Temporal Dissection of β1-Integrin Signaling Indicates a Role for p130Cas-Crk in Filopodia Formation

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Invasin-promoted spreading of β1-integrin-deficient cells, transfected with the β1A- or β1H-integrin splice variants, were used to dissect early β1-integrin signaling events. The β1H isoform, which has a different membrane-distal part of the cytoplasmic tail from β1A, is defective in signaling and function. When plated on surfaces coated with the high affinity ligand invasin, β1A-integrin-expressing cells spread by forming filopodia with distinct adhesive phosphotyrosine complexes at the tips, without signs of lamellipodia. This suggested that the β1H-integrin mediated a partial signaling sufficient for formation of filopodia but insufficient for lamellipodia formation. When screening for proteins present in the distal filopodial phosphotyrosine complexes of β1H cells, p130Cas and the filopodia proteins vasodilator-stimulated phosphoprotein and talin were found, whereas the typical focal complex proteins focal adhesion kinase, paxillin, and vinculin were not. Invasin-promoted adhesion induced complex formation of p130Cas and the adapter Crk. Moreover, Crk together with Dock180 were present at the filopodial tips of β1H-integrin-expressing cells, and there was a prominent Rac1 activation. Expression of dominant negative variants of p130Cas or CrkII blocked β1H-integrin-mediated filopodia formation, indicating that this signaling scaffold is central in this process.

Integrins are a large family of at least 24 heterodimeric transmembrane receptors formed by 18 α and 8 β subunits (1). They are involved in bidirectional signaling across the cell membrane and influence several cellular events including cell spreading, cell migration, and cell survival (1). Integrin receptors link the extracellular matrix to the cytoskeleton at sites known as cell-matrix adhesions, which are multimolecular structures where the extracellular matrix, integrins, filamentous actin (F-actin), F-actin-stabilizing proteins, and proteins involved in cell signaling are found (2, 3). Cells form different types of cell-matrix adhesions that are morphologically distinct and are divided into subtypes such as focal contacts/focal adhesions and focal complexes. Focal contacts represent the relatively large and arrow-shaped cell-matrix adhesion structures mainly found at the basolateral plane of the cell, whereas focal complexes represent the small round-shaped cell-matrix adhesions usually present at the rim of lamellipodia (4). One protein family that regulates the cell-matrix adhesions and the actin cytoskeleton during cell spreading and migration is the Rho family of GTPases that includes Rho, Rac, and Cdc42 (5, 6). During stimulated migration of NIH3T3 cells, Cdc42 is activated and induces formation of thin protrusions rich in actin, known as microspikes or filopodia (7, 8). Activation of Rac1 promotes formation of lamellipodia, which are sheath-like protrusions (9). Both Cdc42 and Rac1 regulate protrusive events and promote formation of the smaller focal complexes (5). RhoA is also activated and mediates formation of stress fibers and focal contacts that firmly anchor the cell to the substratum and allow retraction of the rear end of the cell (5, 10, 11). Among the proteins found in cell-matrix adhesions are FAK, Src, Crk-associated substrate (p130Cas), talin, vasodilator-stimulated phosphoprotein (VASP), paxillin, and vinculin (12). Vinculin plays a central role in the mechanical coupling of integrins to the cytoskeleton as well as in related control of cytoskeletal mechanics, cell shape, and motility (13–16). Talin is responsible for a conformational change of integrins to an active state, and it also links the receptor to the actin cytoskeleton (17–20). VASP, which also is found in the tip of filopodia, is involved in regulation of actin-based types of cell movement and promotes the formation of long actin filaments over branching (21, 22). Paxillin can interact with a variety of signaling proteins and is presumed to be involved in several signaling pathways as well as in anchoring of the actin cytoskeleton (23). FAK is a tyrosine kinase that is activated early in response to integrin stimulation and is involved in regulating the turnover of cell-matrix adhesions (24, 25). This kinase, together with members of the Src family of cytosolic tyrosine kinases, binds and phosphorylates the docking proteins p130Cas and paxillin (26, 27). Phosphorylated p130Cas can interact with Crk, which in turn binds Dock180, which is an unconventional Rac-guanine nucleotide exchange factor that activates Rac1 (28–32).

In a previous study (33), we showed that the high affinity β1-integrin ligand of Yersinia pseudotuberculosis, invasin (34–36), was unable to bind to a β1-integrin-deficient cell line, GD25, unless this cell line had been transfected with either of two splice variants of β1-integrin, β1A or β1H. These splice variants are identical in the extracellular, transmembrane, and membrane-proximal 20 amino acids of the cytoplasmic tail but differ at the end of the cytoplasmic tail (37). The distal part of the β1A tail contains 21 amino acids encompassing two NPXY
motifs, a serine and a double threonine phosphorylation site. The unique part of the β1 tail does not contain any known motifs important for the function of β1 when it localizes to the cell surface although there is a double lysine motif that can cause intracellular accumulation of β1 (37, 38). The β1 spliced variant is most commonly expressed and can cluster, form cell-matrix adhesions, and mediate signaling upon binding to fibronectin or laminin. The β1 spliced variant, on the other hand, is only expressed in a few human cell types and is unable to bind fibronectin unless stimulated with manganese ions that cause intracellular accumulation of this ligand (33). GD25 cells complemented with β1A (GD25β1A), on the other hand, exhibited reduced binding and a spreading that was limited to distinct peripheral assemblies where β1-integrin co-localized with tyrosine-phosphorylated proteins (33). The finding of the Tyr(P) complexes indicated that the β1B-integrin retained some signaling capacity, and in this study, we have used this to explore the signaling associated with this event of spreading.

EXPERIMENTAL PROCEDURES

Materials—The following antibodies were purchased: mouse anti-FAK2A7 (Upstate Biotechnology, Inc., Lake Placid, NY), mouse anti-paxillin, mouse anti-Crk, rabbit anti-Tyr(P), mouse anti-Tyr(P) (PY20), mouse anti-Rac1, mouse anti-Cdc42, mouse anti-p130Cas, Tyr(P)-RC20-HRP (Transduction Laboratories), goat anti-DOCK180 (N-19), goat anti-DOCK180 (C-19) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse anti-human VASP (1E273; ALEXIS Biochemicals), mouse anti-talin, mouse anti-human vinculin (Sigma), fluorescein isothiocyanate-conjugated donkey anti-mouse, fluorescein isothiocyanate-conjugated donkey anti-rabbit, rhodamine-conjugated donkey anti-mouse, rhodamine-conjugated donkey anti-rabbit, and horse-radish peroxidase-conjugated sheep anti-mouse (Jackson Immunoresearch Laboratories Inc.). Polyclonal anti-p130Cas was kindly provided by Dr. J. T. Parsons (Department of Microbiology, University of Virginia, Charlottesville, VA). Alexa Fluor® 488 phallolidin, Alexa Fluor® 568 phallolidin, and wheat germ agglutinin conjugated to lysianne were from Molecular Probes (Leiden, The Netherlands). Other reagents used were PP1 (Alexis Corp.), PP3 (Calbiochem), platelet-derived growth factor (R&D Systems), and the glycine-arginine-glycine-aspartic acid-serine (GRGDs) peptide (Bachem Feinchemicalien AG). The GST-p130Cas Is Required for Filopodia Formation

Cell Spreading and Immunofluorescence Staining—Cell spreading and immunofluorescence staining were performed as previously described (33). In brief, coverslips were coated with GST-invα11 (10 μg/ml) overnight at 4°C and blocked with 1% heat-treated bovine serum albumin in phosphate-buffered saline at 37°C for 1 h. Cells were detached and incubated in serum-free DMEM containing cycloheximide (25 μg/ml; Sigma) on bovine serum albumin-blocked Petri dishes for 40 min. Thereafter, the GRGDs peptide (0.1 μg/ml) was added, and the cells were incubated for an additional 20 min. Cycloheximide and GRGDs was used to inhibit the GD25 fibroblasts from synthesizing extracellular matrix proteins and to block binding to α2β1-integrins, respectively. After this, 2 x 10^5 cells were allowed to attach on invasin-coated coverslips at 37 °C for 3 h. Unattached cells were washed away, and remaining cells were fixed in 2% paraformaldehyde for 10 min. The fixed cells were permeabilized with 0.5% Triton X-100 and further processed for double or triple immunofluorescence labeling. Samples were mounted onto microscope slides using ProLong Antifade (Molecular Probes). The pictures were captured using a microscope (Zeiss Axioskop 50) and a CCD camera (ORCA; Hamamatsu) and processed using Adobe software (Adobe).

The inhibition of Src family kinases by PP1 or the negative control, PP3, was performed as described above with the exception that the 60-min pretreatment with cycloheximide was exchanged with a 45-min pretreatment with PP1 or PP3 at various concentrations prior to seeding.

Immunoprecipitation and Western Blotting—Cells were trypsinized, washed once with phosphate-buffered saline, and resuspended in serum-free DMEM containing cycloheximide (25 μg/ml; Sigma) before being plated on GST-invasin (10 μg/ml) or bovine serum albumin. The cells were allowed to attach at 37 °C for 60 min and thereafter were lysed in radioimmunoprecipitation assay buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl, 1 mM EGTA, 1 mM NaVO4, and Complete™ protease inhibitor mixture mix (Roche Molecular Biochemicals)) for 15 min on ice. The lysates were clarified by centrifugation at 14,000 x g for 15 min and precleared with Protein G-Sepharose beads (Amersham Biosciences) coated with mouse γ-globulin (Jackson Immunoresearch Laboratories) for 1 h at 4°C. After washing, the supernatant was incubated with Protein G-Sepharose beads precoupled to anti-Crk antibodies. The beads were washed twice with radioimmunoprecipitation assay buffer and subjected to SDS-PAGE followed by semidy transfer to Immobilon-P transfer membrane (Millipore Corp.). Tyrosine-phosphorylated proteins were detected with anti-Tyr(P) antibodies conjugated with horseradish peroxidase (RC20) and then enhanced chemiluminescence (Amersham Biosciences). The membrane was stripped by incubation in 1 mM glycine, pH 2.5, for 30 min, whereafter it was divided and subjected to Western blot with anti-p130Cas or anti-Crk antibodies.

Pull-down—The plasmid-cured Y. pseudotuberculosis strain YPIII (47) was used to stimulate β1-integrins to investigate Cdc42 and Rac1 activation. An overnight culture of YPIII, grown in Luria broth at 28 °C, was centrifuged down, resuspended in antibody-free cell culture medium, and incubated for 1 h at 26 °C. This culture was then used to infect a 15-cm dish with cells at 75% confluence (starved for 17 h), at a multiplicity of infection of 5000:1. The infection was carried out at 37 °C in an atmosphere of 5% CO2. The cells were lysed in the pull-down lysis buffer containing 50 mM Tris pH 7.5, 1% Triton X-100, 100 mM NaCl, 15 mM MgCl2, 1 mM EGTA, pH 8.0, and Complete™ protease inhibitor mixture mix. As a positive control for Rac1 activation, the cells were treated with platelet-derived growth factor (3 ng/ml) for 2 min prior to lysis. The lysates were centrifuged at 12,000 × g for 5 min, and the supernatants were saved as the cell lysates. The β1A and β1B cell lysates were analyzed for their protein concentration was determined using the Bio-Rad protein assay, and 1300 μg of the cell lysate was incubated with 30 μg of GST-PK-CRIB (purified as described previously (44)) immobilized on glutathione-Sepharose 4B beads (Amersham Biosciences) for 30 min at 4 °C. The beads were washed four times in wash buffer (10 mM Tris, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM MgCl2), washed with bound CTP-RC20 together with aliquots of cell lysates were subjected to SDS-PAGE (12%) and Western blot with anti-Rac1 antibodies.

RESULTS

β1B-Integrins Have a Partial Signaling and Mediate Formation of Filopodia When Binding to Invasin—To characterize the β1-integrin-mediated spreading, β1Aα5 and β1Bα5-expressing GD25 cells were allowed to spread on invasin-coated plates; triple-stained with phallolidin, wheat germ agglutinin, and anti-Tyr(P) antibodies; and subjected to both fluorescent and phase
contrast light microscopy (Fig. 1A). Fluorescent phalloidin and wheat germ agglutinin were employed to visualize the F-actin architecture and cell edges, respectively. Cells expressing β1A-integrins became flat and spread with the typical star-like morphology of fibroblasts where lamellipodia and distinct cell-matrix adhesions were apparent (Fig. 1, A and B). On the other hand, cells expressing β1B-integrins had a round cell body with long extended F-actin structures consisting of fine threads that resembled filopodia, and there were no detectable lamellipodia-like F-actin structures or focal contacts (Fig. 1, A and B). Moreover, the tips of the thin filopodial extensions contained distinct assemblies of tyrosine-phosphorylated proteins (Fig. 1A). There were no such long filopodia-like structures in the GD25β1A cells; the protrusive elements were instead broader lamellipodia-like extensions and some short filopodia-like structures (Fig. 1A). The initial spreading by β1B-integrin-expressing cells was delayed compared with that of cells expressing the β1A variant but was equal after 1 h, where 60% of all attached cells exhibited spread morphology with filopodia or lamellipodia, respectively (Fig. 1B; Supplementary Fig. 1). Maximal spreading of β1B cells was detected after 2 h, but after longer periods of time cells started to detach (Fig. 1B), where very few cells adhered after 6 h (data not shown). Coating density of invasin did not influence the observed spreading behavior; 1–25 μg/ml of invasin resulted in similar spreading for both GD25β1A and GD25β1B cells (data notshown). Upon plating on poly-l-lysine, no spreading at all could be observed (data not shown), which confirmed that the filopodia formed by the β1B-expressing cells was an effect of invasin binding to the β1-integrin receptor. This indicates that the β1B-integrin receptor can mediate spreading with filopodia when binding to invasin but that signaling for lamellipodia formation is impaired. This system therefore provides a unique tool to dissect the complex signaling associated with filopodia formation, which may allow identification of molecular events in spreading that takes place without the formation of lamellipodia. Interestingly, GD25β1B cells that had spread with filopodia and then been ripped off during sample preparation left remnants of the phosphotyrosine-containing tip complexes on the coverslip (Fig. 1C). This shows that the tips of these filopodia are indeed attached to the invasin-coated coverslips, which is supported by our previous finding that the β1-integrins localize to these filopodial tips (33).

Src family kinases, especially Src and Fyn, are associated with integrin signaling and are activated upon integrin activation (48). To investigate whether Src family kinases were im-

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**Fig. 1.** Invasin-stimulated spreading of β1A- and β1B-integrin-expressing cells. GD25β1A and GD25β1B cells suspended in serum-free DMEM, were allowed to spread on GST-invasin-coated glass coverslips for 3 h (A and C) or the indicated times (B). A, cells stained for F-actin (phalloidin), cell membrane (wheat germ agglutinin; WGA), and Tyr(P) (pY). The arrows indicate filopodial extensions, and the arrowheads indicate a protrusive element in GD25β1A. B, attached cells were quantified for spreading and characterized into three groups: cells not spread, cells with only filopodia, and cells having any kind of lamellipodia-like spreading. The values represent percentages of cells in each category, and 65 cells per time were analyzed. C, remnants of Tyr(P)-stained filopodial tip complexes on the coverslip, following ripping off of a GD25β1A cell. D, GD25β1B cells were pretreated with different concentrations of the Src inhibitor PP1 or the negative control PP3 for 45 min prior to spreading on invasin for 3 h. Attached GD25β1B cells were quantified for spreading (filopodia), and the values were normalized to untreated spread cells. The values represent spread cells in relation to control ± S.E. of four separate experiments. Size bars (A and C), 10 μm.
important for the observed invasin-promoted filopodia formation by GD25β1B cells, the Src inhibitor PP1 was employed. This inhibitor blocked filopodia spreading of GD25β1B (Fig. 1D) and reduced the amount of detectable Tyr(P) in the tip complexes at lower concentrations (data not shown). As expected, PP1 also blocked spreading of GD25β1A cells (data not shown), whereas the negative control, PP3, did not affect the spreading of the cells (Fig. 1D).

To explore the partial signaling from the β1-integrin receptor, we aimed to identify proteins present within the Tyr(P) complexes at the filopodial tips. GD25β1B cells spread on invasin were subjected to a screen of immunostaining with antibodies directed against different proteins that are known to localize to focal contacts (Fig. 2A and Supplementary Fig. 2). This screening revealed that VASP and p130Cas were present at sites enriched in Tyr(P), at the tips of filopodia in GD25β1B cells (Fig. 2A). FAK, vinculin, and paxillin were completely absent from these sites and mainly localized to a perinuclear region of the cytoplasm (Fig. 2B), suggesting that the filopodial tip complexes are distinct from focal contacts and that FAK, vinculin, and paxillin are dispensable for filopodia formation. Talin could also be detected in association with the sites enriched in Tyr(P), but the localization was not as distinct as that seen with p130Cas and VASP, since talin also was present in punctate structures along the filopodial extensions (Fig. 2A).

However, a closer examination of all detectable filopodial Tyr(P) complexes of the stained cells revealed that the majority of these contained talin (77%) as well as VASP (74%) and p130Cas (82%) (Fig. 2B). In GD25β1A cells that were stained in parallel, all of the focal adhesion markers including FAK, vinculin, and paxillin localized to distal protrusive structures, but also here talin was less distinct (Fig. 2A). Hence, the Tyr(P) complexes at the tips of β1-integrin-mediated filopodia contain only a subset of proteins that can be found in focal contacts. However, a closer examination of all detectable filopodial Tyr(P) complexes of the stained cells revealed that the majority of these contained talin (77%) as well as VASP (74%) and p130Cas (82%) (Fig. 2B). In GD25β1A cells that were stained in parallel, all of the focal adhesion markers including FAK, vinculin, and paxillin localized to distal protrusive structures, but also here talin was less distinct (Fig. 2A). Hence, the Tyr(P) complexes at the tips of β1-integrin-mediated filopodia contain only a subset of proteins that can be found in focal contacts. However, a closer examination of all detectable filopodial Tyr(P) complexes of the stained cells revealed that the majority of these contained talin (77%) as well as VASP (74%) and p130Cas (82%) (Fig. 2B). In GD25β1A cells that were stained in parallel, all of the focal adhesion markers including FAK, vinculin, and paxillin localized to distal protrusive structures, but also here talin was less distinct (Fig. 2A). Hence, the Tyr(P) complexes at the tips of β1-integrin-mediated filopodia contain only a subset of proteins that can be found in focal contacts. However, a closer examination of all detectable filopodial Tyr(P) complexes of the stained cells revealed that the majority of these contained talin (77%) as well as VASP (74%) and p130Cas (82%) (Fig. 2B). In GD25β1A cells that were stained in parallel, all of the focal adhesion markers including FAK, vinculin, and paxillin localized to distal protrusive structures, but also here talin was less distinct (Fig. 2A). Hence, the Tyr(P) complexes at the tips of β1-integrin-mediated filopodia contain only a subset of proteins that can be found in focal contacts. However, a closer examination of all detectable filopodial Tyr(P) complexes of the stained cells revealed that the majority of these contained talin (77%) as well as VASP (74%) and p130Cas (82%) (Fig. 2B). In GD25β1A cells that were stained in parallel, all of the focal adhesion markers including FAK, vinculin, and paxillin localized to distal protrusive structures, but also here talin was less distinct (Fig. 2A). Hence, the Tyr(P) complexes at the tips of β1-integrin-mediated filopodia contain only a subset of proteins that can be found in focal contacts.

**p130Cas-Crk Is Required for Filopodia Formation in GD25β1B Cells**—The previous finding that p130Cas was tyrosine-phosphorylated in GD25β1B cells upon adhesion to invasin (33), together with the present observation that p130Cas localized to the tip of invasin-promoted filopodia, suggested a role for this docking protein in filopodia formation. To investigate this, we transfected GD25β1B cells with GFP-tagged full-length p130Cas and a GFP-tagged dominant negative variant of p130Cas (ΔSDp130Cas), which previously has been shown to impair cell migration (49). In nearly 50% of the adherent transfected cells, expression of full-length p130Cas induced formation of lamellipodia-like structures between the bases of extending filopodia, which, as in the corresponding mock-transfected cells, contained distinct peripheral Tyr(P) complexes (Fig. 3, A and F). Interestingly, the expression of ΔSDp130Cas in GD25β1B cells completely blocked the filopodia spreading and assembly of Tyr(P) seen in nontransfected or GFP-transfected GD25β1B cells (Figs. 1 and 3, A and F). This suggests that signaling by p130Cas is crucial for invasin-stimulated filopodia formation by GD25β1B cells. Expression of ΔSDp130Cas also affected GD25β1A cells, which spread less efficiently, where 70% of the cells were more round in shape than corresponding mock-transfected cells (Fig. 3A). However, this effect was less dramatic than the total block seen in GD25β1B cells, where 88% of the transfected cells failed to spread (Fig. 3F).

Due to the lack of the substrate domain, ΔSDp130Cas is deficient in binding to the adaptor protein Crk. In analogy with our finding that the p130Cas substrate domain is required for filopodia formation, the p130Cas-Crk scaffold has been shown to participate in cell migration, being important for pseudopodial extension (49, 50). Therefore, immunostainings were performed to explore whether also Crk was associated with the Tyr(P)-rich filopodial tips of GD25β1B cells. The data obtained
confirmed our assumption, showing a distinct localization of Crk to the Tyr(P) complexes (Fig. 3, B and C). In addition, Crk also localized to the tips of protrusions in GD25β1A cells (Supplementary Fig. 3B). Moreover, immunoprecipitation of Crk from lysates of nonstimulated and invasin-stimulated GD25β1B cells revealed that adhesion to invasin induced complex formation of Crk with tyrosine-phosphorylated p130Cas, as in GD25β1A cells (Fig. 3D). This was associated with tyrosine phosphorylation of p130Cas (33) and dephosphorylation of Crk, the latter seen as a clear shift in SDS gel migration of Crk.
from cells stimulated with invasin (Fig. 3D). Dephosphorylation of CrkII has previously been observed to increase the CrkII-p130Cas association and subsequent cell migration (51). To investigate the importance of Crk in β1-integrin-mediated filopodia formation, GD25β1A cells were transfected with GFP-tagged full-length CrkII, GFP-tagged CrkII SH2 domain (SH2) domain, or GFP-tagged CrkII SH3 domain 1. Similar to what was seen for p130Cas, expression of the truncated dominant negative variants, the Cas-binding CrkII SH2 domain, or the downstream signaling CrkII SH3 domain 1 caused blockage of filopodial spreading by GD25β1A cells, whereas the full-length variant promoted formation of lamellipodia-like structures in nearly 40% of adherent cells (Fig. 3, E and F).

In addition to binding p130Cas via the SH2 domain, Crk can bind Dock180, which is a guanine nucleotide exchange factor for Rac1, via its first SH3 domain (32, 52, 53). These interactions create the p130Cas-Crk-Dock180 scaffold, which has been shown to stimulate Rac1 (52). Staining for Dock180 in non-transfected GD25β1A cells revealed that also this signaling molecule was present within the Tyr(P)-rich filopodial tips (Fig. 3, B and C). A weak staining of DOCK180 could also be seen in GD25β1A protrusions, but here DOCK180 was also diffusely spread in the cytoplasm (Supplementary Fig. 3B). Hence, all of the components of this plausible Rac1-activating pathway are present at the filopodial tips of GD25β1A cells.

The Invasin-β1 Integrin Interaction Mediates Activation of Rac1— Rac1 has been shown to regulate lamellipodia formation in various cell lines (4). To investigate the involvement of Rac1 during invasin-stimulated spreading, GD25β1A and GD25β1A cells were transfected with GFP-tagged constitutively active (L61Rac1) or dominant negative (N17Rac1) variants of Rac1 before plating onto invasin. The GD25β1A cells were affected as anticipated based on previous work (5) in that expression of L61Rac1 resulted in increased spreading, where all transfected cells formed actin-containing ruffles and were larger than corresponding mock transfectants (Fig. 4A). Expression of N17Rac1 in these cells slightly reduced spreading but had no obvious effect on the cell morphology. A majority (84%) of the L61Rac1-expressing GD25β1A cells formed lamellipodia-like structures similar to that seen upon expression of full-length p130Cas or CrkII (Fig. 3, A, E, and F, and Fig. 4, A and B). There was, however, no inhibitory effect of N17Rac1 on β1-integrin-mediated filopodia formation (Fig. 4, A and B); rather, there were more filopodia formed, which suggests that this filopodia formation not depends on Rac1.

Given that p130Cas, Crk, and Dock180 co-localized to the TyrP) assemblies at the filopodial tips of β1-integrin-expressing cells representing a locally assembled scaffold with a guanine nucleotide exchange factor that can activate Rac1, we decided to evaluate the effect of the invasin-β1 integrin ligation on the activation state of Rac1. To accomplish this, the GST-tagged CRIB of PAK, which interacts with active GTP-bound Rac, was used in a pull-down assay. However, it was not feasible to use adhesion of invasin to stimulate cells for the GST-PAK-CRIB pull-down, since this pull-down assay requires a substantial amount of cell material, which is difficult to obtain when plating cells onto dishes. We therefore took advantage of the specific binding of invasin-expressing bacteria to β1-integrin receptors (34). We have previously shown that the Y. pseudotuberculosis plasmid-cured strain YP11111 not bind GD25 cells unless they are transfected with β1A- or β1-integrins (33), showing that this interaction is β1-integrin-specific. Accordingly, YP11111 was used to infect GD25β1A cells for different periods of time following GST-PAK-CRIB pull-down. Surprisingly, despite the lack of invasin-induced lamellipodia in the β1A-expressing cells, Rac1 became active after invasin stimulation (Fig. 4C). Thus, GD25β1B cells can activate Rac1 upon binding to invasin. This activation does not lead, however, to lamellipodia formation, which suggests that Rac1 either is active at a wrong location or that some additional factor, which the β1-integrin is unable to recruit, is needed for Rac1 to induce lamellipodia.

β1 Integrin-Mediated Filopodia Formation Is Independent of Cdc42—Previous studies have shown that Cdc42 regulates filopodia formation in certain cell types (5), and we intended to investigate whether this was the case also for invasin-stimulated filopodia formation in GD25β1A cells. GD25β1A and GD25β1B cells were transfected with the GFP-tagged dominant negative (N17Cdc42) or constitutively active (L61Cdc42) Cdc42 before plating on invasin. However, the dominant negative variant did not inhibit the filopodia formation, and neither did the constitutively active variant seem to induce more filopodia in GD25β1B cells, which suggests that these filopodia form independent of Cdc42 activity (Fig. 5, A and B). The N17Cdc42 construct used has been shown to have a dominant negative
effect in other situations (54). To exclude possible functional effects from the GFP tag, we also analyzed the effects of expressing another variant of dominant negative Cdc42, but also a FLAG-tagged N17Cdc42 was without effect on invasin-stimulated filopodia formation in GD25β1B cells (data not shown).

**DISCUSSION**

In this paper, we show that GD25 cells expressing the β1-integrin splice variant form filopodia-like structures that contain Tyr(P) complexes at the tips without signs of lamellipodia formation. This system was here used to study the molecular events of filopodia spreading in isolation. The Tyr(P)-containing tip complexes, which adhered to the underlying substrate, were found to contain the p130Cas-Crk-Dock180 scaffold, together with talin and VASP. In agreement with the presence of p130Cas with dephosphorylated Crk (40, 49, 51). The p130Cas was found to contain the p130Cas-Crk-Dock180 scaffold, together with talin and VASP. In agreement with the presence of p130Cas or Crk is sufficient to induce cell migration, indicating that the p130Cas-Crk complex also triggers actin polymerization by a Rac-independent mechanism. Initially, we thought that this could be by Cdc42, but surprisingly the filopodia formed upon invasin stimulation of β1-integrins were unaffected by expression of dominant negative Cdc42. Thus, it seems likely that the filopodia formed by GD25β1B cells plated onto invasin are regulated by another mechanism. These filopodia, which are uniformly distributed around the cells that appear totally nonpolarized, might differ from those regulated by Cdc42 that are thought to determine cell spreading direction and polarity (57). Moreover, invasin-promoted internalization of *Yersinia*, which is a Rac1-dependent process, has also been shown to occur independently of Cdc42 in HeLa and COS-1 cells (43, 58), indicating that Cdc42 is dispensable for the cellular responses induced by invasin. Other GTPases than Cdc42 have been shown to induce filopodia formation in cells, and Cdc42 do not mediate filopodia formation in all types of cells. Recently, Aspenstrom et al. (59) showed that overexpression of constitutive active forms of the GTPases Wrch, Rhod, and Rif induced thin filopodia in porcine aortic endothelial cells, whereas overexpression of active L61Cdc42 instead induced lamellipodia. Moreover, the GTPase TC10, which is a member of the Cdc42 family, has also been shown to induce filopodia in cells (60). Interestingly, TC10 has also been shown to be activated downstream of Crk in response to insulin or osmotic shock (61, 62). Hence, speculatively then, TC10 could be responsible for formation of filopodia downstream of Crk in GD25β1B cells. However, the downstream pathway from Crk to filopodia formation is still elusive.

The invasin-induced activation of Rac1 was not associated with formation of any detectable lamellipodia structures. In this situation, Rac1 become GTP-bound due to stimulation of the β1-integrin, but this is obviously not enough to initiate lamellipodia spreading. On the other hand, transfection with constitutively active Rac1 resulted in lamellipodia-like spreading in GD25β1B cells showing that Rac1 can induce lamellipodia in these cells but fail to do so when it is activated through...
invasin-β1A-integrin stimulus. The small lamellipodia-like structures formed by the Rac1-transfected GD25β1B cells are probably caused by global activation of Rac1 that occasionally interacts with the right partners, possibly represented by proteins found at the cell periphery, and this spreading is probably occurring independent of signals from the integrin receptor. The inability of GD25β1B cells to form lamellipodia through activation of the integrin receptor suggests that there are factors additional to assembly of the Cas-CrkII-Dock180 scaffold and subsequent Rac1 activation that are required for formation of lamellipodia. This can be a matter of subcellular localization of key components, where some important Rac1 effectors are absent from the β1B-integrin-induced filopodia, whereas the β1A-integrins, which mediate formation of lamellipodia, can recruit these factors and establish the cytoskeletal connections required. However, the possibility cannot be excluded that ligated β1A-integrins, which do not form clusters as the β1A variant (37, 39), generate a signal that is too “weak” for induction of lamellipodia despite activation of Rac1.

It is noteworthy that the adhesion structure component vinculin, which was missing from the invasin-induced adhesion complexes in β1B-integrin-induced filopodia, has been implicated in lamellipodia formation, since it can mediate activation of Rac1 and also recruit the Arp2/3 complex to newly forming integrin contacts (16, 63). Likewise, the vinculin-deficient cell line 5.51 has been shown to be capable to form filopodia while being unable of forming lamellipodia (13, 14). It is therefore possible that the inability of β1B-integrins to recruit vinculin contributes to the defect-spreading behavior, due to reduced localization of the actin assembly apparatus to the peripheral adhesion sites. However, although the failure of β1B-integrins to recruit vinculin is a plausible “missing link” that is responsible for the incomplete spreading, it does not exclude the possibility that additional players, which also are missing from the invasin-β1B-integrin assembled scaffold, are required for lamellipodia spreading.

We show here that the β1B-integrin variant has a partial signaling capacity, but how the signal is transduced from the receptor to p130Cas is, however, still elusive. In β1B-expressing cells, there is no phosphorylation of FAK in response to invasin ligation (33); nor is FAK recruited to filopodia. It is therefore not likely that this kinase is involved in phosphorylation and recruitment of p130Cas, and instead Src family kinases might play a role. Members of this kinase family are activated and relocalized to cell-matrix adhesions early upon integrin stimulation and are involved in formation of cell-matrix adhesions and actin cytoskeleton modulations (64–67). In accordance with this, the Src family kinase inhibitor PP1 inhibited filopodia spreading by β1B-expressing cells and normal spreading by cells expressing the β1A-integrin.

Recently, Svitkina and co-workers (68) elegantly showed that filopodia arises from reorganization of the actin dendritic network, where a distinct signaling complex at the filopodial tips was suggested to drive filopodia formation, wherein fascin promotes filament bundling, and VASP, which is known to localize to filopodial tips (69, 70), mediates filament elongation. VASP decreases the branching of F-actin and increases the rate of actin polymerization, leading to the formation of long non-branched actin filaments (22, 71) as those seen in filopodia. The initial signals that start this reorganization of the actin network are, however, still elusive. In our study, we (as did Svitkina et al. (68)) found a distinct signaling complex that contained VASP at the filopodial tips. Talin, another protein previously shown to filopodial tips (72), was also present, and we also show, for the first time, the presence of p130Cas, Crk, and DOCK180 at this location. This tip complex is probably involved in driving filopodia formation and is clearly different from the common adhesive focal complex structures, which, in addition to the above, contain FAK, vinculin, and paxillin. In agreement with the study by Svitkina et al. (68), we have data implying that VASP is important for the formation of filopodia in the GD25β1B cells spreading on invasin, and we show here that the p130Cas-CrkII complex is required. Whether VASP is downstream of the activity of the Cas-Crk complex or in a separate parallel pathway remains to be elucidated. Nevertheless, our data imply that the tip complex indeed serves to drive filopodia formation. In addition, we show that this signaling complex can adhere with β1-integrins to the substrate, which we base on the requirement for invasin and expression of β1-integrins to observe these complexes, the localization of β1-integrins to the complexes (33), and the fact that these complexes remain on the substrate after cells have been ripped off. Talin could be implicated in the adhesive role of the complex, since it is known to be crucial for integrin function by linking it to the cytoskeleton (73) and activating integrins by separating the integrin α and β cytoplasmic tails (18, 19). Both biochemical data and NMR studies have shown that talin can bind to two sites on the β1-integrin cytoplasmic tail (3, 19, 20, 74, 75), where one of these binding sites is in the membrane-proximal region of the integrin. This region is common for all β1-integrin splice variants and thus also present in β1B. The presence of talin at the β1-integrin-induced filopodial tips might therefore reflect this binding. Thus, the tip complex of filopodia might serve a dual function, where VASP and the p130Cas-Crk complex are involved in driving filopodia formation. VASP might be involved by its direct activity on the actin polymerization rate, whereas the exact role of p130Cas-Crk might be to mediate activation of a particular GTPase. Talin possibly is involved in activating and anchoring β1-integrins to F-actin even if the role for talin still has to be formally proven.

Our observation that β1-integrin-mediated signaling can be dissected by comparing responses after stimulation of cells expressing β1A- and β1B-integrin isoforms was here shown to be useful to study filopodia formation in isolation. The partial outcome of β1B-integrin ligation is probably due to failure to activate signaling pathways and make cytoskeletal connections. The data presented in this study provide new information about molecular events involved in filopodia formation, but the data also open new questions on mechanisms behind the observed effect. The β1-integrin A and B splice variants have identical membrane-proximal regions in the cytoplasmic tail but differ in the carboxyl-terminal membrane-distal part (37). It therefore remains to be determined whether it is the common region or the unique 12 amino acids of β1B that mediates the observed signal response that results in recruitment of talin, VASP, and the p130Cas-Crk-Dock180 scaffold and activation of Rac1. Neither can it be excluded that the α-chain of the integrin receptor contributes to the observed effects, since α-chains also mediate certain signaling (1, 3, 76). It is also possible that the state of integrin activation and/or the clustering capacity influences the outcome of receptor ligation. However, although these questions remain to be answered, this paper provides new knowledge indicating that filopodial tips adhere to the substrate and that the p130Cas-Crk complex is a crucial player in filopodia formation, which might well represent an initial phase of cell spreading. It is also clear from our data that the membrane-distal 21 amino acids of the β1A-integrin cytoplasmic tail are important for efficient β1-integrin spreading. Among the proteins that have been shown to interact with this region are ICAP-1, Grb-2, Shc, and talin (1, 3). Consequently,

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A. Gustavsson and M. Fullman, unpublished data.
