alpha_6\beta_4$ to Ras signaling mediated by Shc participates in the control of cell cycle progression in normal epithelial cells.

The adaptor protein Shc contains two separate domains involved in the recognition of tyrosine-phosphorylated sequence motifs: an N-terminal phosphotyrosine-binding (PTB) domain and a C-terminal Src homology 2 (SH2) domain (Pawson, 1995). GST fusion protein binding experiments have suggested that both domains can interact with the tyrosine-phosphorylated β_4 tail (Mainiero *et al.*, 1995). The results of this study are in accordance with the notion that the recruitment of Shc to $\alpha_6\beta_4$ is mediated by the cytoplasmic domain of β_4 and suggest that this event is important for the subsequent tyrosine phosporylation of Shc, which is presumably mediated by the integrin-associated kinase. Upon phosphorylation, Shc combines with the other adaptor protein Grb2. Since Grb2 is constitutively associated with the Ras-GTP exchange factor mSOS, the recruitment of Grb2 to the plasma membrane potentially links $\alpha_6\beta_4$ to Ras. In accordance with this hypothesis, our current results show that ligation of $\alpha_6\beta_4$ results in a significant activation of Ras.

In contrast to the recruitment of Shc and Grb2 which could be observed in suspended keratinocytes cross-linked with anti-$\alpha_6\beta_4$ antibodies, full activation of Ras required physical attachment and/or spreading on a substratum coated with $\alpha_6\beta_4$ ligands. Since it has been suggested that proper targeting of the Grb2–mSOS complex to Ras may require an interaction of the Grb2 SH3 domains with the cortical cytoskeleton (Bar-Sagi *et al.*, 1993), it is possible that such targeting is defective in suspended keratinocytes cross-linked with anti-$\alpha_6\beta_4$ antibodies. Alternatively, since the pleckstrin homology domain of mSOS may bind to phosphatidylinositol(4,5)bisphosphate ($PtdInsP_2$) in the plasma membrane (Lemmon *et al.*, 1996) and it is known that $PtdInsP_2$ levels decline in suspended cells (McNamee *et al.*, 1993), it is possible that Ras activation by $\alpha_6\beta_4$ requires a threshold concentration of $PtdInsP_2$ in the plasma membrane which is not available in suspended keratinocytes. Future studies will be required to resolve this issue.

The results of this study indicate that ligation of $\alpha_6\beta_4$ results in the stimulation of both Ras–Erk and Rac–Jnk MAP kinase signaling pathways. The activation of Erk by $\alpha_6\beta_4$ was suppressed by dominant-negative versions of both Shc and Ras, indicating that the coupling to Ras mediated by Shc is the major mechanism by which $\alpha_6\beta_4$ controls Erk activation. Interestingly, Erk activation was also inhibited by dominant-negative RhoA. This result, which is in agreement with the recent observation that Rho activity is required for full activation of Erk in response to various extracellular stimuli (Hill *et al.*, 1995), suggests that this G protein also participates in signaling by $\alpha_6\beta_4$. The activation of Jnk by $\alpha_6\beta_4$ was inhibited by dominant-negative Ras and Rac1, but not by dominant-negative RhoA and Cdc42. In addition, it was suppressed by nanomolar concentrations of the PI-3K inhibitor Wortmannin. Since there is evidence that PI-3K is a downstream target effector of Ras and is involved in the activation of Rac (Rodriguez-Viciana *et al.*, 1994; Nobes *et al.*, 1995; Klippel *et al.*, 1996), it is likely that $\alpha_6\beta_4$ stimulates the Rac–Jnk pathway via Ras. Thus, the coupling of $\alpha_6\beta_4$ to Ras mediated by Shc leads to the activation of both Ras–Erk and Rac–Jnk signaling pathways.

In accordance with the observation that Erk stimulates transcription of the immediate-early gene *fos* (Treisman, 1995), the results of our study indicate that adhesion mediated by $\alpha_6\beta_4$ is sufficient to promote transcription from the Fos SRE. Interestingly, while treatment with mitogens caused a significant elevation of Fos SRE activity in cells plated on the $\alpha_6\beta_4$ ligand laminin 5, it was ineffective in cells adhering to the $\alpha_2\beta_1$ ligand collagen I, indicating that the expression of Fos in response to mitogens requires ligation of a specific integrin, such as $\alpha_6\beta_4$. Future studies will be required to examine further the mechanism by which $\alpha_6\beta_4$ controls immediate-early

gene expression. For example, it is known that Rho family proteins can activate the Fos promoter by stimulating the serum response factor (Hill *et al.*, 1995). The ability of $\alpha_6\beta_4$ to stimulate Rac may thus contribute to the activation of Fos promoter in response to laminin 5. In addition, since it is well established that Jnk controls the activity of the Jun promoter (Karin, 1995), it is likely that $\alpha_6\beta_4$ also regulates the expression of the immediate-early expression gene Jun. Taken together, these observations suggest that $\alpha_6\beta_4$ is a crucial regulator of immediate-early gene expression.

What is the biological significance of $\alpha_6\beta_4$ signaling? The results of our cell proliferation analysis indicate that $\alpha_6\beta_4$ signaling promotes transit through G_1 in keratinocytes and other $\alpha_6\beta_4$-expressing cells exposed to mitogens. In this respect, $\alpha_6\beta_4$ appears to be functionally distinct from other integrins, such as $\alpha_3\beta_1$ and $\alpha_2\beta_1$, which do not appear to be able to do so. In fact, it is quite remarkable that keratinocytes plated on the $\alpha_2\beta_1$ ligand collagen I adhere and spread but do not enter into the S phase despite being exposed to otherwise mitogenic concentrations of EGF. We have observed recently that a class of β_1 and α_v integrins, which include $\alpha_1\beta_1$, $\alpha_5\beta_1$ and $\alpha_v\beta_3$, but not $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_6\beta_1$, are also linked to the Ras–Erk pathway and the control of cell cycle progression by Shc (Wary *et al.*, 1996). Taken together, these observations suggest that the ability of extracellular matrix to control cell proliferation, thereby mediating anchorage-dependent cell growth, depends on its composition and the repertoire of integrins on the responding cell.

What is the mechanism by which $\alpha_6\beta_4$ signaling regulates cell proliferation? Previous studies have indicated that the cell cycle of normal cells contains an anchorage-dependent transition in early–mid G_1. In fact, adhesion to the extracellular matrix is required for translation of cyclin D_1 in cells exposed to mitogens, suggesting that integrin- and growth factor-dependent signals converge prior to the induction of cyclin D_1 to control progression through G_1 (Fang *et al.*, 1996; Zhu *et al.*, 1996). Our results suggest that these signals are integrated before the induction of immediate-early gene expression. The simplest hypothesis is that in normal cells growth factor receptors and specific integrins cooperate to activate MAP kinase beyond the threshold level required for immediate-early gene expression. Since most dominant oncogenes, including Shc (Pelicci *et al.*, 1992), induce neoplastic transformation by constitutively activating the Ras–MAP kinase pathway, this model also explains why neoplastic cells usually display anchorage-independent growth.

In conclusion, the results of this study indicate that the coupling of $\alpha_6\beta_4$ integrin to Ras–Erk and Rac–Jnk pathways mediated by Shc regulates immediate-early gene expression and cell cycle progression in response to mitogens. Since the major keratinocyte integrins, in addition to $\alpha_6\beta_4$, are $\alpha_2\beta_1$ and $\alpha_3\beta_1$, which are not coupled to Shc, the signaling function of $\alpha_6\beta_4$ may explain why cell proliferation is restricted to the basal layer in the epidermis and other stratified epithelia. In addition, since exit from the cell cycle is a prerequisite for differentiation, our results may also explain why the onset of keratinocyte differentiation is coupled to the detachment from the basement membrane (Hall and Watt, 1989). Finally, the ability of $\alpha_6\beta_4$ to stimulate cell growth suggests that its overexpression in squamous carcinoma (Kimmel and Carey, 1986; Wolf *et al.*, 1990) may contribute to tumor progression.

Materials and methods

Antibodies and extracellular matrix molecules

The specificity of anti-β_4 Mab 3E1 was described previously (Giancotti *et al.*, 1992). The polyclonal antiserum to the β_4 ectodomain was generated by immunizing a rabbit with a GST fusion protein comprising amino acids 31–217. The inhibitory anti-β_4 Mab A9 was obtained from Tom Carey (Comprehensive Cancer Center, University of Michigan at Ann Arbor). Hybridomas producing the anti-α_1 Mab TS2/7 and anti-β_1 Mab TS2/16 were obtained from ATCC (Rockville, MD). The anti-α_2 Mab P1E6, anti-α_3 Mab P1B5 and anti-α_5 Mab P1D6 were from Gibco-BRL (Gaithersburg, MD). The anti-β_1 Mab 4B4 was from Coulter (Hialeah, FL). The anti-MHC Mab W6.32 reacts with human and cultured rodent cells. The anti-FLAG M2 Mab and anti-HA peptide tag Mab 12CA5 were purchased from Eastman Kodak Company (New Haven, CT) and Boehringer Mannheim (Indianapolis, IN), respectively. The anti-Shc Mab and the recombinant horseradish peroxidase (HRP)-conjugated anti-P-Tyr Mab RC20 were from Transduction Laboratories (Lexington, KY). Anti-Erk2 and anti-Grb2 polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Human fibronectin and collagen I were purchased from Gibco-BRL. Laminin 5 matrices were prepared as described previously (Sonnenberg *et al.*, 1993; Spinardi *et al.*, 1995).

Cell lines, constructs and transfections

HeLa cells were cultured in Dulbecco's modified minimal essential medium (DMEM) with 10% fetal calf serum (FCS). Primary human keratinocytes were cultured in keratinocyte serum-free medium (K-SFM) supplemented with bovine pituitary extract (50 μg/ml) and human recombinant EGF (5 ng/ml) (Gibco BRL). NIH 3T3 cells expressing recombinant human β_4 were maintained in DMEM containing 10% bovine calf serum (BCS) and 3 mM L-histidinol (Sigma, St Louis, MO). Primary dermal fibroblasts were obtained from Clonetics (San Diego, CA) and cultured in fibroblast basal medium (FBM) supplemented with 2% FCS, 2 ng/ml bFGF and 10 μg/ml insulin.

To generate pLXSHD-β_4, the full-length human β_4 cDNA was subcloned in the *Eco*RI site of pLXSHD, a derivative of the Moloney leukemia virus-derived retroviral vector pLXSN containing as a selection marker the histidinol dehydrogenase gene (Dusty Miller and Rosman, 1989). The recombinant virus was produced by transiently transfecting 293-T cells with 10 μg of pLXHD-β_4 and 10 μg of packaging-defective ecotropic virus (Landau and Littman, 1992). Subconfluent NIH 3T3 cells were infected with a dilution of the culture supernatant collected 48 h after transfection. Cell lines expressing recombinant human β_4 were isolated by L-histidinol selection and identified by FACS analysis. Rat 804G cells expressing a recombinant wild-type human β_4 subunit were previously described (Spinardi *et al.*, 1993). To generate a cytomegalovirus (CMV)-driven eukaryotic expression vector encoding a tail-less β_4 subunit, the 2.3 kb *Eco*RI–*Sca*I fragment of β_4 cDNA was ligated into *Eco*RI–*Bam*HI-digested pRK-5. The 2.3 kb *Eco*RI–*Xba*I fragment of the resulting plasmid was then ligated into *Eco*RI–*Xba*I-linearized pRc-CMV. The recombinant β_4 subunit encoded by this vector is truncated immediately after Lys734, which marks the boundary between the transmembrane and intracellular domains of the polypeptide. Clone L cells were generated by transfecting parental 804G cells with the above vector according to previously published protocols (Spinardi *et al.*, 1993). FACS analysis was used to verify that clone A and clone L cells had comparable levels of expression of recombinant β_4. Metabolic labeling with [^{35}S]methionine/cysteine (Translabel, ICN, Costa Mesa, CA) and immunoprecipitation were used to verify the correct assembly of recombinant β_4 with endogenous α_6 in both NIH 3T3 and 804G transfectants.

The CMV promoter-based expression vectors encoding HA-tagged Erk2, dominant-negative p52^{Shc} (Y317F) and dominant-negative Ras (N17) were obtained from Edward Scolnik (NYU School of Medicine). The vectors encoding GST–Jun and Flag-tagged Jnk1 were described previously (Hibi *et al.*, 1993; Derijard *et al.*, 1994). The cDNAs encoding dominant-negative RhoA (N19) and Rac1 (N17) (Khosravi-Far *et al.*, 1995) were subcloned in pcDNA3. Dominant-negative Cdc42 (N17) in pCMV5 was obtained from Jonathan Chernoff (Fox Chase Cancer Center, Philadelphia, PA). The Fos-SRE–Luc reporter plasmid was from

Joseph Schlessinger. Vectors were transiently transfected in HeLa and NIH 3T3 cells by the lipofectamine method (Gibco-BRL).

Biochemical methods

To obtain ligation of integrins in the absence of any co-stimulus, the cells were growth factor starved for 36 h, detached, and resuspended in serum-free medium. The cells were then either incubated in suspension with polystyrene beads (2.5 μm diameter, IDC, Portland, OR) coated with anti-integrin Mabs (Mainiero *et al.*, 1995; Wary *et al.*, 1996) or plated onto dishes coated sequentially with affinity-purified goat anti-mouse IgGs and anti-integrin Mabs or extracellular matrix proteins. At the coating concentrations used, the cells attached and spread equally well on laminin 5 and collagen I, attached and partially spread on the anti-β_4 Mab 3E1, and attached without spreading on poly-L-lysine and the control Mab W6.32. As a positive control for Jnk activation, adherent cells were exposed to UV radiation as previously described (Hibi *et al.*, 1993; Derijard *et al.*, 1994). At the end of the incubation, the cells were extracted and subjected to biochemical analysis.

To immunoprecipitate Shc and $\alpha_6\beta_4$, primary human keratinocytes were extracted in Triton lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1% Triton X-100) containing 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 25 mM sodium fluoride, 0.01% aprotinin, 4 mg/ml pepstatin A, 10 mg/ml leupeptin and 1 mM phenylmethanesulfonyl fluoride (PMSF) (all from Sigma) for 30 min on ice. Immunoprecipitation, SDS–PAGE and immunoblotting analysis were performed as previously described (Giancotti and Ruoslahti, 1990; Mainiero *et al.*, 1995). Nitrocellulose-bound antibodies were detected by chemiluminescence with ECL (Amersham Life Sciences, Little Chalfont, UK).

To examine Erk activity, cells were extracted with NP-40 lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM EDTA) containing phosphatase and protease inhibitors for 30 min on ice. Endogenous and recombinant tagged Erks were immunoprecipitated with anti-Erk2 or anti-HA Mab, respectively, and subjected to *in vitro* kinase assay. The kinase reaction was initiated by adding to the beads 25 μl of kinase buffer (25 mM Tris pH 7.5, 12.5 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 20 μM cold ATP, 0.5 mM sodium orthovanadate) containing 5 μCi of [γ-^{32}P]ATP (4500 Ci/mmol, ICN Biomedicals Inc.) and 2.5 μg of myelin basic protein (Sigma). After 30 min of incubation at 30°C, the samples were boiled in sample buffer and separated by SDS–PAGE.

To analyze the activation of Jnk, cells were extracted for 30 min on ice with modified Triton lysis buffer (25 mM HEPES pH 7.5, 300 mM NaCl, 0.1% Triton X-100, 0.2 mM EDTA, 20 mM β-glycerophosphate, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol) containing phosphatase and protease inhibitors. Endogenous Jnk was precipitated with 3 μg of GST–Jun fusion protein coupled to glutathione–agarose beads. After washing, the beads were incubated with 25 μl of kinase buffer containing 5 μCi of [γ-^{32}P]ATP. Recombinant Flag-tagged Jnk1 was immunoprecipitated with the anti-Flag Mab M2 and incubated with 25 μl of kinase buffer containing 2.5 μg of GST–Jun and 5 μCi of [γ-^{32}P]ATP. After 30 min of incubation at 30°C, the samples were boiled in sample buffer and separated by SDS–PAGE.

To estimate Ras activation, primary human keratinocytes were starved for 48 h in K-SFM and labeled for 12 h with [^{32}P]orthophosphate (0.5 mCi/ml, ICN) in phosphate-free DMEM supplemented with 0.1% phosphate-free BCS. After stimulation, the cells were extracted and the samples subjected to Ras-GTP loading assay as described previously (Gale *et al.*, 1993). Nucleotides bound to Ras were analyzed by TLC on PEI-cellulose plates in 0.75 M K_2HPO_4, pH 3.5. Radioactivity in GDP and GTP was estimated by Phosphorimager analysis.

To measure trascription from the Fos SRE, HeLa cells and NIH 3T3 transfectants were transiently transfected with the reporter plasmid Fos-SRE–Luc. After 24 h of growth factor starvation, the cells were detached and plated on dishes coated with laminin 5, collagen I or poly-L-lysine for 30 min. The HeLa cells were then either left untreated or exposed to 50 ng/ml EGF, 20 ng/ml PDGF-BB, 2 ng/ml bFGF and 10 μg/ml insulin for 10 min. The NIH 3T3 transfectants were either left untreated or exposed to 10 ng/ml PDGF-BB, 5 ng/ml bFGF and 10 μg/ml insulin for 10 min. Luciferase activity in cell lysates was estimated as previously described (Brasier *et al.*, 1989).

Measurement of cell cycle progression

To monitor progression through G_1 and entry into S phase, the cells were starved by incubation in medium devoid of serum and growth factors for 48 h, detached and plated at low density on microtiter plates or glass coverslips coated with 10 μg/ml poly-L-lysine, laminin 5, collagen I or a mixture of 10 μg/ml fibronectin and 10 μg/ml poly-L-lysine. The keratinocytes were incubated in K-SFM supplemented with 5 ng/ml human recombinant EGF. The NIH 3T3 transfectants were incubated in FBM supplemented with 20 ng/ml PDGF, 2 ng/ml bFGF and 10 μg/ml insulin. Primary dermal fibroblasts were incubated in FBM with 2 ng/ml bFGF and 10 μg/ml insulin. The media were supplemented with 10 μM BrdU and, when indicated, with the inhibitory anti-β_1 Mab 4B4, the inhibitory anti-β_4 Mab A9 or the control anti-MHC Mab W6.32. After 22 h of incubation, the cells were fixed in 70% ethanol, 50 mM glycine, pH 2.0 for 30 min at –20°C and stained with anti-BrdU Mab and alkaline phosphatase-conjugated secondary antibodies (Boehringer Mannheim, Indianapolis, IN). The percentage of labeled nuclei was determined by scoring at least 500 cells from five different microscopic fields.

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