THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 13, Issue of March 26, pp. 8524–8530, 1999

Printed in U.S.A.

Activation of the Cdc42-associated Tyrosine Kinase-2 (ACK-2) by Cell Adhesion via Integrin β_1*

(Received for publication, July 10, 1998, and in revised form, December 3, 1998)

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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 β_1 antibody and RGD peptides inhibited the activation of ACK-2 by cell adhesion. In addition, ACK-2 was co-immunoprecipitated with integrin β_1. Overall, these findings suggest that ACK-2 interacts with integrin complexes and mediates cell adhesion signals in a Cdc42-dependent manner.

Rho family small GTP-binding proteins have been implicated in a diversity of biological activities including cell morphology, cytoskeletal organization, cell adhesion, and gene transcription (1–7). We have focused on one member of the Rho family, Cdc42, which has been shown to induce cell filopodia formation, activate the c-Jun kinase (JNK1),[1] and cause cell anchorage-independent growth (2, 5, 6, 8–11). A number of downstream targets of Cdc42 have been characterized and can be classified into two groups. One class represents the CRIB (for Cdc42/Rac-interactive binding) motif-containing proteins that includes the PAKs (1), ACKs, Wiscott-Aldrich syndrome proteins, and mixed lineage kinases (12–19). The second class of targets lack CRIB motifs and include the IQGAPs, p70 S6 kinase, and the 85-kDa regulatory subunits (p85) of phosphatidylinositol 3-kinase (20–25). Functional analyses of these targets indicate that some are involved in cytoskeletal organization, such as the Wiscott-Aldrich syndrome proteins, IQGAPs and PAKs, whereas others regulate JNK activation or mitogenesis, including the PAKs, mixed lineage kinase-3, p70 S6 kinase, and phosphatidylinositol 3-kinase. The specific cellular activities of Cdc42 that are mediated by these targets have been variable and sometimes contradictory. Thus, activation of Cdc42 has been suggested both to stimulate (17, 26–28) and inhibit (29, 30) F-actin polymerization, as well as to enhance (24, 25) and inhibit (31) cell growth. The complexity of the effects mediated by Cdc42 may reflect a subtle balance or coordination between cytoskeletal organization and mitogenesis that is necessary for cells to process signals that regulate both cell morphology and cell growth.

It is known that cell adhesion in adherent cells directly regulates both cytoskeletal organization and cell mitogenesis (32–34). Anchorage-independent growth becomes a criterion for cell transformation, indicating that cell adhesion has a key function for normal cell growth. In fact, overexpression of integrin molecules or their associated proteins prevents abnormal cell growth and recovers anchorage-dependent growth (35, 36), suggesting that integrin signaling mediates a balance between cytoskeletal organization and mitogenic progression. However, the connection between these two cellular events is not clear. Previous studies indicate that Rho proteins play important roles in cell adhesion (1, 2, 34, 37, 38). Rho has been shown to regulate focal contact complex assembly and actin stress fiber formation (38). Overexpression of a GTPase-defective mutant, Cdc42(G12V), or a constitutively active mutant, Cdc42(F28L), resulted in anchorage-independent growth (10, 11), suggesting that Cdc42 may mediate cell adhesion signals that regulate both cytoskeletal organization and mitogenesis. Recent studies have shown that Cdc42 mediates integrin β_1 signaling and promotes cell migration (39). The Cdc42 targets that mediate these effects are not known, although a number of Cdc42 targets have been reported to regulate F-actin polymerization and depolymerization (17, 26–30).

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 β_1 antibody inhibit the activation of ACK-2 by cell adhesion, and ACK-2 was co-immunoprecipitated with integrin β_1, indicating a role for integrins in the regulation of this Cdc42 target.

EXPERIMENTAL PROCEDURES

Materials—Fibronectin, RGD peptides, anti-integrin β_1 monoclonal antibody, and anti-$\alpha_5\beta_1$ polyclonal antibody were purchased from Life Technologies, Inc. Polylysine, trypsin inhibitor, cycloheximide, and latex beads (6 μM) were purchased from Sigma. Anti-flag antibody (M5) was purchased from Eastman Kodak Co. Anti-FAK 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.

* This work was supported by National Institutes of Health Grants GM40654, GM47458 (to R. A. C.), and GM52890 (to J. G.) and by the Human Frontiers Science Program (to R. A. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] 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.

Cell Culture and Transfection—COS-7 cells were grown in DMEM plus 10% fetal bovine serum at 37 °C, 5% CO_2. NIH3T3 cells were grown in DMEM plus 10% calf serum at 37 °C, 5% CO_2. The cells were split at 3×10^5/60-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.

Tet-off Inducible Cell Lines—*Hin*dIII/*Eco*RV 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 ptTAK (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 (3×10^5/60-mm dish) in the presence of tetracycline (1 μg/ml). After 48 h, the cells were transferred 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 (5 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 Hepes, 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 Hepes, pH 7.4, 100 mM HCl, 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 BSA (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 described 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 were 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 DMEM plus cycloheximide or anti-integrin antibodies or RGD peptides at 37 °C for 30 min. The cells were finally added onto substratum precoated culture dishes or mixed with latex beads and incubated at 37 °C for the indicated time and directly lysed with lysis buffer.

JNK Assays—The flag-tagged JNK1 was immunoprecipitated with anti-flag antibody (M5) from the lysates of cells that were transiently transfected with pcDNA3 flag-JNK1 or pcDNA3 flag-JNK1 plus pcDNA3 Myc-ACK-2 and/or pcDNA3 HA-Cdc42(T17N). The immunocomplex beads were washed twice with lysis buffer and once with JNK assay buffer (20 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 1 mM sodium orthovanadate) and then were mixed with the kinase assay buffer and GST-Jun (5 μg). The phosphorylation was initiated by adding 5 μCi of [γ-^{32}P]ATP (17 mCi/nmol) and performed at 22 °C for 20 min. The reaction was stopped by adding 2× SDS sample buffer, and the samples were boiled for 7 min and loaded onto a 10% SDS-polyacrylamide gel. The gel was transferred onto a polyvinylidene difluoride membrane that was used for autoradiography and immunoblotting.

RESULTS

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. 1*A*). 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. 1*B*, 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 new synthesis of extracellular matrix proteins, because pretreatment of cells with cycloheximide, an inhibitor of protein synthesis, did not block ACK-2 activity (Fig. 1*C*).

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. 1*D* 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 control cells plated on fibronectin (Fig. 2, compare *lanes 4* 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 adherence to

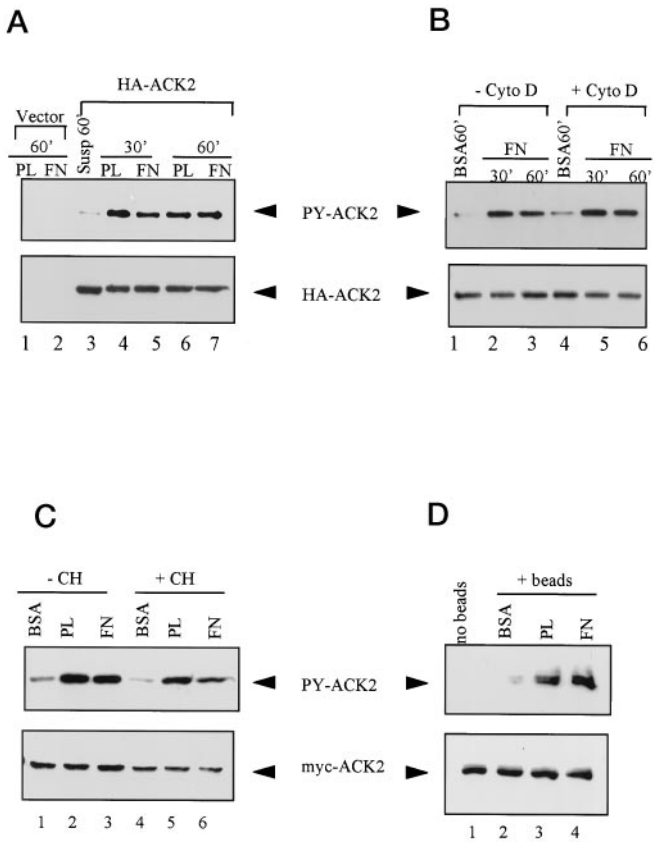

FIG. 1. **ACK-2 is activated by cell adhesion.** *A*, COS-7 cells were transfected with either pcDNA3 (vector) or pcDNA3 HA-ACK-2 (4 μg/60-mm plate) for 48 h and serum-starved overnight. The cells were trypsinized at 37 °C for 10 min, washed in PBS plus trypsin-inhibitor (20 μg/ml) once, and then with PBS, DMEM, and finally resuspended in DMEM. The cell aliquots then were incubated either in an Eppendorf tube (*lane 3*) or replated onto fibronectin- (*lanes 2, 5,* and *7*) or polylysine (*lanes 1, 4,* and *6*)-coated plates at 37 °C in 5% CO_2 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/pTAK 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 ($\sim 10^6$) were mixed with precoated latex beads ($\sim 2 \times 10^8$) 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 with horseradish peroxidase-conjugated anti-phosphotyrosine antibody (*PY20*, Oncogene) (*top panel*) and anti-Myc antibody (*bottom panel*), respectively.

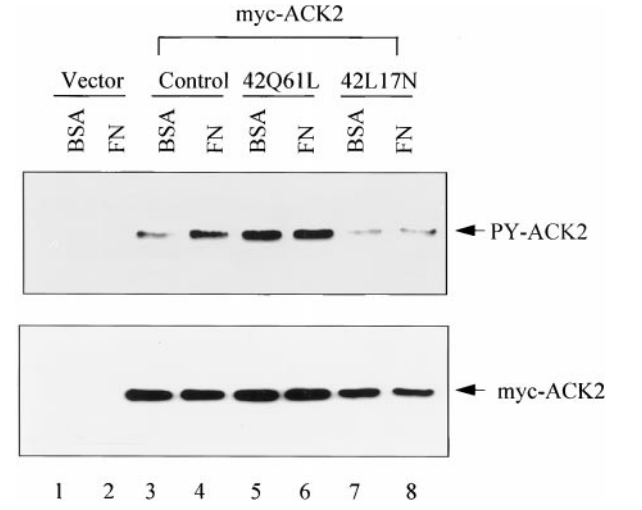

FIG. 2. **Activation of ACK-2 by cell adhesion is Cdc42-dependent.** The experimental procedures were the same as in Fig. 1. ACK-2 was Myc-tagged, and the amounts of either pcDNA3 encoding Myc-ACK-2, pcDNA3 encoding HA-Cdc42(T17N), or pcDNA3 encoding HA-Cdc42(Q61L) for transfection were 2 μg per 60-mm plate. *Top panel,* anti-phosphotyrosine (PY20); *bottom panel,* anti-Myc antibody. *FN*, fibronectin.

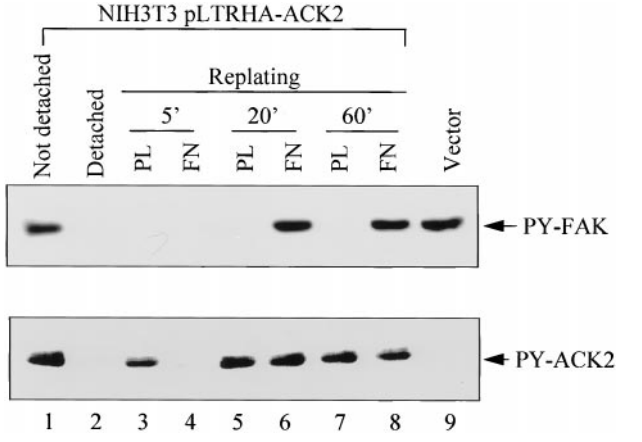

FIG. 3. **Activation of ACK-2 by cell adhesion is distinct from FAK activation.** ACK-2 was activated by cell adhesion on both polylysine and fibronectin, and FAK was activated by cell adhesion on fibronectin. NIH3T3 cells were stably transfected with pLTRHA-ACK-2 and cultured in DMEM + 10% calf serum + G418 (500 μg/ml). The cell adhesion experiments were performed as described under "Experimental Procedures." FAK was immunoprecipitated from cell lysates and blotted with anti-phosphotyrosine (*PY*) antibody. The phosphorylation of ACK-2 was detected from the cell lysates. *Top panel*, tyrosine phosphorylation of FAK upon cell adhesion on either polylysine and fibronectin; *bottom panel*, tyrosine phosphorylation of ACK-2 upon cell adhesion on either polylysine and fibronectin. *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).

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. 4*A*, 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, 4* and *6* 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. 4*B*, 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. 4*C*, 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 β_1—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 β_1 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 β_1 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 β_1-dependent (data not shown). However, treatment with either the anti-integrin β_1 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. 5*A*, the anti-integrin β_1 antibody completely reversed fibronectin-stimulated tyrosine phosphorylation of ACK-2, and at least partially inhibited the polylysine-stimulated tyrosine phosphorylation (Fig. 5*B*). 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

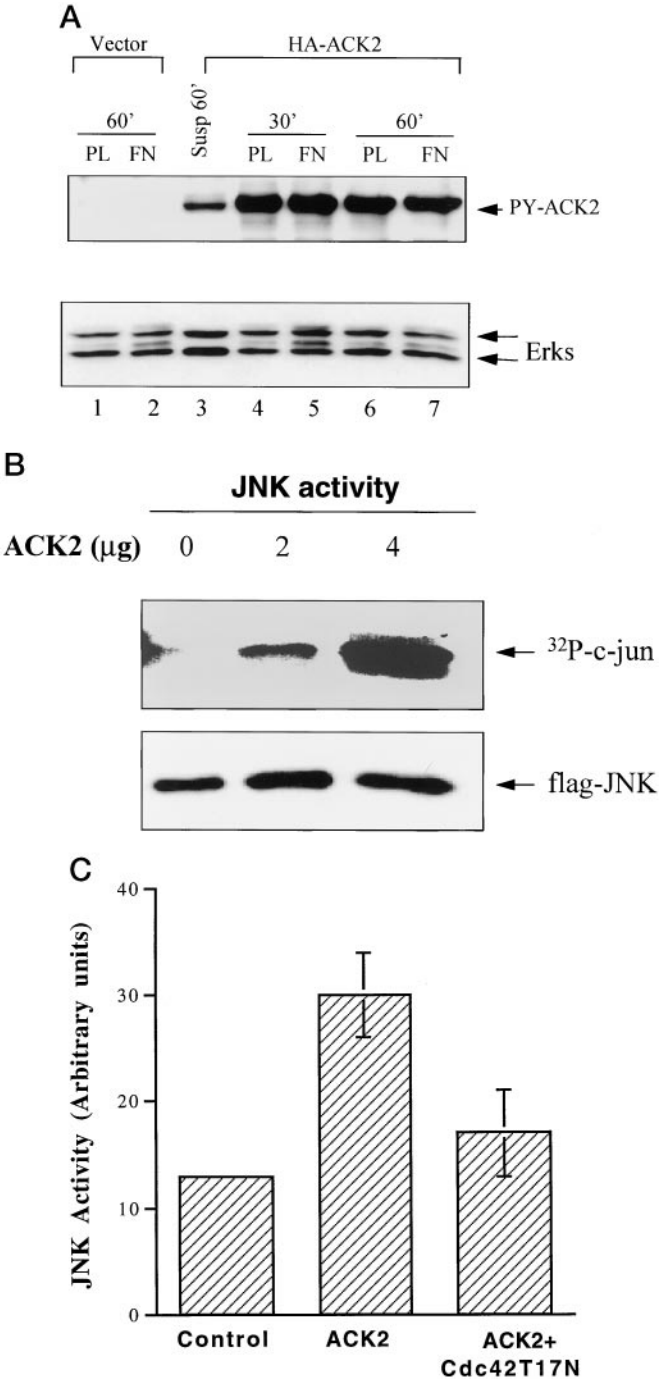

FIG. 4. **Effects of ACK-2 on mitogen-activated protein kinase activities.** *A,* ACK-2 activation is not correlated with Erk activation during cell adhesion. The experimental procedures were exactly as described for Fig. 1. The Erks were immunoblotted with anti-Erk antibody (Santa Cruz Biotechnology). The electrophoretic mobility shifts corresponding to Erk activation are indicated by *arrows*. *B*, ACK-2 activates JNK. The indicated amounts of pcDNA3 HA-ACK-2 were co-transfected with flag-tagged JNK (0.5 μg). The JNK assay was performed following immunoprecipitation with the anti-flag antibody (anti-M5, Kodak). The proteins were fractionated by SDS-PAGE and subsequently transferred onto Immobilon membranes. *Top panel*, autoradiography; *bottom panel*, immunoblot with anti-flag antibody. *C*, pcDNA3 HA-Cdc42(T17N) (2 μg) was co-transfected along with pcDNA3-HA-ACK-2 and flag-tagged JNK1 (0.5 μg). The JNK kinase assay, protein fractionation, and blotting were the same as above. JNK activity was quantitated from the radioactivity with a PhosphorImager.

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. 5*C, 4th* and *5th*

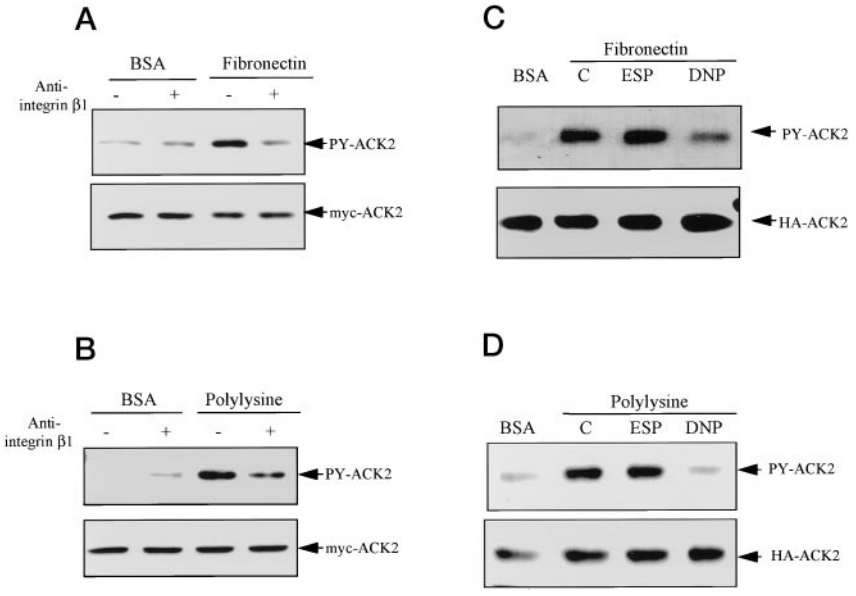

FIG. 5. **Inhibitory anti-integrin β_1 antibody and RGD peptide block activation of ACK-2 by cell adhesion on both polylysine and fibronectin.** The cell adhesion procedures were the same as described for Fig. 1*B* except that the antibody (1:50 dilution) or RDG peptide (1 mM) was preincubated with cells in an Eppendorf tube at 37 °C and 5% CO_2 for 30 min before replating. *A* and *B*, effects of anti-integrin β_1 on tyrosine phosphorylation of ACK-2 upon cell adhesion on fibronectin and polylysine, respectively; *C* and *D*, effects of RGD peptide on tyrosine phosphorylation of ACK-2 upon cell adhesion on fibronectin and polylysine, respectively. *ESP* represents the GRGESP control peptide; *DNP* represents the GRGDNP peptide. In all figures, the *top panel* is blotted with anti-phosphotyrosine (*PY*); the *bottom panel* is blotted with anti-Myc or anti-HA.

lanes; Fig. 5*D, 4th lane*). These data strongly suggest that ACK-2 is activated upon cell adhesion onto a substratum via integrin β_1. Although cell attachment to polylysine was not dependent on integrins, the ability of polylysine to stimulate ACK-2 activity is integrin-dependent. This indicates some type of functional coupling between cell-surface receptors that bind polylysine and integrin β_1 which can in turn influence ACK-2 activity.

ACK-2 Is Constitutively Associated with the Integrin β_1 Complex and the Association Is Independent of ACK-2 Tyrosine Kinase Activity—The implication that integrin β_1 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 β_1 with an anti-integrin β_1 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 β_1 (Fig. 6*A, 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 β_1. 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 β_1 complex was totally independent of its kinase activity or tyrosine phosphorylation (Fig. 6*A, 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 substratum if it is constitutively associated with integrin β_1. The data presented in Fig. 6*B* 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. 6*B* (*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. 6*B, left panel, 3rd* and *4th lanes*). The *right panel* of Fig. 6*B* 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

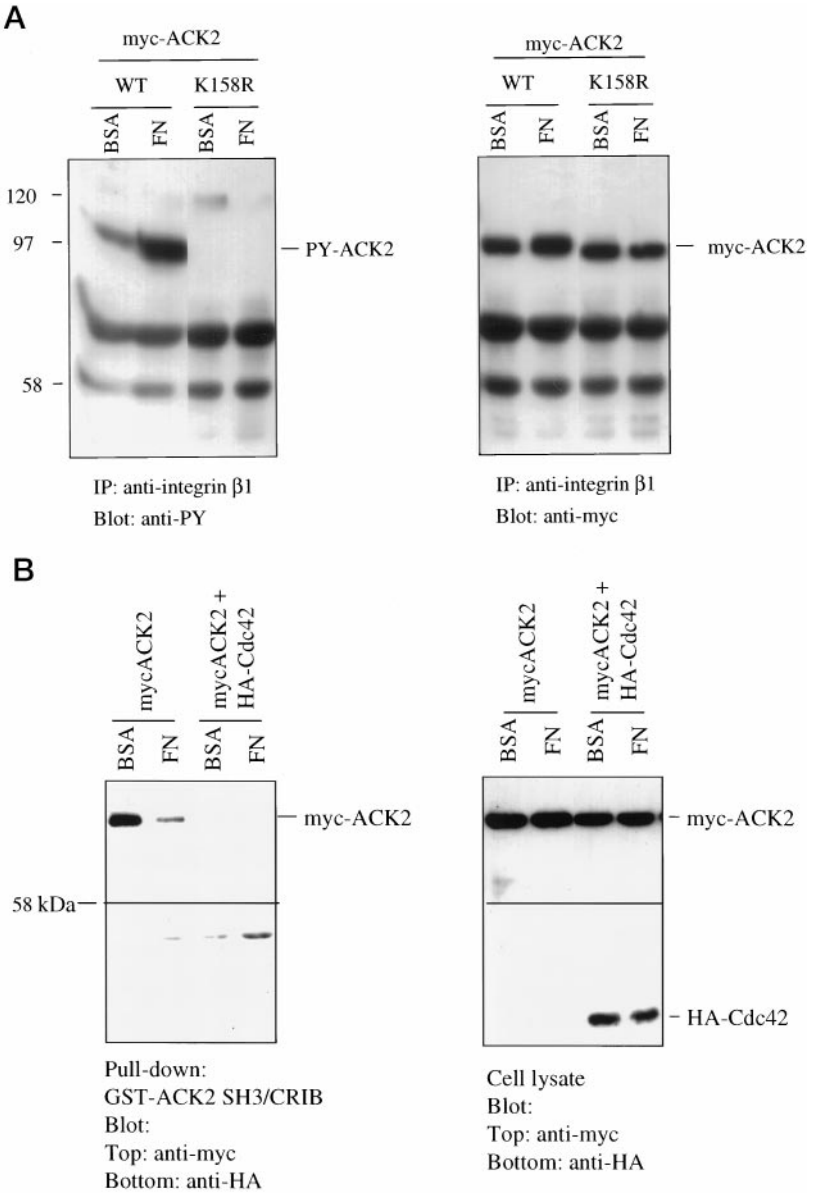

FIG. 6. **ACK-2 is directly associated with the integrin β_1 complex.** *A*, coimmunoprecipitation of ACK-2 with integrin β_1. COS-7 cells transfected with Myc-tagged ACK-2 or Myc-tagged ACK-2-(K158R), a kinase-defective mutant, were lysed with RIPA buffer after replating onto BSA or fibronectin-coated plates. Immunoprecipitation of integrin β_1 was performed by adding anti-integrin β_1 antibody (10 μl) and 10 μl of protein G beads into 500–1000 μg of lysate protein and incubated at 4 °C for 3 h with rocking. The immunoprecipitated sample was divided into 2 parts and blotted with either anti-phosphotyrosine (*PY*) (*left panel*) or with anti-Myc antibody (*right panel*). *B*, self-association of ACK-2 is inhibited by cell adhesion on fibronectin or by cotransfection with Cdc42. COS-7 cells were transfected with pcDNA3 encoding Myc-ACK-2 or pcDNA3 encoding Myc-ACK-2 plus pcDNA3 encoding HA-Cdc42 and lysed with lysis buffer after replating onto BSA or fibronectin-coated plates. In the *left panel*, immobilized GST-ACK-2 SH3/CRIB domain (20 μg/sample), which contains both the SH3 and Cdc42-binding motifs, was incubated with cell lysates, and the precipitated proteins were separated by SDS-PAGE and blotted with anti-Myc (*upper part*, >58 kDa) or anti-HA (*lower part*, < 58 kDa). In the *right panel*, to determine the expression levels of ACK-2 or Cdc42, the cell lysates were blotted with anti-Myc (*upper part,* >58 kDa) or anti-HA (*lower part*, <58 kDa).

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 β_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 β_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 β_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 β_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

described as point contacts that are distinct from focal contacts (49, 50). It has been further proposed that in fibroblasts, $\alpha_1\beta_1$ and $\alpha5\beta_1$ integrin heterodimers first accumulate in point contacts followed by their redistribution into focal contacts (in astrocytes, the accumulation of $\alpha_1\beta_1$ 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 integrins/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 (52–55). Moreover, as yet, we have not been able to show a significant activation of JNK activity upon cell attachment. Thus, either one or more of the signaling participants that may connect cell adhesion to JNK activation was limiting in our experiments, or the observation that overexpression of ACK-2 resulted in JNK activation reflected an aberrant signaling pathway triggered by the higher than normal levels of ACK-2. We suspect that ACK-2 may play some specialized roles in cell differentiation, since it is highly enriched in brain and skeletal muscle (15). One interesting possibility is that ACK-2 may be activated by extracellular matrix proteins that guide neurite outgrowth of neuronal cells or the differentiation of muscle cells. Future efforts will be directed toward determining the *in vivo* function of ACK-2 in specific tissues and identifying its downstream targets.

Acknowledgments—We thank Dr. Shubha Bagrodia for helpful discussions regarding JNK kinase assays and for providing the flag-tagged JNK plasmid. We appreciate the help of Cindy Westmiller in the preparation of this manuscript.

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