The Nonreceptor Tyrosine Kinase ACK2, a Specific Target for Cdc42 and a Negative Regulator of Cell Growth and Focal Adhesion Complexes*

ACK2 (activated Cdc42-associated tyrosine kinase-2) is a nonreceptor tyrosine kinase that is a specific target/effector for the GTP-binding protein Cdc42. Thus far the biological function of this tyrosine kinase has not been determined. Using an inducible eukaryotic expression system in fibroblasts, we demonstrate that ACK2 can strongly influence cell shape and growth as well as focal complex formation. ACK2 was found to associate with the focal adhesion complex components talin and vinculin, but not with the focal adhesion kinase (FAK), in a kinase-independent manner. The tyrosine kinase activity of FAK was also inhibited in cells overexpressing both wild-type and kinase-defective ACK2. This may be due to a competition between ACK2 and FAK for Src, which is an essential cofactor for FAK activation, as we have found that ACK2 specifically binds Src in cells. The ACK2-Src interaction appears to be mediated by the SH3 domain of Src, and the phosphorylation of ACK2 is enhanced in cells overexpressing the hyperactivated Src(Y527F) mutant. Overexpression of both wild-type and kinase-defective ACK2 also results in a severe inhibition of cell growth. In addition, ACK2 dissolves actin stress fibers and disassembles focal complexes but in a kinase-dependent manner. These results, taken together with previous studies demonstrating an association of ACK2 with integrin β1 (Yang, W., Lin, Q., Guan, J.-L., Cerione, R. A. (1999) J. Biol. Chem. 274, 8524–8530) and clathrin (Yang, W., Lo, C. G., Dispensa, T., and Cerione, R. A. (2001) J. Biol. Chem. 276, 17468–17473), suggest that the binding and protein tyrosine kinase activities of ACK2 coordinate changes in cell morphology and growth with the disassembly of focal adhesion sites, perhaps to organize new integrin complexes that are required for endocytosis and/or for cellular differentiation.

ACKs† (activated Cdc42-associated tyrosine kinases) are a family of nonreceptor tyrosine kinases that specifically interact with the GTP-bound, active form of Cdc42, a Rho family GTPase (1, 2). We have cloned and characterized one member of this tyrosine kinase family, ACK2 (1, 3). While Cdc42 has been shown to play important roles in a variety of cellular activities including actin cytoskeletal organization, cell adhesion, intracellular trafficking, DNA biosynthesis, and cell growth regulation (4–10), little is known about the function of the ACKs in cells.

Our initial studies indicated that ACK2 was involved in cell adhesion signaling (3). Cell attachment strongly enhanced the tyrosine phosphorylation of ACK2. Unlike the focal adhesion kinase (FAK), which has a tyrosine kinase activity that is specifically increased by cell adhesion on fibronectin-coated plates, the tyrosine kinase activity of ACK2 is stimulated when attaching cells to either fibronectin- or polylysine-coated plates. Interestingly the stimulation of the tyrosine kinase activity of ACK2 by adhesion is, at least partially, mediated by integrin β1. Data from immunoprecipitation studies indicated that ACK2 associated with an integrin β1 complex (3), thus suggesting that this tyrosine kinase may have a regulatory role in the assembly or disassembly of focal adhesion complexes.

To further address the cellular function of the Cdc42-specific target ACK2, we used a Tet-off inducible eukaryotic cell expression system. Here we show that the overexpression of ACK2 in NIH3T3 cells strikingly affected cell morphology as well as inhibited the tyrosine kinase activity of FAK, perhaps as an outcome of the binding of ACK2 to c-Src. These ACK2-mediated effects were all largely independent of ACK2 tyrosine kinase activity as was a marked inhibition of cell growth that accompanied the inducible expression of ACK2. The expression of ACK2 also resulted in the disruption of actin stress fibers and focal complexes, although these effects were dependent on ACK2 tyrosine kinase activity. Overall the data indicate that the Cdc42 target ACK2 participates in the down-regulation of focal adhesion complex organization and acts as a negative regulator of mitogenic signaling activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Puromycin was purchased from Calbiochem, tetracycline and anti-vinculin antibody were obtained from Sigma, and Texas red-conjugated phalloidin was from Molecular Probes. The Tet-off inducible expression system was described previously (11).

**Selection of Tet-off Inducible Cell Lines**—The HindIII/EcoRV-digested Myc-tagged ACK2 cDNA from pcDNA3 Myc-ACK2 and the corresponding Myc-tagged ACK2 (K158R) cDNA were cloned into the pTet-splice vector to obtain pTet Myc-ACK2 or pTet Myc-ACK2 (K158R). We then co-transfected pTkTAK (3 μg/60-mm dish) with either the pTet-splice (control) vector, the pTet Myc-ACK2, or the pTet Myc-ACK2 (K158R) (3 μg/60-mm dish) together with the puromycin-resistant gene plasmid (0.3 μg/60-mm dish) into NIH3T3 cells (3 × 10^5 cells/60-mm dish) in the presence of tetracycline (1 μg/ml). After 48 h, the cells were transferred to 100-mm dishes and cultured overnight in DMEM plus 10% calf serum (CS) and 1 μg/ml tetracycline. The colony selection was performed by adding puromycin (5 μg/ml) into the culture medium. The positive colonies were identified following removal of

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‡ The abbreviations used are: ACK, activated Cdc42-associated tyrosine kinase; FAK, focal adhesion kinase; BrdUrd, deoxybromouridine; SH3, Src homology 3; DMEM, Dulbecco's modified Eagle's medium; CS, calf serum; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; Tet, tetracycline.
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ACK2 was cleared by centrifugation at 14,000 rpm for 2 min, and an equal volume of lysate was then incubated with an equal volume of binding buffer (40 mM Hepes, pH 7.4, 100 mM NaCl, 1% Triton X-100, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM EDTA, 10 μg/ml leupeptin, and 10 μg/ml aprotinin), and 20 μg of lysate proteins were loaded onto a 10 SDS-polyacrylamide gel. The proteins were transferred to a polyvinylidene difluoride membrane and blotted with an anti-Myc antibody.

Apoptosis Assay—Apoptosis assays were performed using an apoptosis detection kit with annexin V-FITC staining purchased from Calbiochem (Oncogene). Cells that were stably transfected with Tet (vector) or Tet-Myc-ACK2 were cultured on coverslips in DMEM plus 10% calf serum and 1 μg/ml tetracycline overnight. Tetracycline was then removed from the culture medium for 28 h to overexpress ACK2. As a positive control, cells containing the pTet vector were treated at the same time with 200 ng/ml Fas ligand for 12 h to induce apoptosis. Untreated cells containing the pTet vector served as negative controls. The annexin V-FITC staining procedures were modified based on the instructions of the manufacturer (Calbiochem). Briefly, the cells were washed with DMEM serum-free medium twice and incubated with annexin V-FITC (0.5 μg/ml) in the binding buffer (40 mM Hepes, pH 7.4, 150 mM NaCl, 2.5 mM CaCl2, 1 mM MgCl2, 4% bovine serum albumin) at 22 °C for 15 min. The cells were then washed with DMEM plus 5 mM CaCl2 three times and incubated in propidium iodide (30 μg/ml in PBS) in the binding buffer at 22 °C for 5 min to stain dead cells. After washing (three times) with DMEM plus 5 mM CaCl2, the cells were fixed with 3.7% formaldehyde plus 5 mM CaCl2 at 22 °C for 10 min and followed subsequently by three washes with DMEM plus 5 mM CaCl2. The staining was observed under a fluorescent microscope.

Immunofluorescence—Cells that were precipitated on glass slips with or without induction were rinsed with PBS twice, fixed in 3.5% formaldehyde for 10 min, and then permeabilized with 0.2% Triton X-100 for 10 min. The cells were blocked with 0.2% bovine serum albumin in PBS for 5–10 min and then incubated with primary antibodies for 30 min at 37 °C (three times). After washing (three times) with PBS, the cells were incubated with secondary antibodies and phalloidin (conjugated to fluorescent dyes) for 30 min at 37 °C. The cells were then washed with PBS (three times), mounted on a glass slide, and observed under a fluorescence microscope.

Immunoprecipitations—The 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) by rocking for 15–30 min at 4 °C. The lysate was cleared by centrifugation at 14,000 rpm for 2 min, and an aliquot of the lysate (200–500 μl) was used for immunoprecipitation. After the primary antibody (1 μg/400 μg of lysate) was incubated with the lysate on ice for 30 min, protein A beads (Sigma) (1:1) were added, and the mixture was rocked at 4 °C for 1 h. The beads were washed twice with 700 μl of the lysis buffer and finally resuspended in 20 μl of 2× SDS-PAGE sample buffer. The immunoprecipitated proteins were separated by SDS-PAGE.

BrdUrd Incorporation—The BrdUrd incorporation experiments were performed as described previously (11). Briefly, the cells were synchronized at G0 phase by serum starvation, and the expression of ACK2 and the kinase-dead mutant was induced for 24 h before labeling the cells with 100 μM BrdUrd in regular medium for 18 h. The cells were then fixed and stained with anti-BrdUrd and Hoechst as described in the preceding section. The percentage of incorporation of BrdUrd was determined by the ratio of the number of cells showing anti-BrdUrd staining to the cells showing Hoechst staining from five to eight microscopic fields (×200). For quantitation of BrdUrd incorporation, the BrdUrd-labeled cells were collected in 40 μl of kinase buffer including 10 μg of [γ-32P]ATP with (for assaying the phosphorylation of exogenous substrates) or without (for autophosphorylation assays) 5 μg of the exogenous substrate poly(Glu,Tyr) (E2Y) for 20 min at 25 °C. The reactions were stopped by adding 10 μl of 5× SDS-PAGE sample buffer.

RESULTS

ACK2 Causes Cell Shape Changes—To begin to examine the cellular effects of ACK2, we cloned a Myc-tagged cDNA of ACK2 and its kinase-defective mutant ACK2(K158R) into pTet-spllice, a eukaryotic expression vector that contains a tetracycline transactivator regulatory sequence upstream from a minimal human cytomegalovirus promoter. After co-transfection of pTet-ACK2 or pTet-ACK2(K158R) with pTet-tTA, the tetracycline transactivator plasmid, and pMet-puro (a plasmid that contains a puromycin-resistant gene), positive (NIH3T3) cell clones were selected for overexpression of wild-type ACK2 or ACK2(K158R). The expression of wild-type ACK2 or ACK2(K158R) was induced by removing tetracycline from the medium at various times (Fig. 1A).

When assaying the expression of ACK2 following its induction, for the experiments presented here, ACK2 expression peaked at 24 h (postinduction). Cells overexpressing wild-type ACK2 showed dramatic changes in morphology with a time course mirroring that for ACK2 expression (Fig. 1B). The cells that overexpressed wild-type ACK2 showed a significant degree of branching (Fig. 1B, a–c), whereas after 24 h of induction ACK2 was dramatically inhibited by cell-cell contact, whereas the branching structure of those cells expressing wild-type ACK2 expression (Fig. 1B, e–g) followed subsequently by three washes with DMEM plus 5 mM CaCl2. The staining was observed under a fluorescent microscope.

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substrates, as assayed with the tyrosine-containing polymer E_{Y}, with a corresponding inhibition of the autophosphorylation of FAK. In different experiments, we have found that the extent of inhibition ranged from 50–75%. Previous studies have shown that cytochalasin D, an F-actin depolymerization reagent, also inhibits the tyrosine phosphorylation of FAK (12), suggesting that ACK2-mediated effects on the integrity of the actin cytoskeleton may at least in part account for the inhibition of FAK autophosphorylation.

However, the inhibition of FAK activity by ACK2 may also involve c-Src. While screening for potential binding partners for ACK2, we found that the SH3 domain of Src was highly effective in its ability to bind to the proline-rich carboxy-terminal domain of ACK2. The upper panel in Fig. 4A compares the abilities of different GST-SH3 domains to co-precipitate Myc-tagged ACK2, while the middle panel compares the binding of the GST-SH3 domain constructs to dynamin, and the lower panel compares the relative amounts of the GST-SH3 domains that were assayed. We have found that the carboxy-terminal SH3 domain of Grb2 is capable of a weak interaction with ACK2, although in some experiments we have detected no interaction, and in all cases it was at least 50% less effective than the SH3 domain of c-Src. Note that under the same conditions the SH3 domains of both Grb2 and Src bind equally well to dynamin. Thus far we have not found any other SH3 domain-containing protein to be capable of associating with ACK2. When Src(Y527F), a constitutively active Src mutant, was co-transfected with Myc-tagged ACK2ΔNT, an amino-terminal truncation mutant of ACK2, in COS7 cells, the tyrosine phosphorylation of ACK2ΔNT was dramatically increased (Fig. 5A), demonstrating that Src either directly, or through another tyrosine kinase, phosphorylates ACK2 in vivo. Stable complex formation between Src and ACK2ΔNT in vivo was also detected following the immunoprecipitation of either Myc-tagged ACK2ΔNT with an anti-Myc antibody (Fig. 4B, lane 1) or endogenous c-Src with an anti-Src antibody (Fig. 4C, lane 2). In these experiments, we estimated that ~5% of the total ACK2 was co-immunoprecipitated with Src. Although the tyrosine phosphorylation of wild-type ACK2 was also dramatically enhanced by co-transfection with Src(Y527F), we have not been able to consistently detect a stable association between wild-type ACK2 and c-Src in cells (data not shown). These results are reminiscent of the binding interaction between ACK2 and clathrin, which is significantly stronger when assaying the ACK2ΔNT mutant (13). This may mean that the amino-terminal end of ACK2 plays some sort of regulatory role that weakens the affinity of ACK2 for Src, perhaps in a manner similar to its effects on the interaction of ACK2 with clathrin.

ACK2 Inhibits Cell Growth—Another striking effect that accompanied the overexpression of ACK2 was a significant inhibition of cell growth. We determined the growth rates of the different cell lines by either (i) inducing ACK2 expression for different periods of time and then counting cell numbers (where in each case cells were counted for a total of 3 days) (Fig. 5A) or (ii) by inducing the cells for various times and then counting the cells at the different time periods of induction (Fig. 5B). In both cases, the growth rates for cells overexpressing wild-type ACK2 were dramatically reduced. The induced expression of ACK2(K158R) also resulted in a reduction in cell growth, although the growth inhibition was less severe than that caused by wild-type ACK2. Similar results were obtained when assaying DNA synthesis by deoxybromomurdine incorporation (Fig. 5C). Expression of wild-type ACK2, and to a slightly lesser extent ACK2(K158R), resulted in an inhibition of serum-stimulated DNA synthesis.

The inhibition of cell growth by ACK2 was not the outcome of

![Fig. 1. Overexpression of ACK2 causes morphology changes in NIH3T3 cells. A, expression of Myc-ACK2 and Myc-ACK2(K158R) in tetracycline-inducible (Tet-off) cell lines. The induction of protein expression was performed as described previously (9). The proteins were blotted with an anti-Myc antibody (9E10). The numbers at the top of the panel represent the time of induction. B, ACK2 induces cell branching. The cells were seeded in a 12-well plate at a density of 10^5 cells/well and cultured overnight in DMEM plus 10% CS and 1 μg/ml tetracycline. For induction, the cells were rinsed once with DMEM plus 10% CS and replenished with DMEM plus 10% CS. After the indicated times of induction (to the left), the cells were photographed under a microscope. Upper three panels (a, g, and m) represent no induction. Panels a–f, pTet (vector control) cell line; panels g–l, pTet-ACK2 cell line; panels m–r, pTet-ACK2(K158R) cell line.](http://www.jbc.org/)
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Fig. 2. Co-immunoprecipitation of ACK2 with vinculin and talin. The Tet-off inducible cells that were stably transfected with pTet-Myc-ACK2 or pTet-Myc-ACK2(K158R) were cultured in 100-mm dishes with DMEM plus 10% CS plus 1 μg/ml tetracycline overnight, and the expression of ACK2 proteins was induced by removing tetracycline from the medium for 24 h. The cells were then lysed in 1 ml of the radioimmune precipitation lysis buffer for each 100-mm dish. 300 μg of lysate proteins were used in each sample for immunoprecipitation. The anti-vinculin (2 μg), anti-talin (2 μg), or anti-FAK (2 μg) antibody was incubated with the lysates, and the immunoprecipitation was carried out as described under “Experimental Procedures.” The immunoprecipitation complexes were separated by SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and immunoblotted by either anti-phosphotyrosine antibody or anti-Myc antibody. IP, immunoprecipitation; PY, phosphotyrosine; MW, molecular weight marker.

Fig. 3. ACK2 inhibited tyrosine phosphorylation and kinase activity of FAK. U, uninduced (+tetracycline); I, induced (−tetracycline). The upper right panel shows the quantitation of FAK activity by phosphorimage analysis of the autoradiograph shown in the upper left panel. The lower right panel shows inhibition of FAK autophosphorylation by ACK2. The lower left panel compares the relative amounts of FAK that were assayed for the different conditions. WT, wild type; IP, immunoprecipitation.

programmed cell death (apoptosis). Fig. 6 shows that unlike the Fas ligand, which gives rise to a strong apoptotic response as assayed by staining dead cells with annexin V-FITC, the overexpression of ACK2 does not induce programmed cell death. At the present time, we also do not believe that the inhibitory effects of ACK2 on cell growth can be entirely attributed to the ACK2-mediated inhibition of FAK and/or binding of Src. We have generated a number of ACK2 mutations, and thus far we have only found that perturbing the SH3 domain of ACK2 alters its ability to inhibit cell growth (data not shown). This perturbation should not affect the ability of ACK2 to bind Src or inhibit FAK. However, it does strongly inhibit the binding of ACK2 to clathrin, presumably by preventing an intramolecular interaction that allows ACK2 to adapt the appropriate conformation for binding to clathrin (13). This may mean that the participation of ACK2 in the endocytosis of cell surface receptors (e.g., integrins and/or growth factor receptors) has a strong role in the ACK2-mediated inhibition of cell growth.

ACK2 Influences the Actin Cytoskeleton and Focal Adhesion Complexes in a Kinase-dependent Manner—To further examine the cellular effects caused by overexpression of ACK2, actin cytoskeletal staining was performed. As shown in Fig. 7A, in the absence of induction (i.e. plus tetracycline), the cytoskeletal structures for all three cell lines were similar such that each cell line showed intact actin stress fibers (Fig. 7A, see panels a–c). After 48 h of induction (i.e. minus tetracycline), cells expressing wild-type ACK2 were either completely lacking or had markedly reduced amounts of actin stress fibers (Fig. 7B, panel b). Both control cells and cells that express kinase-defective ACK2(K158R) showed intact actin stress fibers (Fig. 7B, panels a and c).

Focal adhesion complexes are the docking sites for stress fibers (14). We have shown that ACK2 is activated by cell attachment (1, 3). The disassembly of stress fibers by ACK2 may be the result of interactions between ACK2 and focal adhesion complexes. When we stained control cells with an antibody against vinculin, a known component of focal adhesion complexes, we observed that the focal adhesion complexes were located at the ends of stress fibers (Fig. 7A, panels d–f). In contrast, vinculin staining was diminished in the cells that overexpressed wild-type ACK2 and had a branching structure (Fig. 7B, panel e). Control cells containing vector alone or cells expressing the kinase-defective ACK2 mutant had normal focal adhesion complexes (Fig. 7B, panels d and f). These results suggest that the disruption of focal adhesion complex formation may be the direct cause for the disassembly of actin stress fibers by ACK2.
DISCUSSION

The GTP-binding protein Cdc42 has been implicated in a diversity of cellular activities that ultimately mediate changes in cell shape, motility, cell cycle progression, differentiation, and cell survival (15, 16). Among the major questions concerning the underlying mechanisms for these different functions are the cellular roles played by the individual targets of Cdc42. Here we have examined the cellular effects of the highly specific Cdc42 target ACK2 and found that it has a significant influence on cell shape, cell cycle progression, and the actin cytoskeleton. Specifically both wild-type and kinase-dead ACK2 induce cell extensions and inhibit cell growth. The effects are somewhat enhanced when expressing a kinase-competent ACK2 compared with the kinase-defective version. Nonetheless, the latter causes clear changes in cell shape and retards cell growth, arguing that it is the binding ability (rather than the tyrosine kinase activity) of ACK2 that is mainly responsible for these effects. However, there is a significant

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FIG. 4. ACK2 interacts with Src and is phosphorylated by Src(Y527F). A, ACK2 specifically interacts with the Src-SH3 domain. Immobilized GST-Src-SH3, Grb2 carboxyl-terminal SH3, and Crk amino-terminal SH3 domains (20 μg each) on glutathione beads were incubated with Myc-tagged ACK2-transfected COS7 cell lysates (200 μg) at 4 °C for 2–3 h. The SH3 domain-associated ACK2 protein was detected by immunoblotting with anti-Myc antibody (upper panel). The middle panel shows the results of immunoblotting with anti-dynamin antibody. The lower panel shows the relative amounts of the different GST fusion proteins used in the experiments. B, ACK2ΔNT is co-immunoprecipitated with and phosphorylated by Src(Y527F). pcDNA3-Myc-ACK2ΔNT (2 μg/60-mm dish) was co-transfected with pcDNA3-Src(Y527F) (2 μg/60-mm dish) into COS7 cells. The cells were lysed with a Triton X-100-based lysis buffer. The immunoprecipitations were performed as described under “Experimental Procedures” with an anti-Myc antibody (9E10). The immunoprecipitated proteins were immunoblotted with either an anti-phosphotyrosine, an anti-Src, or an anti-Myc antibody as indicated in the figure. Lanes 1 and 3, co-transfection of pcDNA3-Myc-ACK2ΔNT with pcDNA3-Src(Y527F); lanes 2 and 4, pcDNA3-Myc-ACK2ΔNT alone. C, ACK2ΔNT is associated with endogenous c-Src. pcDNA3-Myc-ACK2ΔNT (2 μg/60-mm dish) was transfected into COS7 cells. The cells were lysed with a Triton X-100-based lysis buffer. The immunoprecipitation was performed with an anti-Src antibody. The immunoprecipitated proteins were immunoblotted with either an anti-Myc antibody or anti-Src antibody as indicated in the figure. Lane 1, control cells (no transfection); lane 2, pcDNA3-Myc-ACK2ΔNT-transfected cells. PY, phosphotyrosine; IP, immunoprecipitation.

FIG. 5. ACK2 inhibits cell growth. The cells were seeded in a 12-well plate and cultured in DMEM plus 10% CS and 1 μg/ml tetracycline overnight. The initial cell density was 5 × 10^3 in A and 6 × 10^4 in B. Induction of the expression of ACK2 or ACK2(K158R) was performed in two ways: either induction at the different indicated time points and then counting the cell numbers (in all cases, cells were grown for 3 days) (A) or cells were induced for different time periods and then counted (B). The cell growth rate was determined by counting the cell numbers under a microscope with a manual cell counter. The numbers in the figures are the averages from two independent sets of culture. C, ACK2 inhibits DNA synthesis. The pTet, pTet-Myc-ACK2, or pTet Myc-ACK2(K158R) stably transfected NIH3T3 cells were seeded at 10^5/35-mm dish and cultured in DMEM plus 10% CS plus 1 mg/ml tetracycline overnight. The cells were synchronized at G0 phase by serum starvation for 24 h, and the expression of ACK2 was induced at the same time by removing tetracycline in the culture medium. The cells were subsequently cultured in regular culture medium plus BrdUrd (BrdU) (100 μM) for 18 h, and then fixed and stained with anti-BrdUrd antibody and Hoechst. The percentage of BrdUrd incorporation was determined by the ratio of the number of cells showing BrdUrd staining to cells showing Hoechst staining from five to eight microscopic fields (110–200 cells). The variation was calculated from the differences between microscopic fields.
requirement for the kinase activity of ACK2 in its ability to
dissolve actin stress fibers and disassemble focal complexes.
Thus, one can envisage a model where the binding of ACK2 to
integrin/ and/or vinculin, talin, and c-Src initially results in
a modulation of FAK activity and cytoskeletal alterations that
lead to cellular extensions. The activation of ACK2 tyrosine
kinase activity apparently then gives rise to the phosphoryla-
tion of a focal contact or cytoskeletal-associated component(s)
that directs focal adhesion disassembly and the dissolution of
actin stress fibers.

All of this makes it attractive to think of ACK2 as playing a
key regulatory role both through its binding partners and ty-
rosine phosphosubstrate(s) in coordinating changes in cell
shape with a slowing of cell cycle progression. The coordination
of these activities becomes important during cellular differenti-
ation. This is especially the case when considering the shape
changes and effects on actin stress fibers that accompany neuro-
nal differentiation. Previous studies have shown that the inhibi-
tion of actin stress fiber formation results in cell membrane
protrusion or neurite outgrowth (17). Microinjection of the con-
stitutively active Rho mutant Rho(G14V) or the catalytic domain
of the p160 RhoA-binding kinase ROK/ into rat pheochromocy-
toma (PC12) cells rapidly induced neurite retraction (18), while
introduction of the C3 exoenzyme, which is a specific inhibitor for
Rho activation, promoted neurite outgrowth in PC12 cells (17).
However, the microinjection of a dominant-negative mutant of
Cdc42 (Cdc42(T17N)) inhibited neurite outgrowth completely,
indicating that active Cdc42 is required for this neuronal activity.
Microinjection of activated Cdc42 into PC12 cells leads to a loss of
actin stress fibers and focal complexes. Thus, ACK2, which is
capable of inducing cellular extensions and disrupting actin
stress fibers, may be responsible for mediating these Cdc42-
stimulated events during a differentiation response.

FIG. 6. Overexpression of ACK2 did not induce apoptosis. The
cells were cultured, treated, and stained as described under "Experi-
mental Procedures." The expression of Myc-tagged ACK2 was induced
by removing tetracycline from the culture medium for 28 h before
annexin V-FITC staining. The positive control was the pTet (vector)-
transfected cells that were treated with 200 ng/ml Fas ligand (FasL) for
12 h, and the negative control was the pTet (vector)-transfected cells
without any treatment. The green fluorescence is annexin V-FITC
staining. The phase contrast image of the cells was captured from the
same field for the annexin V staining. Notice that Fas ligand induced
apoptosis in about 30% of total cells, while overexpression of ACK2 did
not result in any apoptosis.

FIG. 7. ACK2 disassembles actin stress fibers and focal adhe-
sion complexes. Cells were cultured in 100-mm dishes with DMEM
plus 10% CS and 1 μg/ml tetracycline overnight, and then for induction
of ACK2, cells were cultured without tetracycline for 24 h. The cells
were added to glass coverslips in a six-well plate and cultured under the
same conditions (as described above) to continue the induction for
another 24 h. The cells were then fixed and stained as described under
"Experimental Procedures." A, without induction (+Tet). B, with induc-
tion for 48 h (−Tet). In both A and B, the panels on the left (a, b, and c)
were stained with phallolidin for F-actin (red) and the panels on the
right (d, e, and f) were stained with anti-vinculin (green) for focal
adhesion complexes. Panels a and d represent the pTet (vector control)
cell line, panels b and e represent the pTet-Myc-ACK2 cell line, and
panels c and f represent the pTet-Myc-ACK2(K158R) cell line.

It has been well established that a FAK-Src complex plays a
pivotal role in integrin-mediated cell growth (19–21). Thus, it
seems likely that some of the effects of ACK2 on cell growth are
through its interactions with Src, perhaps by blocking the
proper activation of FAK and preventing its transmission of
mitogenic signals through the Grb2/Ras/extracellular signal-regulated kinase pathway (21, 22). However, thus far we have not been able to draw a direct correlation between the ACK2-Src interaction and ACK2-mediated growth inhibition. We have found that an ACK2 SH3 domain mutant that is incapable of binding clathrin is also unable to inhibit cell growth (data not shown). This provides us with a clue that the ACK2-clathrin interaction is somehow linked to the ACK2-mediated effects on cell cycle progression. Such a possibility is interesting as proteins that participate in the endocytosis of growth factor or other mitogenic receptors are often growth inhibitory. The potential regulatory effects of ACK2 on growth factor receptor and/or integrin endocytosis giving rise to a down-regulation of these receptors could strongly contribute to the effects of ACK2 on cell shape and the actin cytoskeleton as well as cell cycle progression and thereby serve a multipurpose function toward the generation of a differentiation signal. It will be interesting to see how general a role ACK2 plays in cellular differentiation processes and whether such roles are connected to the regulation of ACK2 by integrins and other cell adhesion molecules. Such a connection may be manifested by the involvement of ACK2 in the disruption of focal adhesion complexes and the organization of new integrin complexes necessary for clathrin-coated vesicle endocytosis. Future studies will be directed toward testing these ideas.

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