Activation of the Cdc42-associated Tyrosine Kinase-2 (ACK-2) by Cell Adhesion via Integrin β1*

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Activated Cdc42-associated kinase-2 (ACK-2) is a non-receptor tyrosine kinase that appears to be a highly specific target for the Rho-related GTP-binding protein Cdc42. In order to understand better how ACK-2 activity is regulated in cells, we have expressed epitope-tagged forms of this tyrosine kinase in COS-7 and NIH3T3 cells. We find that ACK-2 can be activated by cell adhesion in a Cdc42-dependent manner. However, unlike the focal adhesion kinase, which also is activated by cell adhesion, the activation of ACK-2 is F-actin-independent and does not require cell spreading. In addition, overexpression of ACK-2 in COS-7 cells did not result in the stimulation of extracellular signal-regulated kinase activity but rather activated the c-Jun kinase. Both anti-integrin β1 antibody and RGD peptides inhibited the activation of ACK-2 by cell adhesion. In addition, ACK-2 was co-immunoprecipitated with integrin β1. Overall, these findings suggest that ACK-2 interacts with integrin complexes and mediates cell adhesion signals in a Cdc42-dependent manner.

The ACKs are members of a family of non-receptor tyrosine kinases that specifically interact with Cdc42 (14, 15). Here we demonstrate that ACK-2 is activated by cell adhesion on a substratum in a Cdc42-dependent manner. The activation does not require cell spreading. The RGD peptide and an anti-integrin β1 antibody inhibit the activation of ACK-2 by cell adhesion, and ACK-2 was co-immunoprecipitated with integrin β1, indicating a role for integrins in the regulation of this Cdc42 target.

**EXPERIMENTAL PROCEDURES**

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‡ The abbreviations used are: JNK, c-Jun kinase; ACK, activated Cdc42-associated tyrosine kinase; FAK, focal adhesion kinase; Erk, extracellular signal-regulated kinase; PAK, p21-activated kinase; SH3, Src homology 3; DMEM, Dulbecco's modified Eagle's medium; HA, hemagglutinin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis.

Materials—Fibronectin, RGD peptides, anti-integrin β1 monoclonal antibody, and anti-α,β, polyclonal antibody were purchased from Life Technologies, Inc. Polysine, trypsin inhibitor, cycloheximide, and latex beads (6 μm) were purchased from Sigma. Anti-flag antibody (M5) was purchased from Eastman Kodak Co. Anti-PAK antibody was prepared and used as described previously (40). Anti-phosphotyrosine (4G10) was purchased from Upstate Biotechnology Inc., and horseradish peroxidase-conjugated anti-phosphotyrosine (PY20) was purchased from Oncogene; anti-Erk was obtained from Santa Cruz Biotechnology, and cytochalasin D was from Calbiochem.
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Cell Culture and Transfection—COS-7 cells were grown in DMEM plus 10% fetal bovine serum at 37 °C, 5% CO₂. NIH3T3 cells were grown in DMEM plus 10% calf serum at 37 °C, 5% CO₂. The cells were split at 3 × 10⁶/60-mm dish within 24 h before transfection. DNA transfections were performed using LipofectAMINE according to the manufacturer's standard protocols (Life Technologies, Inc.). For transient transfections in COS-7 cells, the cDNAs for ACK-2 and Cdc42 were expressed using the pcDNA3 vector. For stable transfections, the expression vector was pLTR. To select stable cell lines for ACK-2, the pLTRHA-ACK-2 (HA-tagged) was co-transfected with a plasmid carrying the neomycin-resistant gene into NIH3T3 cells. G418 (500 µg/ml)-resistant cell colonies were selected. The expression of HA-tagged ACK-2 in each colony was determined by immunoblotting the cell lysates with anti-HA antibody.

Test of Inducible Cell Lines—Inducible EcR/DV digested Myc-tagged ACK-2 cDNA from pcDNA3 Myc-ACK-2 was cloned into the pTet-splice vector (Life Technologies, Inc.) to obtain pTet Myc-ACK-2. We then co-transfected ptPTK (3 µg/60-mm dish) and pTet-splice (vector alone) or pTet Myc-ACK-2 (3 µg/60-mm dish) with a puromycin-resistant gene plasmid (0.3 µg/60-mm dish) into NIH3T3 cells (3 × 10⁶/60-mm dish) in the presence of tetracycline (1 µg/ml). After 48 h, the cells were transfected to a 100-mm dish and cultured overnight in DMEM plus 10% calf serum and 1 µg/ml tetracycline. Colony selection was performed by adding puromycin (1 µg/ml) to the culture medium. Positive colonies were determined by immunoblotting with anti-Myc antibody.

Immunoprecipitation—Confluent cells in 60-mm dishes were lysed in 500 µl of lysis buffer (40 mM Heps, pH 7.4, 100 mM NaCl, 1% Triton X-100, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 µg/ml leupeptin, and 10 µg/ml aprotinin) or RIPA buffer (40 mM Heps, pH 7.4, 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 25 mM β-glycerophosphate, 1 mM EDTA, 1 mM sodium orthovanadate, 10 µg/ml leupeptin, and 10 µg/ml aprotinin) with rocking for 15–30 min at 4 °C. The lysates were cleared by centrifugation at 14,000 rpm for 2 min. Aliquots of the lysates (200–500 µl) were used for immunoprecipitation. After the primary antibody was incubated with lysates on ice for 30 min, protein A or protein G beads (Sigma) were added, and the mixture was rocked at 4 °C for 1 h. The beads were washed twice with 700 µl of lysis buffer and finally resuspended in 20 µl of 2× SDS-PAGE sample buffer. The immunoprecipitated proteins were separated by SDS-PAGE.

Coating Plates or Latex Beads with Polylysine or Fibronectin and Cell Adhesion—Polylysine (10 µg/ml) or fibronectin (10 µg/ml) in PBS was added to plates (2 ml/35-mm plate) and incubated at 4 °C overnight. The plates were subsequently washed (3 times) with PBS (2 mg/ml) in PBS and then incubated at 37 °C with 2 ml of BSA (2 mg/ml) in PBS for 1.5–2 h. The plates were then washed (3 times) with PBS and ready for use. For BSA control plates, treatment procedures were the same as described above. Cells remained in suspension and did not attach to BSA-coated plates. The coating of latex beads was performed essentially as previously (41). Briefly, 20 µl of the latex beads were incubated with either polylysine (50 µg/ml) or fibronectin (50 µg/ml) in PBS overnight at 4 °C with rotation. After washing with PBS (3 times), the beads were incubated with BSA (2 mg/ml) at 37 °C for 2 h, washed with PBS (2 times), resuspended in 200 µl of DMEM, and then ready for use. For cell adhesion, the cells were trypsinized, resuspended with trypsin inhibitor solution, and washed twice with serum-free DMEM medium. The cells were then resuspended in DMEM. In some cases, the cells were preincubated with Cdc42 or ACK-2-activating agents. For experiments requiring ACK-2 activation by cell adhesion, the cells were plated onto polylysine or fibronectin, the adherence of cells to either polylysine- or fibronectin-coated beads strongly activates ACK-2.

Cell Adhesion Stimulates Tyrosine Phosphorylation of ACK-2 That Is Independent of Cell Spreading—Our previous studies have shown that cell attachment activated ACK-2, whereas cell detachment resulted in its dephosphorylation (15). To investigate the relationship between cell adhesion and the activation of ACK-2 further, we precoated plates with either polylysine or fibronectin and then added cells expressing ACK-2 onto the plates. When the cells were plated onto polylysine or fibronectin for either 30 or 60 min at 37 °C, the tyrosine phosphorylation of ACK-2 was markedly increased (Fig. 1A). On polylysine-coated plates, the cells were firmly attached within 5 min and maintained a round shape for at least 30 min before they began to flatten and spread (not shown). This suggests that the activation of ACK-2 by cell adhesion does not require cell spreading or focal adhesion complex assembly.

To confirm further that activation of ACK-2 by cell adhesion does not require cell spreading, we treated the cells with cytochalasin D, a reagent that disrupts F-actin. This caused the cells to round-up and lose their ability to spread. However, cytochalasin D did not affect cell attachment onto either polylysine- or fibronectin-coated plates, suggesting that such treatment did not disrupt the interaction of integrins with fibronectin. As shown in Fig. 1B, treatment with cytochalasin D also did not affect the activation of ACK-2 upon the attachment of cells to fibronectin-coated plates. Thus, ACK-2 activation by cell adhesion is only correlated with cell attachment and not with cell spreading. In addition, the activation of ACK-2 by attachment onto polylysine-coated plates is not due to the synthesis of extracellular matrix proteins, because pretreatment of cells with cycloheximide, an inhibitor of protein synthesis, did not block ACK-2 activity (Fig. 1C).

In order to examine the effects of cell adhesion on the activation of ACK-2 further, we performed cell adhesion experiments with extracellular matrix molecule-coated latex beads (diameter 6 µm) as described by Miyamoto et al. (41). The data presented in Fig. 1D show that like the case when cells are plated onto polylysine or fibronectin, the adherence of cells to either polylysine- or fibronectin-coated beads strongly activates ACK-2.

**The Activation of ACK-2 Tyrosine Phosphorylation by Cell Adhesion Is Cdc42-dependent**—To determine whether the activation of ACK-2 by cell adhesion requires Cdc42, we co-transfected ACK-2 with either the wild type, constitutively active, or dominant negative forms of Cdc42 in COS-7 cells. After 48 h, the cells were plated onto BSA- or fibronectin-coated plates, and the tyrosine phosphorylation of ACK-2 was detected by immunoblotting with anti-phosphotyrosine antibody. When vector (pcDNA3) was co-transfected with ACK-2, the tyrosine phosphorylation of ACK-2 was enhanced by cell adhesion onto fibronectin-coated plates, compared with the phosphorylation detected in suspended cells (i.e. BSA-coated plates) (Fig. 2, lanes 3 and 4). When ACK-2 was co-transfected with the GTPase-defective Cdc42(Q61L) mutant, the tyrosine phosphorylation of ACK-2 showed a slight enhancement relative to the case when cells are plated onto polylysine or fibronectin (Fig. 2, lanes 5 and 6). Even in suspended cells, the tyrosine phosphorylation of ACK-2 was enhanced upon the expression of Cdc42(Q61L) (compare lanes 3 and 5). When Cdc42(T17N), a dominant negative mutant, was co-transfected with ACK-2, the tyrosine phosphorylation of ACK-2 was strongly inhibited (Fig. 2, lanes 7 and 8). These data indicate that the stimulation of ACK-2 activity upon cell adhesion was dependent on Cdc42.

**The Activation of ACK-2 by Cell Adhesion Is Distinct from the Activation of FAK**—It is well known that FAK, a non-receptor tyrosine kinase, is specifically activated upon cell adhesion to
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Fig. 1. ACK-2 is activated by cell adhesion. A, COS-7 cells were transfected with either pcDNA3 (vector) or pcDNA3 HA-ACK-2 (4 μg/60-mm plate) for 48 h and serum-starved overnight. The cells were trypsinized at 37°C for 10 min, washed in PBS plus trypsin-inhibitor (20 μg/ml) once, and then with PBS, DMEM, and finally resuspended in DMEM. The cell aliquots then were incubated either in an Eppendorf tube (lanes 3) or replated onto fibronectin-(lanes 2, 5, and 7) or polylysine (lanes 1, 4, and 6)-coated plates at 37°C in 5% CO₂ for the indicated times. Both the resuspended and adhered cells were collected and lysed. The HA-tagged ACK-2 was immunoblotted with either anti-phosphotyrosine antibody (PY20) (top panel) or anti-HA antibody (12C5) (bottom panel). B, the experimental procedure was basically the same as above except the control for cell adhesion was BSA-blocked plates. Cytochalasin D (2.5 μg/ml) or ethanol (solvent for cytochalasin D) was added during cell adhesion. Top panel, blotted with anti-phosphotyrosine (PY; bottom panel, blotted with anti-HA. CytoD, cytochalasin D; PL, polylysine; FN, fibronectin. C, the experimental procedure was basically the same as above except the cells were transfected with pcDNA3 Myc-tagged ACK-2 instead of pcDNA3 HA-tagged ACK-2 and pretreated with cycloheximide (20 μg/ml) at 37°C for 30 min before plating, as well as during plating. The cells were allowed to plate onto precoated plates at 37°C for 30 min. CH, cycloheximide. D, the Tet-off-inducible Myc-tagged ACK-2 cell line was used in this experiment. NIH3T3 cells that were stably transfected with pTet Myc-ACK-2/ptTAK were cultured in DMEM plus 10% calf serum and 1 μg/ml tetracycline (non-induced condition) to 90% confluence and subsequently cultured in DMEM without serum and tetracycline (induced condition) for 20 h. The cells were trypsinized and collected as described above and treated with 20 μg/ml cycloheximide at 37°C for 30 min before incubation with latex beads. Cells (~10⁶) were mixed with precoated latex beads (~2 × 10⁷) in 400 μl of DMEM at 37°C for 30 min with gentle shaking. The cells were lysed with RIPA buffer. Tyrosine phosphorylation of ACK-2 and the amount of Myc-tagged ACK-2 were detected from the cell lysates. Not detached refers to cells that were directly lysed from culture plates. PL, polylysine; FN, fibronectin. Lane 9 represents a vector control (cells were not detached).

ACK-2 and FAK were dephosphorylated (Fig. 3, lane 2). When replated onto polylysine or fibronectin-coated plates for 5 min, the cells attached to polylysine-coated plates but not to the fibronectin-coated plates. Under these conditions, the tyrosine phosphorylation of ACK-2 was stimulated upon plating on polylysine (Fig. 3, bottom panel, lane 3). Neither ACK-2 nor FAK was activated on fibronectin-coated plates because there was no cell attachment at this time point (Fig. 3, lane 4). After replating for 20 min, 90% of the cells adhered to both polylysine- and fibronectin-coated plates. However, only cells attached to fibronectin were able to spread. The cells on polylysine remained round even after having been replated for 60 min (data not shown). ACK-2 was activated on both polylysine- and fibronectin-coated plates (42–44). Thus, we compared the cell adhesion-dependent activation of ACK-2 with that of FAK. As expected, when cells were directly lysed from culture plates (not detached), both ACK-2 and FAK were highly autophosphorylated (Fig. 3, lane 1), whereas when cells were detached and resuspended in BSA-blocked plates for up to 60 min, both ACK-2 and FAK were dephosphorylated (Fig. 3, lane 2). When replated onto polylysine or fibronectin-coated plates for 5 min, the cells attached to polylysine-coated plates but not to the fibronectin-coated plates. Under these conditions, the tyrosine phosphorylation of ACK-2 was stimulated upon plating on polylysine (Fig. 3, bottom panel, lane 3). Neither ACK-2 nor FAK was activated on fibronectin-coated plates because there was no cell attachment at this time point (Fig. 3, lane 4). After replating for 20 min, 90% of the cells adhered to both polylysine- and fibronectin-coated plates. However, only cells attached to fibronectin were able to spread. The cells on polylysine remained round even after having been replated for 60 min (data not shown). ACK-2 was activated on both polylysine-
and fibronectin-coated plates after replating for 20 min (Fig. 3, lanes 5–8, bottom panel), whereas FAK was only activated on fibronectin-coated plates (Fig. 3, lanes 6 and 8). However, when the cells were treated with cytochalasin D during their replating onto fibronectin-coated plates, the cells were not able to spread but rather adhered to the plates with a rounded morphology, and the autophosphorylation of FAK was significantly decreased (data not shown). Thus, whereas FAK activation upon cell adhesion requires cell spreading or actin-cytoskeletal organization, ACK-2 activation appears to only require cell attachment to a substratum.

Previous studies have shown that FAK activation results in a stimulation of Erk activity (45). Therefore, we examined whether the overexpression of ACK-2 could also stimulate Erk activity, by using an anti-Erk antibody and determining whether an activation-dependent change in the electrophoretic mobility of the Erks occurred. As shown in Fig. 4A, consistent with previous studies, adhesion of cells on fibronectin resulted in the stimulation of Erk activity (compare lanes 2, 5, and 7 with lane 3). Adhesion of cells on polylysine also stimulated Erk activity (compare lanes 1 and 4 with lane 3) but to a lesser extent compared with fibronectin. However, we did not observe a significant effect on Erk activity upon expression of ACK-2 (compare lanes 1 and 2 with lanes 6 and 7). This suggests that ACK-2 does not input into the Ras/Raf/Erk pathway.

To determine whether ACK-2 influences the activity of the stress-responsive mitogen-activated protein kinase, the c-Jun kinase (JNK1), we co-transfected the cDNAs encoding ACK-2 and flag-tagged JNK into COS-7 cells and assayed JNK activity after immunoprecipitation with an anti-flag antibody. As shown in Fig. 4B, the expression of ACK-2 was accompanied by a significant activation of JNK activity. In order to determine whether Cdc42 was required for this activation event, we co-transfected the cDNA encoding a dominant-negative mutant of Cdc42 (Cdc42(T17N)) with the cDNAs encoding ACK-2 and flag-tagged JNK. As shown in Fig. 4C, the expression of dominant-negative Cdc42(T17N) inhibited the activation of JNK by ACK-2.

Activation of ACK-2 by Cell Adhesion Is Mediated by Integrin β1—We next examined the molecular basis by which cell adhesion activates ACK-2. The fact that the adhesion of cells onto fibronectin activates ACK-2 suggested that integrins may be involved in the activation process. To examine this possibility, we determined whether treatment with an antibody against integrin β1 or RGD peptides, which block the interaction of fibronectin with integrins, affected the tyrosine phosphorylation of ACK-2 when plating the cells on either polylysine or fibronectin (Fig. 5). When cells were treated with the anti-integrin β1 antibody or the RGD peptides, about 80–90% of the cells were no longer able to adhere onto fibronectin-coated plates, indicating that the ability of the cells to adhere to fibronectin was integrin β1-dependent (data not shown). However, treatment with either the anti-integrin β1 antibody or the RGD peptides did not significantly affect attachment of the cells onto polylysine-coated plates (data not shown), suggesting that the cell adhesion to polylysine can occur via integrin-independent events.

As shown in Fig. 5A, the anti-integrin β1 antibody completely reversed fibronectin-stimulated tyrosine phosphorylation of ACK-2, and at least partially inhibited the polylysine-stimulated tyrosine phosphorylation (Fig. 5B). A similar set of experiments were performed using the RGD peptides (Fig. 5, C and D). We used GRGESP as a control peptide (labeled ESP in the figures) and GRGDNP as an inhibitory peptide (labeled DNP). We found that the control peptide, GRGESP, affected neither cell adhesion nor the tyrosine phosphorylation of ACK-2 upon cell adhesion (Fig. 5, C and D), whereas GRGDNP inhibited the activation of ACK-2 by cell adhesion onto either fibronectin- or polylysine-coated plates (Fig. 5C, 4th and 5th experiments were performed using the RGD peptides (Fig. 5, C and D). We used GRGESP as a control peptide (labeled ESP in the figures) and GRGDNP as an inhibitory peptide (labeled DNP). We found that the control peptide, GRGESP, affected neither cell adhesion nor the tyrosine phosphorylation of ACK-2 upon cell adhesion (Fig. 5, C and D), whereas GRGDNP inhibited the activation of ACK-2 by cell adhesion onto either fibronectin- or polylysine-coated plates (Fig. 5C, 4th and 5th
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ACK-2 Is Constitutively Associated with the Integrin β1 Complex and the Association Is Independent of ACK-2 Tyrosine Kinase Activity—The implication that integrin β1 mediates the activation of ACK-2 then raises the question of whether ACK-2 directly associates with the integrin complex. To address this question, we transfected the cDNA encoding Myc-tagged ACK-2 or a kinase-defective mutant of ACK-2 (ACK-2(K158R)) into COS-7 cells and then replated the cells onto BSA- or fibronectin-coated plates. We then immunoprecipitated endogenous integrin β1, with an anti-integrin β1 antibody and Western-blotted the immunoprecipitated complex with an anti-Myc antibody to detect integrin-associated Myc-tagged ACK-2. Unexpectedly, we found that a similar amount of Myc-tagged ACK-2 was co-immunoprecipitated with integrin β1 (Fig. 6A, right panel, 1st and 2nd lanes) from cells in suspension (BSA-coated plates) and when cells are attached to fibronectin, suggesting that ACK-2 was constitutively associated with the integrin β1. Control experiments with non-immune IgG or using anti-FAK antibody did not immunoprecipitate ACK-2 (data not shown). The association of ACK-2 with the integrin β1 complex was totally independent of its kinase activity or tyrosine phosphorylation (Fig. 6A, left panel, all lanes, and right panel, 3rd and 4th lanes).

These findings raise the question of how ACK-2 transduces signals upon cell adherence to a substrate if it is constitutively associated with integrin β1. The data presented in Fig. 6B begin to point toward a possible explanation. In these experiments, a GST fusion protein encoding the SH3 and CRIB domains of ACK-2 was immobilized on glutathione beads and incubated with lysates from COS-7 cells expressing Myc-tagged full-length ACK-2 or Myc-tagged ACK-2 and HA-tagged Cdc42. These cells had been replated onto BSA- or fibronectin-coated plates. As shown in Fig. 6B (left panel, 1st and 2nd lanes), the GST-SH3/CRIB domain construct bound more effectively to full-length ACK-2 in lysates from suspended cells (BSA) compared with lysates from adherent cells (fibronectin). However, the co-transfection of Cdc42 with ACK-2 totally blocked the binding of the GST-SH3/CRIB domain construct to full-length ACK-2 (Fig. 6B, left panel, 3rd and 4th lanes). The right panel of Fig. 6B shows that equal amounts of ACK-2 and Cdc42 were expressed in each lysate sample. These data suggest that the interaction between the SH3 domain and a proline-rich sequence of ACK-2 is tightly regulated by Cdc42. One possibility is that in the basal state, ACK-2 undergoes an intramolecular interaction between its SH3 domain and a proline-rich sequence which prevents the binding of cellular targets and/or phospho-substrates. However, upon the addition of an excess of the GST-SH3/CRIB domain, one of the proline-rich sequences of ACK-2 may be able to undergo an intermolecular interaction with the GST-SH3/CRIB domain fusion protein, due to an equilibrium between a “closed” state where full-length ACK-2 is engaged in an intramolecular interaction and an “open” state where the proline-rich sequences are accessible to intermolecular interactions. The binding of activated Cdc42 to the CRIB motif, which lies between the SH3 and proline-rich sequences of ACK-2, may then prevent the intramolecular interaction between these domains and thereby allow the binding of cellular target proteins. This in turn would reduce the amount of cellular ACK-2 that is available to bind the GST-SH3/CRIB domain construct. We would further propose that upon cell adhesion and the formation of integrin clusters (Cdc42-activated), ACK-2 molecules are brought into sufficient proximity to one another to undergo trans-phosphorylation, thus accounting for the marked increase in the tyrosine phosphorylation of ACK-2 that occurs under these conditions.

DISCUSSION

It is well known that Rho-related small GTP-binding proteins regulate cytoskeletal organization and cell morphology. Given that cell adhesion induces marked changes in the actin...
cytoskeleton, it seems likely that the Rho-related proteins will also play roles in bridging adhesion-dependent signaling with effects on the cytoskeletal architecture. Along these lines, Cdc42 and Rac have recently been shown to mediate integrin $\beta_1$ signaling in cell migration (39), suggesting that cell adhesion or integrins induce the activation of Cdc42 and Rac (see also Ref. 46). Tiam-1, a guanine-nucleotide exchange factor for Rac, is involved in cell invasion (47), and Rac has been shown to participate in cadherin signaling in epithelial cells and to inhibit Ras-induced cell invasion (48). An obviously important question will be to identify the target molecules for Cdc42 and Rac that mediate the effects of cell adhesion. Based on our initial studies with the non-receptor tyrosine kinase ACK-2 (15), we felt that it was an attractive candidate for such a role. Specifically, we had earlier shown that ACK-2 was activated upon cell adhesion (15). Here we show that ACK-2 can be activated by cell adhesion via the $\beta_1$ integrin in a Cdc42-dependent manner and that ACK-2 appears to associate with an integrin complex.

It is interesting to note that the activation of ACK-2 by cell adhesion clearly differs from that of FAK. Activation of ACK-2 by cell adhesion does not require cell spreading nor an intact F-actin structure, whereas the activation of FAK requires both.

There are two possible explanations for this difference. 1) ACK-2 and FAK participate in distinct integrin signaling pathways, or 2) ACK-2 is upstream from FAK during cell adhesion signaling. However, the latter possibility seems unlikely given that we have not observed that overexpression of ACK-2 enhances FAK tyrosine phosphorylation.

A particularly interesting distinction between ACK-2 and FAK concerns the ability of polylysine to activate ACK-2. Based on the inhibitory effects of anti-integrin $\beta_1$ and GRD peptides on polylysine-induced activation of ACK-2, at least part of the activation of ACK-2 by cell adhesion onto polylysine-coated plates appears to be mediated through $\beta_1$ integrin. However, when cells adhere onto polylysine-coated plates, they can only attach to the plates but are not able to spread, indicating that cells plated on polylysine can not form stress fibers (45). The inability of the cells to spread when plated on polylysine probably explains why FAK is not activated under these conditions. It has been reported that cells adhered onto polylysine form filopodia, suggesting that Cdc42 may be activated upon cell adhesion on polylysine (49) and thus providing a link to ACK-2 activation. We have also observed that some protrusions appear from the bottom of cells that are attached to polylysine-coated plates (data not shown). These protrusions have been
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described as point contacts that are distinct from focal contacts (49, 50). It has been further proposed that in fibroblasts, αβ₁ and α5β₁ integrin heterodimers first accumulate in point contacts followed by their redistribution into focal contacts (in astrocytes, the accumulation of αβ₁ heterodimers in point contacts was shown to occur when cells were plated on either polylysine, fibronectin, or laminin (49)). Thus, cell attachment on either polylysine or fibronectin, leading to an accumulation of integrins in point contacts, may represent an early signal for the activation of Cdc42 and then ACK-2. Met, a receptor tyrosine kinase that is a proto-oncogene and involved in cell invasion and tumor cell metastasis, shows a similar activation behavior as ACK-2 upon cell adhesion, i.e., it is activated upon plating cells on polylysine (51). However, thus far we have not found any signaling connection between ACK-2 and Met.

Overall, the findings reported here now provide a possible molecular basis for the signaling connections between integrin-cell adhesion and Cdc42. The mechanisms underlying the apparent activation of Cdc42 by cell adhesion, which lead to the recruitment and/or activation of ACK-2, remain to be delineated. However, it appears that upon activation, Cdc42 may reverse an intramolecular interaction within ACK-2 which then makes the kinase accessible to interact with other binding partners or possibly substrates. At present, we know relatively little about the downstream signaling pathways that are engaged following ACK-2 activation, although the Raf-Mek-Erk pathway does not appear to be involved. Although we have found that overexpression of ACK-2 stimulates JNK activity, it is difficult to assess the importance of this activation in vivo given that a number of tyrosine kinases including Src, Pyk2, Abl, and Btk have also been shown to stimulate JNK activity, it is activated upon

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