Ack1 Mediates Cdc42-dependent Cell Migration and Signaling to p130Cas*

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We previously showed that activation of the small GTPase Cdc42 promotes breast cell migration on a collagen matrix. Here we further define the signaling pathways that drive this response and show that Cdc42-mediated migration relies on the adaptor molecule p130Cas. Activated Cdc42 enhanced p130Cas phosphorylation and its binding to Crk. Cdc42-driven migration and p130Cas phosphorylation were dependent on the Cdc42 effector Ack1 (activated Cdc42-associated kinase). Ack1 formed a signaling complex that also included Cdc42, p130Cas, and Crk, formation of which was regulated by collagen stimulation. The interaction between Ack1 and p130Cas occurred through their respective SH3 domains, while the substrate domain of p130Cas was the major site of Ack1-dependent phosphorylation. Signaling through this complex is functionally relevant, because treatment with either p130Cas or Ack1 siRNA blocked Cdc42-induced migration. These results suggest that Cdc42 exerts its effects on cell migration in part through its effector Ack1, which regulates p130Cas signaling.

Integrin-mediated cell interactions with components of the extracellular matrix regulate several aspects of both epithelial polarization and migration. Integrins are involved in signaling pathways that include activation of small GT-Pases, scaffolding molecules, and protein kinases (1–3). These signaling pathways regulate various aspects of cell migration on integrin ligands, such as collagen.

Members of the Rho family of GT-Pases, Rho, Rac, and Cdc42, are important regulators of the actin cytoskeleton and have been shown to mediate cell migration (4–10, for review see Refs. 3 and 11). Although much work has focused on understanding the mechanism by which Rho GT-Pases regulate the actin cytoskeleton, relatively less is known about the effects they have on integrin signaling pathways. Ack1 and Ack2 are homologous tyrosine kinases that bind exclusively to activated Cdc42-GTP, but not Rac or Rho (12, 13). The structure of Ack includes a kinase domain, an SH3 domain, a Cdc42/Rac-interactive binding (CRIB) domain, and a proline-rich C terminus, which is the key determinant between Ack1, a 120-kDa protein (12, 14) and Ack2 an 83-kDa isofrom (13). Multiple interactions have been identified for Ack1. In vitro binding to the proline-rich region has been demonstrated for Nck (15), Grb2 (16), Src (15), and Hck (17), whereas the SH3 domain binds HSH2, an adaptor in hematopoietic cells (18). Ack1 can also be co-immunoprecipitated with clathrin (16) and sorting nexin 9 (19), components of vesicle dynamics. Ack kinases have been implicated in a variety of signaling pathways. Tyrosine phosphorylation of Ack1 and Ack2 has been demonstrated downstream of growth factors (13, 16), cell adhesion (14, 20), and muscarinic receptors (21). Ack1 phosphorylates and activates Dbl, an exchange factor for Rho (22), as well as Ras/GRF1, an exchange factor for Ras (23). Ack1 also mediates p130Cas phosphorylation downstream of melanoma chondroitin sulfate proteoglycan (24), whereas Ack2 is implicated in modulating cell spreading, migration, and focal adhesion dynamics (20, 25).

Recent data demonstrate an important role for Ack1 in cancer cell survival (26–28), as well as in tumor formation and metastasis (29–31). MacKeigan et al. (27) identified Ack1 as an anti-apoptotic gene in an RNAi screen targeted against human kinases, while Nur-e-Kamal et al. (26) demonstrated that Ack1 is required for survival of v-Ras-transformed cells. Analysis of prostate cancer specimens reveals an amplification of the Ack1 gene (30) and an increased phosphorylation and presumably activation of Ack1 (29). Additionally, introduction of activated Ack1 enhances anchorage-independent growth in vitro and accelerates tumor formation in nude mice (29). Overexpression of Ack1 in cancer cell lines results in a more invasive phenotype both in vitro and in vivo (30). Finally, microarray analysis identified an increase in Ack1 expression in mammary tumors from MMTV-neu transgenic mice (31).

p130Cas is one of the key components of integrin-mediated signaling pathways involved in cell migration (32–34). Overexpression of p130Cas promotes cell migration, and conversely, p130Cas knock-out cells exhibit minimal migration on variety of extracellular matrix components (35, 36). p130Cas is an adaptor molecule composed of an SH3 domain, a proline-rich region, a substrate domain, a serine-rich domain, and a C-terminal Src binding domain. p130Cas phosphorylation is crucial for its association with other signaling molecules, and its function in cell enhanced green fluorescent protein; GST, glutathione S-transferase; Ack, activated Cdc42-associated kinase; HEK, human embryonic kidney; siRNA, short interfering RNA.

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2 The abbreviations used are: SH, Src homology domain; CRIB, Cdc42/Rac-interactive binding; HA, hemagglutinin; SD, substrate domain; EGFP, enhanced green fluorescent protein; GST, glutathione S-transferase; Ack, activated Cdc42-associated kinase; HEK, human embryonic kidney; siRNA, short interfering RNA.
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migration (35, 37, 38). Thus far, Src has been the major tyrosine kinase shown to phosphorylate p130Cas (39, 40). Once phosphorylated, the YXXP motifs within the substrate domain can bind SH2-containing proteins, including Crk (37, 41). The interaction of p130Cas with Crk has been described as a “molecular switch” for cell migration (35).

We previously showed that activation of Cdc42 induces cell migration on collagen (4) and wanted to further explore signaling pathways responsible for this effect. Here we demonstrate that Ack1 is a Cdc42 effector that mediates Cdc42-induced migration by modulating p130Cas signaling.

EXPERIMENTAL PROCEDURES

Tissue Culture—T47D cells were maintained in RPMI (Cellgro, Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 8 µg/ml insulin. HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium (Cellgro, Invitrogen) supplemented with 10% fetal bovine serum. Both cell lines were obtained from ATCC. Stable transfectants of T47D cells expressing Cdc42(12V) or control pZIP vector have been previously described (4).

DNA Constructs and siRNA—pEGFP was purchased from Clontech. pXJ-HA-Ack1 and pXJ-EGFP-Ack1 constructs were kindly provided by Dr. Ed Manser. pKH3-p130CasSH3 was a generous gift from Dr. Jun-Lin Guan. pRK, pRK-p130Cas wild type, p130CasΔSH3, p130CasΔSD were kindly provided by Dr. Amy Bouton. pKH3- and pCDNA3- Cdc42 wild type, 61L, and 17N constructs, as well as GST-PBD were a generous gift from Dr. Rick Cerione. To create GST fusion proteins, SH3 domains of Ack1, p130Cas, and Crk were amplified by PCR using the wild-type constructs as templates and subcloned into the BamHI and EcoRI sites of the pGEX-4T1 vector (Amersham Biosciences). pGEX-SrcSH3 was a gift from Dr. Brian Kay. siRNA oligonucleotides targeting the C terminus of Ack1 (AAGGTCAGCAGCACCCACTAT) was designed with the assistance of and purchased from Qiagen-Xeragon. Predesigned siRNA targeting p130Cas (HS_BCAR1_3) and non-silencing control oligonucleotides were purchased from Qiagen-Xeragon.

Antibodies—Primary antibodies were purchased from the following companies: mouse monoclonal antibodies to p130Cas and Crk from Transduction Labs, rabbit polyclonal anti-Ack1 from Santa Cruz Biotechnology, mouse monoclonal anti-Src from Upstate Biotech, mouse monoclonal anti-HA from Sigma, rabbit polyclonal phosphospecific anti-p130Cas (pY165, pY249, pY410) antibodies from Cell Signaling Technology. Polyclonal anti-Ack2 antibody was a gift from Dr. Richard Cerione. Secondary anti-mouse and anti-rabbit horseradish peroxidase antibodies were from Jackson Laboratories.

Transfections—HEK cells were transfected for 24–36 h in 100-mm dishes with 4–6 µg of DNA using Lipofectamine Plus (Invitrogen) according to the manufacturer’s directions. T47D cells were transfected for 48 h with Ack1, p130Cas, or control siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s directions.

Migration Assays—T47D cells stably expressing Cdc42(12V) or control pZIP vector were transiently transfected with 50 nm Ack1 siRNA, 200 nm p130Cas siRNA, or control oligonucleotide at appropriate concentrations. 48 h after transfection, cells were detached with 0.05% trypsin, counted, and equal cell numbers were seeded into transwells (Costar) coated on the underside with 100 µg/ml collagen I (BD Bioscience). Cells were allowed to migrate for 16 h. Cells that migrated to the underside were fixed and stained using Diff-Quik (Dade Behring) and mounted using Vectashield (Vector Laboratories). Experiments were performed in triplicate with 6–8 fields counted for each transwell, and the total number of cells per transwell was calculated for each condition. Results are reported as averages of the three experiments relative to control pZIP cells, or pZIP cells treated with control siRNA where appropriate.

Immunoprecipitation and Immunoblotting—Subconfluent cells were detached with Versene (phosphate-buffered saline + 0.5 mM EDTA), washed and resuspended in medium containing 5 mg/ml fatty acid free bovine serum albumin. Cells were counted and volumes adjusted such that equal numbers were used for each immunoprecipitation. Cells were stimulated in suspension for 10–20 min at 37 °C with 0.5 mg/ml collagen I that had been neutralized by addition of 2× phosphate-buffered saline + 100 mM Hepes pH 7.3. Cells were then lysed with 2× lysis buffer containing 100 mM Hepes pH 7.3, 150 mM NaCl, 2% Nonidet P-40, 0.5% deoxycholate, 2 mM EDTA, 2 mM NaF, 2 mM pervaNate, and 0.1% protease inhibitor mixture III (Calbiochem) for immunoprecipitation experiments, or 100 mM Hepes pH 7.3, 150 mM NaCl, 20% glycerol, 2% Nonidet P-40, 2 mM NaF, 2 mM pervaNate, and 0.1% protease inhibitor mixture III for co-immunoprecipitations. Lysates were incubated at 4 °C for 15–30 min, and then centrifuged at 14,000 rpm. Immunoprecipitations were performed from cleared lysates by the addition of 2–4 µg of appropriate antibody and 30 µl of Gammabind beads (Amersham Biosciences) at 4 °C for 2 h to overnight, washed three times with 1× lysis buffer, and processed for SDS-PAGE. Proteins were transferred to polyvinylidene difluoride filters (Immobilon-P; Millipore), and the filters were blocked overnight in 5 mg/ml milk, or 5 mg/ml bovine serum albumin for phosphotyrosine blots. Membranes were immunoblotted for phosphotyrosine content or co-immunoprecipitated proteins and subsequently stripped (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-Cl, pH 6.8) and rebotted for loading. Quantification was performed by measuring the band density of scanned images using ImageJ software.

SH3-GST Pulldowns—GST-tagged SH3 domains were expressed in Escherichia coli and purified as described in Ref. 42. Cells were lysed in 2× lysis buffer containing 100 mM HEPES, 150 mM NaCl, 0.5% deoxycholate, 2% Nonidet P-40, 2 mM EDTA, 2 mM NaF, 0.2% SDS, 2 mM pervaNate, 0.1% protease inhibitor mixture III, and lysates, or 1× lysis buffer alone, were incubated with 30 µg of the GST probes for 30 min at 4 °C. Samples were washed three times, bound proteins eluted with Laemmli buffer, resolved on SDS-PAGE, followed by immunoblotting with appropriate antibodies.

RESULTS

Cdc42 Induces Cell Migration through p130Cas—We previously reported that stable expression of constitutively active Cdc42(12V) caused increased migration of T47D breast epithe-
lial cells across collagen (4). p130Cas becomes phosphorylated upon integrin activation and is one of the key signaling molecules involved in cell migration. Phosphorylated p130Cas binds its effectors, such as Crk, which is required for its effects on cell migration (35). First, we wanted to know whether p130Cas was required for Cdc42-induced cell migration. Control (pZIP vector only) and Cdc42(12V)-expressing T47D cells were transiently transfected with siRNA directed against p130Cas or control siRNA, and migration across collagen-coated filters was determined in a transwell assay (43). Cdc42(12V)-expressing cells exhibited a 2–3-fold increase in migration on collagen. Importantly, p130Cas siRNA inhibited migration induced by Cdc42(12V) to baseline levels (Fig. 1A), demonstrating an important role for p130Cas in this migration.

To determine whether p130Cas signaling was affected by Cdc42, p130Cas phosphorylation, and binding to Crk were assessed. Collagen treatment caused a substantial increase in p130Cas phosphorylation in control T47D cells. In cells stably expressing activated Cdc42(12V) there was an increase in the baseline level (in absence of collagen) of p130Cas phosphorylation (Fig. 1B) compared with cells expressing control vector, suggesting stimulation of p130Cas downstream of Cdc42 activation. Similarly, the amount of Crk that could be co-immunoprecipitated with p130Cas in unstimulated cells was higher in cells expressing activated Cdc42(12V) than in control cells (Fig. 1C). Collagen stimulation could further increase Crk binding to p130Cas in both control and Cdc42(12V)-expressing cells, suggesting that additional signaling pathways downstream of collagen stimulation also contribute to this effect.

As a complementary approach, HEK cells were transfected with either activated (61L) or dominant negative (17N) mutants of Cdc42 and levels of p130Cas phosphorylation and Crk binding were determined in either the absence or presence of collagen stimulation. Transient expression of activated Cdc42(61L) resulted in a substantial increase in the level of p130Cas phosphorylation in the absence of collagen. Such an increase was not p130Cas or control siRNA, and migration determined in a Boyden Chamber assay. Cdc42(12V)-expressing cells exhibit a 2–3-fold increase in migration as compared with control cells (vector). Treatment with p130Cas siRNA reduces this enhancement to control level migration. Data are presented as the mean ± S.D. of three independent experiments performed in triplicates. *, p < 0.001 relative to pZIP + control siRNA; **, p < 0.001 relative to Cdc42(12V) + control siRNA. B, Cdc42 enhances p130Cas phosphorylation. Control and Cdc42(12V)-expressing T47D cells were left untreated or stimulated with collagen for 15 min, lysed, and p130Cas was immunoprecipitated. The phosphorylation of p130Cas was determined by immunoblotting with anti-phosphotyrosine (pY) antibody and subsequent reblotting with anti-p130Cas antibody. Bar graph shows the amount of p130Cas phosphorylation/total amount of p130Cas and represents the mean of two experiments normalized internally to unstimulated control cells. Error bars represent the range. C, Cdc42 enhances Crk binding to p130Cas. The amount of p130Cas bound to Crk was determined in a parallel immunoprecipitation by immunoblotting with anti-p130Cas antibody and anti-Crk antibody for loading. Bar graph demonstrates quantification of two experiments, as in B. D and E, HEK cells transiently transfected with control vector, activated HA-Cdc42(61L), or dominant negative HA-Cdc42(17N) were stimulated with collagen for 20 min, lysed, and either p130Cas (D) or Crk (E) was immunoprecipitated. Phosphorylation of p130Cas and the amount of p130Cas co-immunoprecipitated with Crk were determined by immunoblotting with anti-pY antibody. Results shown are representative of three similar experiments and demonstrate that Cdc42 activation leads to increased p130Cas phosphorylation and Crk binding to p130Cas.
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noted in cells expressing control vector or dominant negative Cdc42(17N) (Fig. 1D). Furthermore, the amount of Crk that could be co-immunoprecipitated with p130Cas was increased in unstimulated cells expressing activated Cdc42(61L) (Fig. 1E). Collagen stimulation further increased p130Cas phosphorylation and Crk binding in Cdc42(61L)-expressing cells. In cells expressing control vector or inactive Cdc42(17N), p130Cas phosphorylation, and Crk binding were induced only in response to collagen stimulation. Taken together, these results demonstrate that Cdc42 activation can stimulate p130Cas signaling.

The Cdc42 Effector, Ack1, Mediates Cdc42 Effects on p130Cas Phosphorylation and Migration—Ack is the only known tyrosine kinase effector of Cdc42. To determine whether Ack1 mediated the effects of Cdc42 on p130Cas phosphorylation, cells were treated with Ack1 siRNA. siRNA reduced Ack1 expression levels in T47D cells by 50–80% but did not affect the expression of Ack2, or the unrelated tyrosine kinase, Src (Fig. 2A). p130Cas was immunoprecipitated from control and Cdc42(12V)-expressing cells treated with control or Ack1 siRNA, and phosphorylation on tyrosine determined. As already noted in Fig. 1B, Cdc42 activation induced p130Cas phosphorylation even in the absence of collagen (Fig. 2B). This effect was decreased in cells transfected with Ack1 siRNA, as compared with those treated with non-silencing control siRNA (Fig. 2B). Additionally, Ack1 siRNA also resulted in a decrease in collagen-driven p130Cas phosphorylation in control cells (Fig. 2B). These results suggest that Ack1 mediates Cdc42 signaling to p130Cas.

To determine whether Ack1 was required for Cdc42-driven cell migration, control and Cdc42(12V)-expressing T47D cells were transiently transfected with siRNA to Ack1, or control siRNA, and migration across collagen-coated filters determined in a transwell assay. Cdc42(12V)-expressing cells exhibited a 3-fold increase in migration on collagen. Ack1 siRNA inhibited migration induced by Cdc42(12V) to baseline levels (Fig. 2C). Additionally, treatment with Ack1 siRNA also resulted in a small reduction in migration in control cells (Fig. 2C). These results suggest that Ack1 is required for Cdc42-induced migration.

Cdc42, Ack1, p130Cas, and Crk Form a Collagen-regulated Signaling Complex—Because Cdc42 and Ack1 regulate p130Cas signaling, we wanted to know whether these proteins interact in cells. We were able to co-immunoprecipitate endogenous p130Cas and Cdc42 in T47D cells (Fig. 3A). This interaction was minimal in untreated cells and significantly enhanced upon collagen stimulation. We were also able to co-immunoprecipitate endogenous Ack1 with both p130Cas and its effector Crk (Fig. 3, B and C). In both cases there was a baseline level of interaction and an increase was observed with collagen stimulation. Expression of activated Cdc42(12V) appeared not to have an effect on these interactions, suggesting the formation of these complexes is regulated by additional pathways downstream of integrins. These results suggest that Ack1, Cdc42, and p130Cas are not only functionally linked, but also interact in a complex within cells.

The Interaction of Ack1 and p130Cas Relies on SH3 and Proline-rich Domains—Interactions between a kinase and its substrate often rely partially on the catalytic domain of the kinase and the tyrosine phosphorylation motifs of the substrate. To determine whether the interaction between Ack1 and p130Cas was dependent on Ack1 kinase activity, p130Cas was immunoprecipitated from HEK cells transiently transfected with either wild-type or kinase-dead HA-Ack1, and the amount of co-precipitating Ack1 was determined by immunoblotting. In cells treated with Ack1 siRNA, Ack1 expression decreased significantly as compared with levels of Ack2 and Src. B, Ack1 is necessary for p130Cas phosphorylation. Vector control (pZIP) and Cdc42(12V)-expressing T47D cells transfected with 50 nM control or Ack1 siRNA were unstimulated or stimulated with collagen for 15 min, lysed, and p130Cas was immunoprecipitated. Phosphotyrosine levels were determined by immunoblotting with anti-phosphotyrosine (pY) antibody and reblotting with anti-p130Cas antibody. Results shown are representative of three similar experiments and indicate that Ack1 is required for complete p130Cas phosphorylation in control and Cdc42(12V)-expressing cells. C, Ack1 contributes to cell migration. Control and Cdc42(12V)-expressing T47D cells were treated with 50 nM control or Ack1 siRNA, and 48 h later cell migration was determined in a Boyden Chamber assay. Cdc42(12V)-expressing cells exhibited a 3-fold increase in migration compared with control cells. Treatment with Ack1 siRNA reduces this enhancement to control level migration. Data are presented as the mean ± S.D. of three independent experiments performed in triplicates. *, p < 0.01 relative to pZIP + control siRNA; **, p < 0.01 relative to Cdc42(12V) + control siRNA.
cases the association was collagen-dependent (Fig. 4A), demonstrating that kinase activity is not required for the association of Ack1 with \(p_{130}^{\text{Cas}}\).

The substrate domain (SD) of \(p_{130}^{\text{Cas}}\) is its predominant phosphorylated domain. To determine whether the substrate domain is required for the interaction with Ack1, a \(p_{130}^{\text{Cas}/\text{H9004}}\) mutant in which the substrate domain has been deleted was overexpressed in HEK cells. \(p_{130}^{\text{Cas}/\text{H9004}}\) could be co-immunoprecipitated with Ack1, but surprisingly this interaction was no longer regulated by collagen (Fig. 4B). Although, it was difficult to obtain equal expression levels of the \(p_{130}^{\text{Cas}/\text{ASD}}\), the amount that co-immunoprecipitated with Ack1 was always substantially increased compared with full-length \(p_{130}^{\text{Cas}}\) and \(p_{130}^{\text{Cas}/\text{ASH3}}\). Full-length \(p_{130}^{\text{Cas}}\), as well as a mutant lacking the SH3 domain also bound to Ack1, and these interactions were still increased following collagen stimulation.

Both Ack1 and \(p_{130}^{\text{Cas}}\) contain SH3 domains and proline-rich regions (Fig. 5A). To determine whether the interaction between \(p_{130}^{\text{Cas}}\) and Ack1 occurred through their SH3 domains, GST pull-down assays were performed. Ack1, \(p_{130}^{\text{Cas}}\), Src, and both Crk SH3 domains were expressed as GST fusion proteins, purified and adjusted to equal concentrations (Fig. 5B). The Ack1 GST-SH3 domain was able to pull-down endogenous \(p_{130}^{\text{Cas}}\) and the interaction was stimulated by collagen. Both HEK cells transfected with full-length \(p_{130}^{\text{Cas}}\), \(p_{130}^{\text{Cas}/\text{ASH3}}\), or \(p_{130}^{\text{Cas}/\text{ASD}}\) were unstimulated or stimulated with collagen for 20 min, lysed, and endogenous Ack1 immunoprecipitated. Levels of \(p_{130}^{\text{Cas}/\text{H9004}}\) mutants that co-precipitated were determined by immunoblotting with anti-\(p_{130}^{\text{Cas}}\) and reblooming with anti-Ack1 antibody. Endogenous Ack1 could also be pulled down with the Ack1 SH3 domain (data not shown).

To determine whether the SH3 domain of \(p_{130}^{\text{Cas}}\) was required for the interaction with Ack1, HEK cells were transfected with wild type (wt) or kinase-dead (kd) HA-Ack-1 were unstimulated or stimulated with collagen for 20 min and lysed. Endogenous \(p_{130}^{\text{Cas}}\) was immunoprecipitated, and the amount of Ack1 co-immunoprecipitated was determined by immunoblotting with anti-HA and subsequent reprobing with anti-\(p_{130}^{\text{Cas}}\) antibody. Lysates were immunoblotted with anti-HA antibody to determine expression. Results indicate that both wt and kd Ack1 co-immunoprecipitate with \(p_{130}^{\text{Cas}}\), and this interaction is stimulated by collagen. 8. HEK cells transfected with full-length \(p_{130}^{\text{Cas}}\), \(p_{130}^{\text{Cas}/\text{ASH3}}\), or \(p_{130}^{\text{Cas}/\text{ASD}}\) were unstimulated or stimulated with collagen for 20 min, lysed, and endogenous Ack1 immunoprecipitated. Levels of \(p_{130}^{\text{Cas}/\text{H9004}}\) mutants that co-precipitated were determined by immunoblotting with anti-\(p_{130}^{\text{Cas}}\) and reblooming with anti-Ack1 antibody. Results indicate that both wt and kd Ack1 co-immunoprecipitate with \(p_{130}^{\text{Cas}}\), and this interaction is stimulated by collagen.
Cdc42 Signals through Ack1 and p130Cas

**A**

Ack1

- Kinase domain
- SH3
- CRIB
- Pro

p130Cas

- SH3
- P
- Substrate Domain
- Ser
- SB

**B**

Ack1 and p130Cas interact through their SH3 domains. A, Ack1 contains a kinase domain, an SH3 domain, CRIB, and a large proline-rich C terminus (Pro); p130Cas has an N-terminal SH3 domain and a short proline-rich domain (P), followed by a substrate domain (SD), a C-terminal serine-rich region (Ser), and a Src binding (SB) domain. B, recombinant GST-SH3 domains were adjusted to equal concentrations and stained with Coomassie Blue.

**C**

WB: p130Cas

**D**

WB: HA (Ack1)

**E**

IP: Ack1

Vector

wt

ΔSH3

WB: p130Cas

WB: Ack1

WB: p130Cas (lysates)

**FIGURE 5.** Ack1 and p130Cas interact through their SH3 domains. A, Ack1 contains a kinase domain, an SH3 domain, CRIB, and a large proline-rich C terminus (Pro); p130Cas has an N-terminal SH3 domain and a short proline-rich domain (P), followed by a substrate domain (SD), a C-terminal serine-rich region (Ser), and a Src binding (SB) domain. B, recombinant GST-SH3 domains were adjusted to equal concentrations and stained with Coomassie Blue. C, HEK cell lysates or 1× lysis buffer (−) were incubated with GST-fused SH3 domain of Src, Ack1, or Crk (N-terminal (Nt) or C-terminal (Ct)) or GST. Precipitates were subjected to SDS-PAGE and immunoblotted with anti-p130Cas. Ack1-SH3, Src-SH3, and Crk-SH3(Nt) but not Crk-SH3(Ct) or GST alone pulled down p130Cas. No cell lysate controls (−) demonstrate that the anti-p130Cas did not bind nonspecifically to the GST-SH3 probes. D, HEK cells transfected with HA-Ack1 were lysed, and lysates were incubated with p130Cas SH3, Src-SH3, Src-SH3(Nt), or GST. Pulled-down proteins were subjected to SDS-PAGE and immunoblotted with anti-HA. Ack1 associates with the SH3 domain of p130Cas and Src but not the N-terminal SH3 domain of Crk(Nt) or GST. No cell lysate controls (−) demonstrate the anti-HA did not bind nonspecifically to the GST-SH3 probes. E, HEK cells were transfected with vector control, full-length p130Cas (wt) or p130CasΔSH3, stimulated with collagen for 20 min, lysed, and endogenous Ack1 was immunoprecipitated. Full-length p130Cas and p130CasΔSH3 immunoprecipitated with Ack1 in a collagen-dependent manner. The amount of p130CasΔSH3 co-immunoprecipitated was reduced as compared with full-length p130Cas. The experiments are representative of at least three similar results and indicate that Ack1 interaction with p130Cas relies in part on their respective SH3 domains.

The p130Cas Substrate Domain Is the Major Site of Ack1-dependent Phosphorylation—To determine whether Ack1-driven p130Cas phosphorylation depends on its SH3 or substrate domain, full-length p130Cas, p130CasΔSH3, and p130CasΔSD were co-transfected with either pEGFP vector or pEGFP-Ack1 in HEK cells. Because overexpression of Ack1 results in increased phospho-Ack1 levels, EGFP tagging was necessary to increase the size of Ack1 and allow its separation from p130Cas. In cells expressing control EGFP vector, phosphorylation of p130Cas and p130CasΔSH3 was increased only upon collagen stimulation. In contrast, in cells expressing EGFP-Ack1, p130Cas, and p130CasΔSH3 phosphorylation levels were dramatically increased even in the absence of collagen stimulation (Fig. 6A). p130CasΔSH3 was phosphorylated to a lesser extent than that of full-length p130Cas (Fig. 6A, lower exposure). Importantly, p130CasΔSD was only minimally phosphorylated in Ack1-expressing cells (Fig. 6A, longer exposure). These results suggest that the major phosphorylation sites regulated by Ack1 lie within the substrate domain of p130Cas.

To map phosphorylation sites in p130Cas, lysates of cells expressing p130Cas and either control vector (EGFP) or EGFP-Ack1 were immunoblotted with antibodies specific for tyrosine sites within the substrate domain of p130Cas: pY165, pY249, and pY410. Collagen induces phosphorylation of all three sites. Significantly, in cells expressing EGFP-Ack1, all three sites were phosphorylated to a much greater extent even in the absence of collagen (Fig. 6B), which suggests that Ack1 enhances phosphorylation of all three of these sites.

**DISCUSSION**

Although Cdc42 has been demonstrated to regulate cell migration, a complete understanding of its various effector pathways has not been fully elucidated. We demonstrate a role for the Cdc42 effector, Ack1, in Cdc42-induced cell migration on collagen and in collagen-mediated signaling pathways. An intriguing finding is that collagen stimulation regulates the formation of a complex involving both Cdc42 and integrin signaling intermediates, because Ack1 as well as Cdc42 could be co-immunoprecipitated with p130Cas and its effector, Crk (Fig. 3). Interestingly, overexpressing activated Cdc42 in the absence of collagen stimulation was not sufficient to induce complex formation between Ack1 and p130Cas or Crk, demonstrating cooperative regulation of Cdc42 effector pathways by integrin signaling. This finding adds further support to the emerging notion of cross-regulation between integrins and small GTPases.

3 P. J. Keely, unpublished data.
Ack1 and p130Cas interact in unstimulated cells and their binding is enhanced upon collagen stimulation (Figs. 3 and 5). The SH3 domains of both Ack1 and p130Cas were involved in facilitating this interaction (Fig. 5). It is interesting that the deletion of the SH3 domain in p130Cas significantly decreased its interaction with Ack1 in unstimulated cells, but collagen stimulation was still able to drive binding (Fig. 6). This suggests that the collagen-induced interaction lies elsewhere, such as within the substrate domain of p130Cas, which becomes heavily phosphorylated on multiple tyrosines following integrin engagement. In fact, similar levels of p130Cas/H9004SD (p130Cas in which the substrate domain was deleted) can be co-immunoprecipitated with Ack1 in the presence or absence of collagen (Fig. 4). Surprisingly, the amount of p130Cas/ASD (p130Cas in which the substrate domain was deleted) can be co-immunoprecipitated with Ack1 in the presence of collagen (Fig. 4). Interestingly, there was only a slight enhancement in phosphorylation of p130Cas/ASD in cells overexpressing Ack1, although its binding to Ack1 was not impaired. These results suggest that the major Ack-1-dependent phosphorylation site(s) lie within the substrate domain of p130Cas. In fact, the

Ack1 not only associated with p130Cas but also induced its phosphorylation. These results are consistent with recent findings that Ack1 can interact with p130Cas and lead to its phosphorylation in response to laminin stimulation (30) and that Ack1 is required for melanoma chondroitin sulfate-induced p130Cas phosphorylation (24). Both full-length and p130Cas/ΔSH3 were hyperphosphorylated in HEK cells overexpressing Ack1 under conditions in which Ack1 and p130Cas were also associated (Fig. 6). This suggests that the interaction between Ack1 and p130Cas drives the phosphorylation and suggests that p130Cas may be a direct substrate for Ack1. Interestingly, there was only a slight enhancement in phosphorylation of p130Cas/ΔSD in cells overexpressing Ack1, although its binding to Ack1 was not impaired. These results suggest that the major Ack-1-dependent phosphorylation site(s) lie within the substrate domain of p130Cas. In fact, the

Crk was also a component of this complex, and there are several potential ways it can interact. Crk binds to the phosphorylated substrate domain of p130Cas (37). We suspect that the interaction of Crk with Ack1 occurs most likely through p130Cas. However, because Ack1 is also phosphorylated upon collagen treatment it is possible that Crk binds via its SH2 domain not only to phosphorylated p130Cas but also to Ack1 directly. Additional binding studies are needed to investigate this possibility.

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Cdc42 Signals through Ack1 and p130Cas

phosphorylation of three sites within the substrate domain was enhanced in cells expressing Ack1: pTyr165, pTyr249, and pTyr410 (Fig. 6). Whereas Src has been shown to phosphorylate sites 249 and 410, tyrosine 165 is thought to be a minor site of Src activity (44). Cells expressing Ack1 exhibited a substantial increase in phosphorylation of site 165 in addition to tyrosine 249 and 410. Phosphorylation on tyrosines 249 and 410 creates binding sites for the Crk SH2 domain, whereas site 165 does not interact with Crk (44). Therefore, enhanced phosphorylation at Tyr249 and Tyr410 in p130Cas is consistent with enhanced binding of p130Cas to Crk, and subsequent enhanced cell migration. One possibility is that Ack1 leads to phosphorylation on some p130Cas tyrosines through Src, thus leading to increased Crk binding. In addition, Ack1 may phosphorylate sites independently of Src and create binding sites for additional SH2-containing molecules. Alternatively, Ack1 may be able to phosphorylate all three sites directly and subsequently lead to association with different downstream effectors. To rule out the role of Src, we considered using PP2 inhibitors in our phosphorylation studies, but determined that data from such experiments would not be conclusive because Src family members are also required for Ack1 activation (17, 21). This is a similar situation to focal adhesion kinase (FAK), whose kinase activity is activated by Src, making it difficult to discern whether it contributes to the phosphorylation of other proteins directly or by scaffolding with Src.

We propose that Ack1 is the kinase that mediates p130Cas phosphorylation in Cdc42(12V)-expressing cells. In these cells, Ack1 would be predicted to exhibit enhanced kinase activity and could potentially phosphorylate p130Cas and prompt Crk binding, even in the absence of collagen stimulation. In control cells, on the other hand, collagen may be required to activate Cdc42 and in turn activate Ack1. In both control and Cdc42-expressing cells, Ack1 is phosphorylated in response to collagen stimulation (data not shown), which should result in further activation of the Ack1-p130Cas pathway.

Collagen stimulation can also lead to p130Cas phosphorylation by other kinases, which could result in a multi-armed stimulation downstream of collagen receptors. Thus far, Src has been the major kinase thought to be responsible for p130Cas phosphorylation. Interestingly, Cdc42(12V)-expressing cells exhibit lower levels of Src expression in comparison with control T47D cells (data not shown). It is possible that in cells expressing activated Cdc42(12V), Ack1 takes over as the major kinase responsible for phosphorylation of p130Cas (and possibly other substrates), and Src levels are diminished.

It is increasingly clear that Rho GTPases play an important role in integrin signaling pathways. Cdc42 can be activated upon integrin engagement with extracellular matrix components (45). We also see Cdc42 activation upon collagen treatment and direct β1 integrin stimulation (data not shown). It is still unclear how Cdc42 becomes activated downstream of integrin activation. One way this can occur is through a recently identified exchange factor PIX. αPIX can be recruited to integrin complexes by associating with β-parvin-ILK complex and lead to activation of both Cdc42 and Rac at sites of integrin clustering (46, 47). Thus, Cdc42 is a key component of integrin signaling pathways leading to cell migration.

A substantial body of evidence suggests a crucial role for p130Cas in the process of cell migration (12–14, 16, 17). We found that migration induced by Cdc42 activation also relied on p130Cas, because this migration could be inhibited by p130Cas siRNA (Fig. 1). We think that the requirement for p130Cas downstream of Cdc42 goes beyond a simple obligate need for this molecule during cell migration, because Cdc42 and p130Cas are also required for Ack1 activation (17, 21). This is a similar situation to focal adhesion kinase (FAK), whose kinase activity is activated by Src, making it difficult to discern whether it contributes to the phosphorylation of other proteins directly or by scaffolding with Src.

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