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Kindlin-1 regulates integrin dynamics and adhesion turnover

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ABSTRACT

Loss-of-function mutations in the gene encoding the integrin co-activator kindlin-1 cause Kindler syndrome. We report a novel kindlin-1-deficient keratinocyte cell line derived from a Kindler syndrome patient. Despite the expression of kindlin-2, the patient’s cells display several hallmarks related to reduced function of β1 integrins, including abnormal cell morphology, cell adhesion, cell spreading, focal adhesion assembly, and cell migration. Defective cell adhesion was aggravated by kindlin-2 depletion, indicating that kindlin-2 can compensate for the loss of kindlin-1 to a certain extent. In the epidermis, kindlin-1 and kindlin-2 are localized in different subcellular compartments, both in the patient and in an unaffected individual. Intriguingly, β1-integrins at the cell-surface were aberrantly glycosylated in the patient’s cells, and β1 expression was considerably reduced, both in vitro and in the patient’s epidermis. Reconstitution with wild-type kindlin-1 but not with a β1-binding defective mutant restored the aberrant β1 expression and glycosylation, and normalized cell morphology, adhesion, spreading, and migration. Furthermore, expression of wild-type kindlin-1, but not of the integrin-binding-defective mutant, increased the stability of integrin-mediated cell-matrix adhesions and enhanced the redistribution of internalized integrins to the cell surface. Thus, these data uncover a role for kindlin-1 in the regulation of integrin trafficking and adhesion turnover.

INTRODUCTION

Integrins are αβ heterodimeric transmembrane glycoproteins that link the extracellular matrix to the cytoskeleton. Integrin-ligand binding triggers the recruitment of a variety of adaptor, structural, and signalling proteins, and the formation of adhesion complexes such as focal adhesions (FAs) (1). Most integrins reside in focal adhesions and connect to the actin cytoskeleton, with the exception of integrin α6β4 which is localized in hemidesmosomes and connects to intermediate filaments (2). Many integrins can tune their affinity for ligand by conformational changes, and the switch from the low- to the high-affinity conformation is called integrin activation (3). Integrin activation is promoted by the binding of talin-1 or talin-2 and any of the 3 kindlin isoforms to the cytoplasmic tail of the β-subunit (3-5). The kindlins consist of an F0-F3 four-point-one/ezrin/radixin/moesin (FERM) domain, that contains the integrin-binding site in F3, and a pleckstrin homology (PH) domain inserted into F2. Kindlin-1 is highly expressed in epithelia, in particular in the epidermis and the gastro-intestinal tract, and loss-of-function mutations in KIND1, the gene encoding kindlin-1, cause Kindler syndrome (KS), a congenital bullous disorder of the epidermolysis bullosa-family (6-8). KS is characterized by skin fragility and blistering, photosensitivity and poikiloderma, and some patients suffer from colitis (9-12). The defects result from reduced β1-integrin-mediated adhesion of the epidermis to the basement membrane (BM), and are reminiscent of the abnormalities in mice lacking the α3 or the β1 subunit in the epidermis, as well as of
patients carrying mutations in the ITGA3 gene encoding α3 (9,10,13-18). *In vitro*, keratinocytes isolated from KS patients or keratinocytes in which kindlin-1 expression is suppressed, display several abnormalities related to defects in β1 integrin function including reduced cell adhesion, cell spreading, and polarity (19-21).

In this study, we describe a novel kindlin-1-deficient keratinocyte cell line derived from an Italian KS patient, which expresses kindlin-2 but not kindlin-1. We study the functional redundancy between the kindlins, and identify a role for kindlin-1 in the regulation of adhesion turnover and integrin trafficking.

**RESULTS AND DISCUSSION**

**Defects in β1 integrin function in Kindler syndrome cells that express kindlin-2 but not kindlin-1.**

We isolated kindlin-1-deficient keratinocytes from a previously described male KS patient from Italy (22). This patient is homozygous for the mutation c.1161delA within exon 10, which is predicted to cause a complete loss of expression due to nonsense-mediated RNA decay, or to cause the expression of a protein product that is truncated in the PH-domain (Figure 1A).

We first investigated kindlin-1 protein expression by Western blotting, using an antibody directed against an epitope in the F1 domain (20). Full-length kindlin-1 was clearly detected at the expected size (~75 kDa) in normal human keratinocytes (NHK), isolated from a healthy individual (23). In KS cells, a very faint band of the same size was detected but smaller proteins were not observed, suggesting that the c.1161delA mutation causes the near-complete loss of expression of full-length kindlin-1, whereas truncated protein products are not synthesized (Figure 1B). Kindlin-2 expression was detected in both NHK and KS keratinocytes (Figure 1B). The morphology of the KS cells was highly aberrant, as compared to that of NHK (Figure 1C). In addition, cell growth was poor and large numbers of dead cells were regularly observed (data not shown). To establish whether the observed abnormalities were due to defects in integrin-mediated adhesion, we analyzed adhesion to collagen (Col)-1 in adhesion assays. Adhesion of KS cells was indeed significantly impaired, and only a fraction of the cells that adhered was able to spread (Figures 1D,E). Consistent with reduced integrin-mediated adhesion, cell motility was significantly enhanced (Figures 1F,G). Finally, we analysed the organization of the actin cytoskeleton and the presence of FAs, using phalloidin and an antibody against phosphotyrosines P(Y). P(Y)-staining was generally weak in KS keratinocytes, and actin filaments appeared abnormal (Figure 1H). In contrast, the assembly of hemidesmosome-like structures upon transfer to Ca²⁺ rich medium, and the synthesis and deposition of laminin (Ln)-332 in KS cells appeared to be normal (Figures S1A,B).

In summary, we have isolated a kindlin-1-deficient keratinocyte cell line that displays defects in β1 integrin function, despite the presence of kindlin-2.
Kindlin-1 and kindlin-2 are partially redundant, and β1 expression is decreased in kindlin-1-deficient keratinocytes and epidermis.

We next investigated the cell-surface expression and activation status of β1-integrins in KS and NHK cells by flow cytometry. Interestingly, β1 cell-surface expression was significantly reduced, whereas the activation status, as judged by the ratio of 9EG7 staining over total β1 staining, was slightly (but not significantly) increased (Figure 2A). Decreased expression of β1 in KS cells was further confirmed by Western blotting (Figure 2B). We then analysed the expression of β1 in skin biopsies of the same patient. Ln-332 staining revealed BM abnormalities and detachment of keratinocytes in the patient’s epidermis (Figure 2C; indicated by arrows), typical of KS (22,24), and the expression of β1 was strikingly decreased (Figure 2C). Thus, whereas there is a clear reduction in β1 expression, both in vivo and in vitro, integrin activation in the KS cells is not impaired. The latter finding is reminiscent of keratinocytes derived from the kindlin-1 knockout mice, in which integrin-mediated cell adhesion and cell spreading were compromised whereas...
there was no significant reduction in integrin activation, due to the expression of kindlin-2 (25). We therefore introduced shRNAs directed against kindlin-2 into KS cells by lentiviral transduction. Depletion of kindlin-2 caused complete detachment of KS cells (Figure 2D). Previous studies have reported both overlapping and distinct functions of kindlin-1 and kindlin-2 in keratinocytes (26,27). Our results are in line with these findings as kindlin-2 can apparently partially rescue cell adhesion in the absence of kindlin-1 in KS cells, but there are still considerable defects in cell adhesion and spreading. In vivo, kindlin-2 cannot completely compensate for the loss of kindlin-1, neither in the epidermis of KS patients, nor in the colon of kindlin-1(−/−) mice (25,28), which is probably due to differences in subcellular localization (8). We therefore also investigated kindlin-2 distribution in vivo. Consistent with its expression in NHK and KS cells, kindlin-2 was detected both in the patient’s epidermis and the epidermis of a normal individual. In basal keratino-

![Figure 2 Decreased integrin expression in the absence of kindlin-1.](image)

(A) FACS histograms and quantification (n=3) of NHK and KS cells showing cell-surface expression of β1 (left) and active β1 (right), as measured by 9EG7 staining. (B) Western blot showing the precursor (110 kDa) and the mature form of β1 (130 kDa) in NHK and KS cells. (C) Expression of β1 (green) and Ln-332 (red) in the skin of an unaffected individual (normal) and the KS patient. Outline indicates the upper border of the epidermis. Bar, 50 μm. (D) Depletion of kindlin-2 in KS cells causes complete cell detachment. Bar, 20 μm. (E) Expression of kindlin-1 (top) and kindlin-2 (bottom) in the skin. Bar, 50 μm. d; dermis, e; epidermis.
cytes kindlin-2 localization was exclusively lateral, while kindlin-1 distribution in normal epidermis was predominantly basal, in line with previous observations (Figure 2E) (8,28). Interestingly, kindlin-2 staining at the lateral membranes was weak and occasionally completely absent from the basal keratinocyte layer of the patient (Figure 2E; indicated by arrows), which most likely reflects defects in cell-cell contacts, as described in Chapter 6.

Together, these data show that β1 expression is reduced in KS cells and epidermis, and that kindlin-2 compensates only partially for reduced cell adhesion in the absence of kindlin-1.

Stable re-expression of full-length kindlin-1 in KS cells restores the defects in integrin function.

The previous sections have shown that in the absence of kindlin-1, integrin-dependent events are disturbed despite the presence of kindlin-2. To investigate whether the observed defects in KS cells are a direct consequence of the loss of kindlin-1, kindlin-1 expression was restored in KS cells by retroviral delivery of eGFP-conjugated kindlin-1 followed by FACS sorting, creating a stable cell line that we designated KSK. Expression of eGFP-kindlin-1 was confirmed by Western blotting (Figure 3A). Re-expression of kindlin-1 reversed the aberrant morphology of KS cells, and significantly enhanced cell proliferation (Figures 3B,C). Kindlin-1 was diffusely distributed in the cytoplasm, while some enrichment in P(Y)-positive FAs was observed. In addition, a re-organization of the actin cytoskeleton into stress fibers and/or circumferential actin bundles was observed in KSK cells (Figure 3D). Re-expression of kindlin-1 clearly enhanced integrin-mediated cell adhesion, both to Col-1 and to a Ln-332-containing matrix derived from Rac-11P cells (Figure 3E). In addition, cell spreading on these substrates was significantly increased (Figures 3F,G) and the hypermotility observed in KS cells was reversed by re-introduction of kindlin-1 (Figures 3H,I). Finally, β1 cell-surface levels were increased in KSK cells with respect to KS, confirming that kindlin-1 enhances β1 expression (Figure 3J). This notion is consistent with several previous studies (29-31), and was further supported by the observation that over-expression of kindlin-1 also enhanced β1 cell-surface expression in NHK cells, which was accompanied by enhanced cell spreading (Figure S2).

Together, these data show that restoration of kindlin-1 expression in KS cells rescues the defects in β1 integrin function.

Regulation of β1 expression and function by kindlin-1 requires the F3 domain.

We next investigated whether the effects of kindlin-1 depend on a direct interaction with the integrin β1-tail. The integrin-binding site in kindlin-1 resides in the C-terminal F3 domain, and a mutation that causes the expression of a protein lacking the F3 and part of the F2 has been identified in a KS patient, demonstrating the vital importance of this region (32). In addition, we have recently isolated a zebrafish mutant with KS-like epidermal defects, which expresses a truncated kindlin-1 protein lacking the F3 domain (described in Chapter 6). To delete the integrin-binding site, we truncated the F3 region after residue 581, and stably expressed
eGFP-kindlin-1 del581 into KS cells, creating a cell line designated KSK del581 (Figure 4A). Western blotting revealed a band of the expected size of ~95 kDa in KSK del581 cells (Figure 4B). Intriguingly, increased expression of mature β1, but not precursor β1, was observed in KS cells but not in KSK del581, suggesting that a direct interaction is required for the stimulation of β1 cell-surface expression by kindlin-1. Furthermore, the mobility of mature β1 in gel electrophoresis was reduced in lysates of KS and KSK del581 with respect to KSK cells (Figure 4C). This was reversed after treatment with neuraminidase, which specifically removes monosaccharides called sialic acids, indicating that kindlin-1 regulates β1 sialylation, depending on the F3 domain (Figure 4D). Flow cytometry analysis confirmed that β1 cell-surface levels were increased in KSK, but not in KSK del581 cells (Figure 4E). Furthermore, the expression of eGFP-kindlin-1 del581 did not promote cell spreading of KS cells (Figure 4F). Consistently, FAs seemed less pronounced in KSK del581 than in KS cells, and the subcellular distribution of mutant kindlin-1 was different from that of full-length kindlin-1; kindlin-1 del581 localization seemed predominantly cytoplasmic, with no clear enrichment in adhesions (Figure 4G). Similarly, kindlin-1 mutants
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carrying a Q611A or W612A mutation, that prevents interaction with integrins, are not targeted to FAs and do not increase cell-surface expression of α5β1, when overexpressed in fibroblasts (29).

Together, these data suggest that a direct interaction between kindlin-1 and β1 is required for the targeting of kindlin-1 to cell-matrix adhesions, and for the effects of kindlin-1 on β1 cell-surface expression and glycosylation.

Kindlin-1 targeting to adhesions and adhesion dynamics depend on the F3 domain.

We next addressed the relationship between kindlin-1 targeting to adhesions and adhesion dynamics in living cells. To this end, we introduced the FA marker vinculin, fused to mCherry, into KS, KSK, and KSK del581 cells by lentiviral transduction. The dynamics of eGFP-kindlin-1 and mCherry-vinculin were then monitored by total internal reflection (TIRF)
microscopy. Consistent with the results of the cell migration assays, KS cells were very motile and displayed many rapid cell shape changes. Imaging of vinculin revealed few FAs that had a high turnover rate (Figure 5A). In contrast, KSK cells were considerably more static, in line with the reduced velocity of migration, and their adhesions were much more stable than those in KS cells (Figure 5B). Interestingly, eGFP-kindlin-1 was clearly enriched in adhesions, some of which were surprisingly large, but many of these clusters did not contain mCherry-vinculin, suggesting that kindlin-1 and vinculin can resides in distinct pools of adhesions. Furthermore, kindlin-1 was strongly concentrated in retraction fibers, consistent with the role of kindlin-1 in delaying cell migration (Figure 5B). In KSK^{del581} cells, we also observed a rapid turnover of mCherry-vinculin-containing adhesions,

Figure 5 Kindlin-1 targeting to adhesions and adhesion stability depend on the F3 domain. (A) Stills from a TIRF movie, showing the dynamics of mCherry-vinculin in KS cells. (B) Dynamics of mCherry-vinculin (top), and eGFP-kindlin-1 (bottom) in KSK cells. (C) Dynamics of mCherry-vinculin (top), and eGFP-kindlin-1 (bottom) in KSK^{del581} cells. Look-up table ‘fire’ was used to enhance visibility of adhesions. Shown are images at 0, 7.5, 15, 22.5, and 30 min. Boxed regions are enlarged. Bar, 10 μm.
as well as fast cell shape changes. Consistent with the images acquired by confocal microscopy, there was some diffuse localization of eGFP-kindlin-1\textsuperscript{del581} at the basal cell-surface, but clearly no enrichment in adhesions or retraction fibers (Figure 5C).

Thus, kindlin-1 controls the dynamics of integrin-mediated cell-matrix adhesions, which is dependent on an intact F3 region.

**Kindlin-1 interaction with β1 regulates integrin traffic**

We next investigated whether integrin trafficking plays a role in the regulation of cell-matrix adhesion dynamics and β1 surface expression by kindlin-1. Integrins undergo continuous internalization, and the recycling of internalized integrins is important for integrin-mediated processes such as cell spreading (33,34). Internalization and recycling were determined according to a well-established protocol (35). First, we labeled cell-surface β1 with the antibody K20, conjugated to DyLight 649 (10 μg/ml), which clearly revealed localization of β1 integrins at the membrane in KS, KSK, and KS cells (Figure 6, top). The cells were then transferred to serum-free medium at 37ºC, which allows internalization but not recycling of internalized integrins. The labeled cell-surface pool underwent internalization in all cell lines, with no apparent differences depending on kindlin-1 (Figure 6, middle panel). Recycling of internalized integrins was subsequently induced by stimulation with 20% FCS, which in KSK cells triggered the rapid return of β1 integrins to the plasma membrane and their delivery to peripheral adhesions (Figure 6, bottom). In contrast, redistribution of the internal integrin pool to the plasma membrane was not evident in KS cells or KS cells expressing truncated kindlin-1, suggesting that kindlin-1 regulates the redistribution of internalized integrins, depending on the F3 domain. We did not detect kindlin-1 in vesicles, in line with similar observations for kindlin-2 (30,36). Therefore, kindlin-1 probably regulates integrin routing indirectly, i.e. by sorting integrins at the plasma membrane to a specific internalization and recycling pathway. This is conceivable as the kindlin-binding site in β1 is largely defined by the membrane-distal NPxY motif, which is also a canonical signal for clathrin-mediated endocytosis.

In summary, the results presented here suggest that kindlin-1 regulates the redistribution of internalized integrins, which requires a direct kindlin-integrin interaction.
Cristina Has), kindlin-2 (Sigma-Aldrich), kindlin-2 (a kind gift from Dr. Reinhard Fassler), Ln-332 (a kind gift from Dr. Takako Sasaki), plectin (a kind gift from Dr. Katsushi Owaribe), and P(Y) (clone 4G10; a kind gift from Dr. Kevin Wilhelmsen). Neuraminidase, puromycin and zeocin were from Sigma-Aldrich. TRITC-, FITC-, and Cy5-conjugated secondary antibodies, phalloidin, and DAPI were purchased from Molecular Probes (Eugene, OR), HRP-conjugated secondary antibodies were from Amersham, and Col-I was from Vitrogen (Nutacon, Leimuiden, The Netherlands). K-20 was conjugated to DyLight 649 (Thermo Scientific) at the NKI.

**Figure 6 Kindlin-1 interaction with β1 regulates integrin trafficking.** Cell-surface β1 integrins on KS, KSK, and KSK_del581 cells were labelled with DyLight 649-conjugated K-20 at 4°C (top panel), after which they were allowed to internalize in serum-free medium at 37°C for 2 hrs (middle panel). Recycling of the internal pool was induced with 20% FCS for 7.5 min (bottom panel). Cells were then fixed and processed for confocal microscopy. β1 is pseudo-colored green, nuclei were counterstained with DAPI (pseudocolored red). Arrows indicate delivery of recycled β1 to adhesions. Bar, 10 μm.

**Patient material, cell culture, cloning, retroviral and lentiviral transductions**
Skin cryosections and primary KS keratinocytes were obtained after informed consent from a previously described patient (22), and immortalized by SV40 infection. NHK cells have been described previously (23). KS and NHK cells were routinely cultured on Col-1 (3 μg/ml) in keratinocyte serum-free medium (K-SFM; Gibco BRL), supplemented with 50 μg/ml bovine pituitary extract, 5 ng/ml EGF,
100 U/ml penicillin and 100 U/ml streptomycin. Rac-11P cells were cultured in DMEM supplemented with 10% FCS, 100 U/ml penicillin and 100 U/ml streptomycin. All cells were maintained at 37°C and 5% CO2. eGFP-kindlin-1del581 was generated using eGFP-kindlin-1 in C1. Full-length or truncated kindlin-1 were recloned into LZRS-IRES-zeo, and transfected into Phoenix packaging cells using the Calcium Phosphate method. Virus-containing supernatant was isolated 48 hrs later and stable expression in KS cells was achieved by retroviral transduction, followed by selection with zeocin and cell sorting. Expression of mCherry-vinculin was established by lentiviral transduction of the pLV-CMV-mCherry-Vinculin-Ires-Puro-construct, followed by selection with 5 μg/ml puromycin.

**Knockdown of kindlin-2 in KS cells**

Short hairpins against human kindlin-2 (target sequence CGACTGATATAACTCCTGAAT), cloned into pLKO.1, were obtained from the TRC shRNA Open Biosystems library and transfected into HEK 293FT cells together with the Virapower™ Packaging mix (Invitrogen), using Lipofectamine 2000 according to the manufacturers’ instructions. Viral supernatant was harvested 48 hrs later, transduced into KS cells, and positive cells were selected with puromycin.

**Flow cytometry and cell sorting**

For flow cytometry and cell sorting, cultured cells were trypsinized, washed twice in PBS containing 2% FCS, and incubated with primary antibodies for 45 min at 4°C. Cells were then washed twice in 2% FCS/PBS, incubated with appropriate secondary antibodies for 45 min at 4°C, washed twice in 2% FCS/PBS, and analyzed on a FACS Calibur (BD Biosciences). Alternatively, the cells were sorted on a MoFlo High Speed Cell Sorter (Beckman Coulter).

**Immunoprecipitations and Western blotting**

Cells were washed in ice-cold PBS and lysed on ice in RIPA buffer (25 mM Tris/ HCl pH 7.6, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS), supplemented with protease inhibitor cocktail (Sigma). Immunoprecipitation of β1 was performed essentially as described earlier (16), using TS2/16. For Western blotting of whole-cell extracts, cell lysates were cleared by centrifugation at 13,000xg, heated at 95°C in SDS sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA, 0.02% bromophenol blue), and proteins were resolved by SDS-PAGE, after which they were transferred to polyvinylidene difluoride membranes (Millipore) and analyzed by Western blotting followed by ECL using the SuperSignal system (Pierce Chemical Co.).

**Microscopy**

Phase-contrast images were acquired on a Zeiss microscope (Axiovert 25) at 10x (NA 0.25) or 20x (NA 0.3) magnification, using a Zeiss CCD camera (Axiocam MRC) and Zeiss Mr. Grab 1.0 software. For confocal microscopy, cryosections of human skin or cells cultured on coverslips were prepared as previously described (16), and images were acquired on an inverted confocal microscope (Leica AOBS) using 20x (NA 0.7) dry, 40x (NA 1.25) oil, and 63x (NA 1.32) oil objectives.
(Leica). For TIRF microscopy, cells were seeded on glass coverslips and videos were acquired using Leica application suite software on a Leica DMI600B system with a 63X objective (NA 1.47), at 37°C in an atmosphere containing 5% CO₂. Images and videos were processed using Photoshop 7.0 and ImageJ 1.44.

Adhesion, migration, cell spreading, and proliferation assays
For adhesion assays, 96-well plates were coated with 2% BSA or 3 μg/ml Col-1 for 1 hr at 37°C. Ln-332-containing matrix was prepared by growing Rac-11P cells to confluency, prior to overnight detachment with 10 mM EDTA at 4°C. The plates were then washed twice with PBS, blocked with 2% BSA for 1 hr at 37°C, and washed twice with PBS before use. Subconfluent cells were trypsinized, resuspended in K-SFM and seeded at a density of 3x10⁴ cells per well. After 30 min at 37°C, nonadherent cells were washed away with PBS. The adherent cells were fixed in 4% PFA, washed with H₂O, stained for 10 min with crystal violet, washed with H₂O, and then lysed in 2% SDS. Absorbance was measured at 490 nm on a microplate reader. Background values (binding to BSA) were subtracted from all other values.

To determine cell spreading, subconfluent cells were trypsinized, resuspended in K-SFM, and then seeded in 12-well plates coated with Col-1 or Rac11P matrix. Cells were photographed on a Widefield CCD system using 10x and 20x dry lens objectives (Carl Zeiss MicroImaging, Inc.). The number of spread cells was counted and expressed as a percentage of the total number of cells. Alternatively, the surface area was determined using ImageJ. Values shown represent the averages of 3 experiments. In each experiment, approximately 500 cells were analyzed for each condition.

For single-cell migration assays, cells were seeded sparsely on 3 μg/ml Col-1, and phase-contrast images were captured every 15 min at 37°C and 5% CO₂ on a Widefield CCD system using a 10x dry lens objective (Carl Zeiss MicroImaging). Migration tracks were generated using ImageJ 1.44, and the average velocity was calculated from approximately 250 cells out of 3 independent experiments.

Proliferation was investigated by seeding cells in 6-well plates, coated with 3 μg/ml Col-1, at a density of 5x10⁴ cells per well, whereafter they were trypsinized and counted every day. Values shown represent the averages of 3 experiments.

Integrin internalization and recycling assays
Integrin internalization and recycling were investigated essentially as described earlier with some modifications (34). Briefly, cells on glass coverslips were incubated for 2 hrs at 37°C in serum-free medium, after which they were washed twice in the same medium at 4°C. Cell-surface β1 was then labeled with DyLight 649-conjugated K-20 (10 μg/ml) for 1 hr at 4°C. Immediately after labeling, some coverslips were fixed, and the rest was transferred to 37°C to undergo endocytosis. After 2 hrs, some coverslips were fixed, and the rest was stimulated with 20% FCS for 7.5 min to stimulate recycling of internalized integrins. The cells were fixed, permeabilized with 0.5% Triton and 0.01% saponin, and then processed for confocal microscopy as described above.
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ABBREVIATIONS

BM, basement membrane; Col, collagen; FA, focal adhesion; FERM, four-point-one/ezrin/radixin/moesin; KS, Kindler syndrome; Ln-332, laminin-332; NHK, normal human keratinocytes; PH, pleckstrin homology; P(Y), phosphotyrosine; TIRF, total internal reflection microscopy

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