Compartmentalization of integrin α6β4 signaling in lipid rafts

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Integrin α6β4 signaling proceeds through Src family kinase (SFK)–mediated phosphorylation of the cytoplasmic tail of β4, recruitment of Shc, and activation of Ras and phosphoinositide-3-kinase. Upon cessation of signaling, α6β4 mediates assembly of hemidesmosomes. Here, we report that part of α6β4 is incorporated in lipid rafts. Metabolic labeling in combination with mutagenesis indicates that one or more cysteine in the membrane-proximal segment of β4 tail is palmitoylated. Mutation of these cysteines suppresses incorporation of α6β4 in lipid rafts, but does not affect α6β4–mediated adhesion or assembly of hemidesmosomes. The fraction of α6β4 localized to rafts associates with a palmitoylated SFK, whereas the remainder does not. Ligation of palmitoylation-defective α6β4 does not activate SFK signaling to extracellular signal–regulated kinase and fails to promote keratinocyte proliferation in response to EGF. Thus, compartmentalization in lipid rafts is necessary to couple the α6β4 integrin to a palmitoylated SFK and promote EGF-dependent mitogenesis.

Introduction

The α6β4 integrin is a laminin-5 receptor with unique functions in epithelial growth and carcinoma invasion (Mainiero et al., 1997; Shaw et al., 1997). The cytoplasmic tail of β4 is large and bears no homology to the short tails of other integrin β subunits. Upon matrix binding, the β4 tail is phosphorylated on tyrosine and interacts with the adaptor Shc, inducing Ras/extracellular signal–regulated kinase (ERK) signaling (Mainiero et al., 1995, 1997). In addition, β4 activates phosphatidylinositol-3-kinase (PI-3K) and Rac (Shaw et al., 1997). Upon dephosphorylation, the β4 tail associates with the keratin cytoskeleton, leading to assembly of hemidesmosomes (Spinardi et al., 1993; Murgia et al., 1998; Dans et al., 2001). α6β4 cooperates with multiple receptor protein tyrosine kinases. Activation of the EGF receptor (EGF-R) and Met enhances phosphorylation of β4 and Shc signaling, causing disruption of hemidesmosomes and increased cell motility and proliferation (Mariotti et al., 2001; Trusolino et al., 2001). Conversely, matrix binding to α6β4 increases activation of ErbB2/Neu (Falcioni et al., 1997). Regulated joint α6β4/receptor protein tyrosine kinase signaling promotes epithelial cell survival, proliferation, and migration (Mainiero et al., 1997; Mariotti et al., 2001; Weaver et al., 2002). Deregulation of this system plays a crucial role in carcinoma invasion and growth (Shaw et al., 1997; Mariotti et al., 2001; Trusolino et al., 2001). Mice with a targeted deletion of the β4 tail display proliferation defects in the epidermis and intestinal epithelium, highlighting the physiological significance of α6β4 signaling (Murgia et al., 1998).

The lipid rafts—subdomains of the plasma membrane enriched in cholesterol and glycosphingolipids—promote membrane compartmentalization of signaling components (Simons and Toomre, 2000). Because of the biophysical properties of their lipid anchor, GPI-linked receptors and...
palmitoylated signaling proteins, such as certain G proteins, H-Ras, many Src family kinases (SFKs), and eNOS, are concentrated in rafts (Resh, 1999). Here, we report that the α6β4 integrin is incorporated in lipid rafts in a palmitoylation-dependent manner, and this is necessary to couple the integrin to a palmitoylated SFK (pSFK) and to promote mitogenic signaling.

Results and discussion

Lipid rafts are resistant to extraction in Triton X-100, and because of their low density, float on sucrose or OptiPrep™ gradients. To examine if α6β4 localizes to lipid rafts, HaCat keratinocytes were solubilized in Triton X-100 and subjected to sucrose gradient fractionation. The rafts were recovered from fractions 2 and 3, as indicated by the relative enrichment of caveolin-1 and the pSFK Yes in these fractions. The Triton X-100–soluble cellular fraction was distributed over fractions 7–10, as shown by blotting with anti-transferrin receptor and anti-tubulin, whereas the insoluble material remained in the pellet fraction, P (Fig. 1 A). Immunoblotting with anti-β4 showed that a significant part of α6β4 (∼15% of the total) cofractionates with the rafts, whereas the remainder is in the Triton X-100–soluble fraction and, to a minor extent, in the pellet fraction (Fig. 1 A). As shown in Fig. 1 B, treatment of HaCat cells with the cholesterol-chelating agents methyl-β-cyclodextrin and saponin disrupted the association of α6β4 with lipid rafts. Thus, a fraction of α6β4 partitions in the low density fractions of sucrose gradients in a cholesterol-dependent manner, as expected of a lipid raft component.

Antibody-mediated cross-linking was used to mimic the effect of ligand-induced aggregation of α6β4 and to study its effect on the incorporation of the integrin in rafts. Fractionation was on OptiPrep™ gradients. Fig. 1 C shows that antibody-mediated ligation of α6β4 greatly increased the amount of integrin recovered from rafts (4–30%), whereas cross-linking of type I MHC did not exert this effect. In addition, the amount of α6β4 recovered from the raft fraction of untreated suspended cells was much lower than that usually obtained from the same fraction of stably adherent cells (compare Fig. 1 C with Fig. 1 A; 4 vs. 15%). These results suggest that ligand-induced aggregation promotes incorporation of α6β4 in lipid rafts.

The juxtamembrane segment of the β4 tail contains a cluster of cysteines, which may be palmitoylated (Fig. 2 A). To examine this possibility, rat bladder 804G cells expressing a wild-type (A) or a tail-less (L) human β4 were metabolically labeled with 16-[125I]iodohexadecanoic acid ([125I]IC16) palmitate analogue or [35S]methionine/cysteine and were immunoprecipitated with the anti–human β4 mAb 3E1. Fig. 2 B shows that β4 incorporated [125I]IC16, but α6 did not. Deletion of the β4 tail prevented palmitoylation of β4. In addition, treatment with alkali released the [125I] radioactive signal from β4, implying that the radioactive palmitate analogue was attached to β4 through a thio–ester bond. Notably, β4 was found to be palmitoylated to a higher apparent stoichiometry in HaCat keratinocytes (Fig. 2 B), primary human keratinocytes, and squamous carci-
noma A431 cells (unpublished data). Although other integrin β subunits do not contain potential palmitoylation sites, the cytoplasmic segments of α3, α6, α8, and αE contain one membrane-proximal cysteine. We did not detect any palmitoylation of α3β1 and α6β1 (unpublished data). Thus, β4 may be the only integrin subunit modified by palmitoylation.

To identify the region of β4 tail modified by palmitoylation, we examined various cytoplasmic deletion mutant forms of human β4 (Fig. 2 A). Metabolic labeling with [3H]palmitate revealed that mutants B and C were palmitoylated as efficiently as wild-type β4. By contrast, mutants L and E incorporated very little [3H]palmitate (Fig. 2 C), possibly because of nonphysiological, partially compensatory palmitoylation of Cys 732, which resides at the boundary between the transmembrane and cytoplasmic domain of β4. These results suggest that the membrane-proximal segment of the tail of β4 comprises the major site(s) of palmitoylation. Next, we introduced alanine permutations at each one of the seven membrane-proximal cysteines. None of these individual mutations reduced palmitoylation of β4 by a significant degree (unpublished data). However, simultaneous replacement of the first three cysteines (β4 Cys 3) reduced the incorporation of [3H]palmitate by ~50%, and permutation of the first five (β4 Cys 5) abolished it almost completely. In contrast, replacement of the last two cysteines (β4 Cys 6–7) did not affect palmitoylation of β4 (Fig. 2 D). These results imply that the first five cysteines in the membrane-proximal segment of β4 tail comprise the major site(s) of palmitoylation. We suspect that mutagenesis did not allow us to identify a single palmitoylation site in β4 because mutation of a specific cysteine in a cluster of potential sites may result in palmitoylation of an adjacent, not necessarily physiological site. Finally, we examined if α6β4 localizes to lipid rafts in a palmitoylation-dependent manner. 804G cells expressing equivalent levels of human β4 or the palmitoylation-defective mutant β4 Cys 5 were subjected to Triton X-100 extraction and sucrose gradient ultracentrifugation. Proteins from each fraction were probed with anti-human β4 N20 or with anti-caveolin N20.
Notably, the β4 Cys 5 mutant was excluded from the lipid raft fraction (Fig. 2 E), indicating that palmitoylation of β4 is required for incorporation of α6β4 in lipid rafts. To examine if palmitoylation of β4 and incorporation of α6β4 in lipid rafts play a role in ligand binding, 293-T cells were transfected with vectors encoding α6 in combination with either β4 or β4 Cys 5. Immunoblotting and FACS analysis showed that the expression levels of wild-type and mutant α6β4 were comparable. The cells were plated on laminin-5 at 4°C because at this temperature the function of α3β1, which also mediates adhesion to laminin-5, is suppressed (Xia et al., 1996). Untransfected 293-T cells did not attach to laminin-5 at 4°C, whereas cells expressing wild-type α6β4 attached to a significant extent, indicating that adhesion to laminin-5 at 4°C requires expression of α6β4. The palmitoylation-defective α6β4 mutant promoted attachment to laminin-5 as effectively as wild-type α6β4 (Fig. 3 A), suggesting that palmitoylation of β4 is not required for α6β4-mediated binding to laminin-5. To assess the ability of the palmitoylation-deficient mutant form of β4 to promote assembly of hemidesmosomes, we introduced β4 and β4 Cys 5 in β4-deficient keratinocytes from a patient affected by junctional epidermolysis bullosa with pyloric atresia (PA-JEB). As reported previously (Gagnoux-Palacios et al., 1997), transient transfection of PA-JEB keratinocytes with wild-type β4 caused assembly of hemidesmosome-like adhesions containing HD-1/plectin (Fig. 3 B) and BPAG-2 (unpublished data). Introduction of β4 Cys 5 resulted in formation of hemidesmosome-like structures similar to those nucleated by wild-type β4 (Fig. 3 B), indicating that palmitoylation of β4 is not required for assembly of hemidesmosomes. To examine the role of β4 palmitoylation in keratinocyte adhesion, we used retroviral transduction to generate PA-JEB keratinocytes expressing similar levels of β4 and β4 Cys 5. PA-JEB keratinocytes expressing β4 Cys 5 adhered to laminin-5 in the presence of anti-α3β1 antibodies as well as PA-JEB keratinocytes expressing wild-type β4 (Fig. 3 C), confirming that palmitoylation of β4 is not required for α6β4-mediated adhesion. Upon assembly of hemidesmosomes, keratinocytes become more resistant to detachment with trypsin/EDTA (Gagnoux-Palacios et al., 2001). Analysis of the kinetics of cell detachment revealed that β4 Cys 5 delays trypsin/EDTA-induced cell detachment as effectively as the wild-type β4 (Fig. 3 D). Thus,
α6β4-mediated adhesion and assembly of hemidesmosomes does not require palmitoylation of β4 and incorporation of the integrin in lipid rafts.

Prior reports have indicated that α6β4 signaling is mediated by a pSFK (Mariotti et al., 2001). We asked whether α6β4 associates with a pSFKs in lipid rafts. After antibody-mediated ligation of α6β4, the lipid raft and the Triton X-100–soluble fractions of HaCat keratinocytes were immunoprecipitated with anti-β4 and probed by blotting with anti-panSrc, which recognizes Src, Fyn, and Yes, and as a control, with anti-β4. We found that the lipid raft fraction of α6β4 is associated with SFKs, but the Triton X-100–soluble fraction, which is much larger, is not (Fig. 4 A). Upon introduction in HaCat cells, a GPI-linked form of GFP localized to lipid rafts, but did not communoprecipitate with SFKs, providing a control for specificity (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200305006/DC1). We were unable to identify Yes in association with α6β4 immunoprecipitated from the lipid raft fraction (unpublished data), possibly due to the low stoichiometry or relative

Figure 4. Compartmentalization of α6β4 signaling in lipid rafts. (A) After antibody-mediated ligation of α6β4, HaCat cells were lysed with Triton X-100 and subjected to OptiPrep™ gradient ultracentrifugation. The lipid raft (R) and Triton X-100–soluble (S) fractions were either immunoprecipitated with mAb 3E1 and probed with anti-β4 or anti-pan-Src, or directly probed with antibodies reacting with Yes or Src. The lipid raft and Triton X-100–soluble fractions were also immunoprecipitated with mAb 3E1 and subjected to kinase assay or immunoblotting with anti-phospho Src Y418. (B) PA-JEB keratinocytes expressing wild-type β4 or β4 Cys 5 were plated on dishes coated with mAb 3E1 for the indicated times. Total lysates were probed with anti-phospho Src Y418 or anti-β4 N20. (C) HUVECs transiently transfected with vectors encoding α6β4 were left untreated (C) or treated with the SFK inhibitor PP2 or the EGF-R inhibitor AG1478. Cells were plated on dishes coated with mAb 3E1 for the indicated times or stimulated with EGF. Total proteins were probed with anti-phospho-ERK and anti-ERK-2. (D) HUVECs were transiently transfected with α6 in combination with wild-type β4, phosphorylation-defective β4 (4YF), β4 Cys 5, or tail-less β4 (L). Cells were plated for the indicated times on dishes coated with mAb 3E1. Blotting was as in B. (E) PA-JEB keratinocytes stably transduced with empty vector (LZRS), wild-type β4, and β4 Cys 5 were deprived of mitogens, detached, and plated on coverslips coated with purified laminin-5 and incubated with mAb 3E1, and were then plated on coverslips coated with anti–mouse IgGs. After 24 h of incubation with EGF, the cells were subjected to anti-BrdU staining. The data represent the average and SDs of values obtained from three experiments and are expressed as percentage of rescue.
instability of the association of α6β4 with Yes and the low affinity of currently available antibodies reacting specifically with this kinase. However, immunoblotting experiments indicated that Yes is highly enriched in the lipid raft fraction of HaCat keratinocytes, whereas Src is not (Fig. 4 A). HaCat keratinocytes express very low levels of the other pSFK, Fyn (unpublished data). These results suggest that the lipid raft fraction of α6β4 is preferentially associated with a pSFK.

To examine if α6β4 activates SFK signaling in lipid rafts, HaCat cells were subjected to anti-B4 cross-linking and sucrose gradient fractionation. The lipid raft and the Triton X-100–soluble fractions were immunoprecipitated with anti-B4 and subjected to kinase assay or blotting with anti-phospho Src Y418, which monitors phosphorylation of tyrosine in the activation loop of SFKs. The α6β4-associated pSFK from the lipid raft fraction underwent autophosphorylation, as shown by increased incorporation of [32P]P and reactivity with the anti-phospho Src Y418 antibody, and it also phosphorylated the exogenous substrate enolase. By contrast, the Triton X-100–soluble fraction displayed low kinase activity (Fig. 4 A). Furthermore, we observed that the B4 Cys 5 mutant promoted activation of SFKs less efficiently than wild-type B4, implying that palmitoylation of B4 is required for activation of SFKs (Fig. 4 B). These findings indicate that α6β4 is coupled to SFK signaling in lipid rafts.

To examine the role of lipid rafts in α6β4 signaling to ERK, we transiently transfected vectors encoding various mutant forms of α6β4 in β4-negative human umbilical venous endothelial cells (HUVECs; Dans et al., 2001). To confirm that α6β4 signaling to ERK is mediated by an SFK, cells transfected with wild-type α6β4 were treated with the SFK inhibitor PP2. As expected, α6β4-mediated activation of ERK was suppressed by PP2, but not by the EGF-R inhibitor AG1478. By contrast, EGF-R–mediated activation of ERK was inhibited by AG1478, but not by PP2 (Fig. 4 C). Then, we asked if localization to lipid rafts is necessary for α6β4-mediated SFK signaling to ERK. Cells were transfected with constructs encoding α6 in combination with wild-type β4, phosphorylation-defective β4 (4F), palmitoylation-defective β4 (Cys 5), or tail-less β4 (L). Immunoblotting showed that the cells expressed comparable amounts of wild-type, palmitoylation-defective α6 and anti-BrdU staining indicated that wild-type B4 significantly enhanced the ability of PA-JEB keratinocytes to progress through the cell cycle on laminin-5. Similar results were obtained after antibody-mediated ligation of α6β4. By contrast, the palmitoylation-defective B4 was not able to rescue EGF-mediated proliferation of PA-JEB keratinocytes (Fig. 4 E), providing evidence that α6β4-dependent mitogenic signaling requires palmitoylation of B4 and incorporation of α6β4 in lipid rafts.

Although α6β4, like other integrins, does not contain a kinase domain, ligation of α6β4 causes phosphorylation of the cytoplasmic tail of B4, and hence, recruitment of Shc and other signal transducers. In order for this to occur, the integrin must associate with a tyrosine kinase. Here, we have shown that compartmentalization in lipid rafts is necessary to couple α6β4 to a pSFK and thus reconstitute its ability to activate signaling and promote epithelial mitogenesis. These results provide direct evidence that compartmentalization in lipid rafts is required for α6β4 signaling. Because it is known that part of the EGF-R localizes to lipid rafts (Waugh et al., 1999), and our prior analyses have indicated that the EGF-R activates B4 signaling through the integrin-associated pSFK (Mariotti et al., 2001), it is possible that incorporation in lipid rafts is also necessary for EGF-R–dependent activation of B4 signaling.

How does matrix binding activate α6β4 signaling? At steady state, only a fraction of α6β4 is palmitoylated, and hence localized to lipid rafts. However, antibody- or ligand-induced oligomerization of α6β4 increases the amount of integrin recovered in the raft fraction, suggesting that matrix binding to α6β4 increases the integrin’s affinity for lipid rafts. In addition, palmitoylation is a reversible process (Resh, 1999), allowing for regulated incorporation of α6β4 in lipid rafts. We envision that matrix-induced aggregation of α6β4-containing rafts promotes signaling by bringing the integrin in close proximity to the pSFK, and possibly by excluding a negative regulatory tyrosine phosphatase, as implied by the observation that vanadate greatly enhances phosphorylation of B4 (Dans et al., 2001). In addition, because H-Ras, which is palmitoylated and localizes to lipid rafts, activates PI-3K more efficiently than other Ras isoforms (Yan et al., 1998; Roy et al., 1999), the association with lipid rafts may explain the ability of α6β4 to activate PI-3K, and hence, Rac, more efficiently than other integrins (Shaw et al., 1997). Thus, compartmentalization in lipid rafts potentially explains several specific aspects of α6β4 signaling.

Although other integrins do not appear to be palmitoylated, prior reports suggest that membrane compartmentalization plays a role in signaling by many integrins. Certain β1 and αβ integrins associate, through caveolin-1, with pSFKs, thereby activating Shc signaling to ERK (Wary et al., 1998). Although these integrins are soluble in Triton X-100, it is possible that they associate with lipid rafts through a Triton X-100–sensitive interaction with uPAR, which is GPI linked and localized to rafts (Wei et al., 1999). The αβ1, αβ1, and certain other integrins associate with tetraspanins (Hemler, 2001). Because many tetraspanins are palmitoylated and also tend to form oligomers, they could promote integrin incorporation in lipid raft-like domains. Accordingly, α6β1 associates with detergent-resistant microdomains to promote survival signaling in oligodendrocytes (Baron et al., 2003). Finally, αβ3, αββ3, and αβ1 combine with the integrin-associated protein in cholesterol-dependent microdomains distinct from classical rafts (Green et al., 1999), and αβ1 and αLI2 have been shown to colo-
calize with the lipid raft marker GM-1 in T cells (Leitter and Hogg, 2002). We anticipate that future experiments will reveal that the mechanism of membrane compartmentalization illustrated here also operates, with some variations, in other integrin-signaling systems.

Materials and methods

Antibodies and chemicals

The antibodies to β4, BPAG-2, and HD-1/plectin were characterized previously (Hieda et al., 1992; Murgia et al., 1998). Other antibodies and chemicals were from New England Biolabs, Inc. (anti-phospho-ERK), Santa Cruz Biotechnology (anti-ERK-2, anti-pan Src SRC2), anti-caveolin-1 N-20, and anti-GFP antibodies. The antibodies were described previously (Spinardi et al., 1993; Mainiero et al., 1997). These antibodies and chemicals were from Sigma-Aldrich (saponin and methyl-β-cyclodextrin), and Boehringer (Brdu) and anti-Brdu mAb.

Cells, constructs, and expression methods

Vectors encoding human α6, β4, and the β4 mutants B, C, E, and L were described previously (Spalding et al., 1993; Mainiero et al., 1997). The β4 Cys mutants were generated with QuikChange® (Stratagene). HaCat, HUVECs, rat bladder 804G, and 293-T HEK cells were transfected as described previously (Dans et al., 2001; Mariotti et al., 2001). Immortalized PA-JEB keratinocytes (Gagnoux-Palacios et al., 1997) were cultured in serum-free keratinocyte growth medium (GIBCO BRL). Retroviral particles were recovered from Phoenix packaging cells transfected with pLZRS-IRES-zeo encoding β4 or the β4 Cys 5 mutant, and were used to transduce PA-JEB keratinocytes.

Biochemical methods

For fractionation, cells were lysed on ice for 30 min with 0.5% Triton X-100, 1% Triton X-100, and 10% sucrose. After lysis, the samples were centrifuged at 100,000g for 1 h at 4°C. The resulting supernatants were collected and used for biochemical analyses. For immunoprecipitation, the lipid raft and soluble fractions were diluted with an equal volume of TNE, 1% Triton X-100, and 10% sucrose. After lysis, the samples were centrifuged at 100,000g for 1 h at 37°C before detergent extraction. To mimic ligand-induced aggregation of α6β4, cells were detached with EDTA, incubated in suspension with the mAb 3E1 and then plated on gelatin or cell adhesion. The relative resistance of PA-JEB keratinocytes to trypsin/EDTA-induced detachment was measured as described previously (Gagnoux-Palacios et al., 2001). The relative resistance of PA-JEB keratinocytes to trypsin/EDTA-induced detachment was measured as described previously (Gagnoux-Palacios et al., 2001). Cells were fixed with 3.7% PFA and permeabilized with 0.1% Triton X-100 before immunofluorescent staining.

Cell cycle progression

PA-JEB keratinocytes stably transduced with pLZRS, pLZRS-B4, or pLZRS-β4 CysS were deprived of growth factors, detached, and either plated on coverslips coated with 5 μg/ml human laminin-5 (CHEMICON International) or incubated in suspension with the mAb 3E1 and then plated on coverslips coated with 10 μg/ml anti–mouse IgGs. Cells were cultured for 16 h in serum-free keratinocyte medium supplemented with 10 ng/ml EGF and 50 μg/ml bovine pituitary extract, labeled with Brdu, and then stained with anti-Brdu mAbs. The results were expressed as percentage of rescue (R), according to the formula R = ([X] - [L] - [F] - [L]) × 100 (where X is the percentage of Brdu-positive cells expressing β4 Cys S; F is the percentage of Brdu-positive cells expressing wild-type β4; and L is the percentage of Brdu-positive cells transduced with plLZRS, on either laminin-5 or the anti-β4 substrate).

Online supplemental materials

For Suppl. Fig. S1 a control for the communoprecipitation of α6β4 with p56Lck from the lipid raft fraction. HaCat keratinocytes were transiently transfected with a vector encoding a GFP-linked form of p56Lck, obtained by fus- ing GFP to the COOH terminus of CD55. After Triton X-100 extraction and sucrose density fractionation, the lipid raft and the soluble fractions were immunoprecipitated with anti-GFP mAbs and subjected to blotting with either anti panSrc or anti-GFP pAbs. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200305006/DC1.

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