Cloning and Characterization of a Novel Cdc42-associated Tyrosine Kinase, ACK-2, from Bovine Brain*

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Cdc42 plays an important role in intracellular signaling pathways that influence cell morphology and motility and stimulate DNA synthesis. In attempts to determine whether nonreceptor tyrosine kinases play a fundamental role in Cdc42 signaling, we have cloned and biochemically characterized a new Cdc42-associated tyrosine kinase (ACK) from bovine brain. This tyrosine kinase, named ACK-2, has a calculated molecular mass of 83 kDa and shares a number of primary structural domains with the 120-kDa ACK (ACK-1). The main differences between the primary structures of ACK-2 and ACK-1 occur in the amino- and carboxyl-terminal regions. Like ACK-1, ACK-2 binds exclusively to 

activated (GTP-bound) Cdc42 and does not bind to its closest homologs, e.g. activated Rac. ACK-2 could not be activated by addition of glutathione S-transferase (GST)-Cdc42(Q61L), a GTPase-defective mutant, or by GTPγS-loaded GST-Cdc42 in in vitro kinase assays. However, ACK-2 was activated when cotransfected with wild type Cdc42 or Cdc42(Q61L) and stably associated with Cdc42(Q61L) in vivo, indicating that ACK-2 interacts with active Cdc42 in cells. Furthermore, the tyrosine kinase activity of ACK-2 was stimulated both by epidermal growth factor and bradykinin, suggesting that ACK-2 may play a role in the signaling actions of both receptor tyrosine kinases or heterotrimeric G-protein-coupled receptors.

Cdc42 is a member of the Rho subfamily of small GTP-binding proteins. Studies of both yeast and mammalian cells have shown that Cdc42 plays crucial roles in yeast budding and mating (1–4), mammalian cell filopodium formation (5, 6), stress-induced signal transduction (7–10), and mating (1–4), mammalian cell filopodium formation (5, 6), and biochemically characterized a new Cdc42-associated tyrosine kinase (ACK) from bovine brain. This tyrosine kinase, named ACK-2, has a calculated molecular mass of 83 kDa and shares a number of primary structural domains with the 120-kDa ACK (ACK-1). The main differences between the primary structures of ACK-2 and ACK-1 occur in the amino- and carboxyl-terminal regions. Like ACK-1, ACK-2 binds exclusively to activated (GTP-bound) Cdc42 and does not bind to its closest homologs, e.g. activated Rac. ACK-2 could not be activated by addition of glutathione S-transferase (GST)-Cdc42(Q61L), a GTPase-defective mutant, or by GTPγS-loaded GST-Cdc42 in in vitro kinase assays. However, ACK-2 was activated when cotransfected with wild type Cdc42 or Cdc42(Q61L) and stably associated with Cdc42(Q61L) in vivo, indicating that ACK-2 interacts with active Cdc42 in cells. Furthermore, the tyrosine kinase activity of ACK-2 was stimulated both by epidermal growth factor and bradykinin, suggesting that ACK-2 may play a role in the signaling actions of both receptor tyrosine kinases or heterotrimeric G-protein-coupled receptors.

Although the different protein interaction motifs on ACK make it an intriguing signaling partner for Cdc42, very little is known regarding how general a role it might play in Cdc42 action (given that Cdc42 is ubiquitously distributed) and/or how this tyrosine kinase might be regulated by Cdc42 or by other signal-transducing proteins in cells. Here we describe the cloning and characterization of another Cdc42-associated tyrosine kinase, ACK-2, that has the same basic structural features as ACK. ACK-2 also has structural domains similar to Tnk1, a nonreceptor tyrosine kinase from stem/progenitor cells, with the exception being that Tnk1 lacks the Cdc42-binding domain (26). We show that ACK-2 is a highly specific binding partner for activated Cdc42 and that its tyrosine kinase activity can be stimulated in cells both by EGF and bradykinin, suggesting that it may serve as a point of convergence between receptor tyrosine kinase or G protein-coupled receptor signaling and Cdc42.

EXPERIMENTAL PROCEDURES

Materials—EGF and PDGF were purchased from Life Technologies, Inc.; interleukin 1 (IL-1) and TNF-α were purchased from Boehringer Mannheim, Bradykinin was obtained from Sigma Chemical Co., anti-EMM antibody (PY20) was from Santa Cruz, horseradish peroxidase-conjugated anti-PY (PY20) was from Transduction Laboratories, and rabbit liver mRNA was obtained from CLONTECH. The bovine brain cDNA library was a kind gift from Dr. Ronald E. Diehl of Merck, Sharp and Dohme Co.

Cloning of ACK-2 cDNAs—To clone ACK cDNAs, two PCR primers, TGCCCATGCCCCAGTG/GATG and GTCAGAGTCGGGGA(C/T)TT(T/C) GG, were generated from the conserved coding region of the tyrosine kinase domain and from the Cdc42-binding domain (CRIB domain) (27) of ACK. A 0.6-kb PCR product was obtained from a rabbit liver cDNA, and the sequence of the PCR product matched the corresponding region of the ACK cDNA. This PCR product was used as the probe for screening a ZAP bovine brain cDNA library. The procedures used to screen the cDNA library were according to the CLONTECH Lambda Library Protocol Handbook. After three rounds of screening, single phase colonies were picked, and the insert cDNA was spliced according to standard protocols (Stratagene) for excising a ZAP II inserts. The excised
plasmids were purified and sequenced by the automatic sequencer at the Cornell DNA Facility.

**Construction of Expression Plasmids**—To make hemagglutinin (HA)-tagged or Myc-tagged ACK-2, a primer, CTTGGATCCATGCAGCCA-GAGGAG, which contains an introduced BamHI site just before the start codon ATG, was used for PCR to generate a 1.7-kb fragment from the ACK-2 cDNA clone 3. The 1.7-kb fragment was digested with BamHI/PflMI and then ligated with a fragment from a PflMI/EcoRI digestion of the ACK-2 cDNA clone 2 into a pcDNA3 HA-tagged or pcDNA3 Myc-tagged vector. The resultant constructs were pcDNA3 HA-ACK-2 or pcDNA3 Myc-ACK-2.

**Multiple Tissue Blots**—The human multiple tissue blot membrane that contains human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas was purchased from CLONTECH. The probe used that contains human heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas.

**Transfection Studies**—COS-7 cells were sustained in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum (FBS) at 37 °C, 5% CO2. The cells were split at 80% confluence and used for transfection. The cDNA transfections were performed by the lipofectamine method according to the manufacturer's standard protocols (Life Technologies, Inc.).

**Immunoprecipitation Experiments**—The 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) 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 was incubated with the lysate on ice for 30 min, a 1:1 slurry of protein A beads (Sigma) was 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.

**Expression and Purification of GST-Fusion Proteins**—All GST-fusion proteins were expressed and purified as described previously (15).

**ACK Tyrosine Kinase Assays**—After being washed twice with lysis buffer, the immunocomplex beads or GST-Cdc42-ACK-2 complex beads were rinsed with 1× TK buffer (50 mM Hepes, pH 7.4, 10 mM NaCl). Then 5 μl of 10× TK buffer (0.5 mM Hepes, pH 7.4, 0.1 mM NaCl), 1 μl of the synthetic polymer substrate E,Y (20 μg/μl), 5 μl of 32P[γ-ATP (10 μCi/μl), 3000 Ci/mmol) were added to the beads. 1× TK buffer was added to initiate the reaction. After incubation at 24 °C for 5 min, the reaction was stopped by the addition of 30 μl of 2× SDS-PAGE sample buffer. Phosphorylated substrates were separated by SDS-PAGE and visualized by autoradiography.

**RESULTS AND DISCUSSION**

Two classes of protein kinase targets for Cdc42 contain a common CRIB domain (27). One of these is the family of p21-activated PAKs, whose members have been shown to initiate a kinase cascade that culminates in the activation of the nuclear mitogen-activated protein kinases (the e-Jun kinase (JNK1) and p38 (7–10)). The second is a tyrosine kinase that binds...
Cdc42 with high specificity and has been designated ACK (17). At present, very little is known about the actions of ACK, although it has been recently appreciated that its tyrosine kinase domain shares some sequence similarity with those for the focal adhesion kinase and PYK-2, a Ca$$^{2+}$$-activated tyrosine kinase that has been implicated in a number of signaling pathways (28–30). As a first step toward better understanding the role of tyrosine kinases in Cdc42 signaling, we set out to determine whether there were ACK-related tyrosine kinases that might serve as putative targets for activated Cdc42.

**Indications for a Family of ACK-related Tyrosine Kinases**—ACK-related tyrosine kinases were cloned using PCR primers that were made to the tyrosine kinase domain and CRIB motif of ACK. After PCR, using rabbit liver cDNA as the template, a 0.6-kb product was obtained. The sequence for this product matched ACK nucleotides 1413–2025. This PCR product was then used to screen a bovine cDNA library. After three rounds of screening, 12 single positive clones were obtained; 5 of these contained sequences that corresponded to those of the original PCR product (Fig. 1A). Clones 2, 3, and 10 have a unique 45-base insert; clone 6 represents bovine ACK; and clone 12 has a partial sequence that matches those of the other clones but has a unique 39-end. In clone 10, there is a 9-base pair deletion. Taken together, these findings indicate that there are at least two different isoforms of tyrosine kinases that contain a tyrosine kinase domain, a CRIB motif, and an SH3 domain that are highly related to ACK. It is not clear whether these isoforms are present in the same or different tissues. However, the tyrosine kinase domain, the SH3 domain, and the CRIB motif are highly conserved (>90% identity) among these different clones.

We next examined the mRNA message levels for ACK-2 and highly related nonreceptor tyrosine kinases among various human tissues using a probe that encoded the conserved CRIB motif, the SH3 domain, and a portion of the tyrosine kinase domain. The mRNA messages for ACK-related tyrosine kinases range in size from 4.0 to 6.0 kb and appear to be largely expressed in brain and skeletal muscle (Fig. 1B). The fact that the size of the message in skeletal muscle is slightly different from that in brain suggests that there may be several different isoforms of ACK in these tissues. Pancreas, heart, placenta, and lung all show detectable amounts of ACK-related message, suggesting that members of this tyrosine kinase family are present in a wide variety of tissues.

**Identification of ACK-2**—Clones 2 and 3 are 100% identical in overlapping regions (676 bp) and both contain the 45-base pair insert (which distinguishes them from the original ACK), suggesting that they are derived from the same transcript. We patched the sequences from the two clones and obtained a 4.3-kb cDNA that contains an open reading frame encoding a 747-amino acid protein (Figs. 1A and 2A). The methionine residue at position 1 (Fig. 2A) is the first methionine after a stop codon at the 5′ end of the cDNA in the open reading frame, indicating that the deduced amino acid sequence is complete.

![FIG. 3. The alignment of ACK-2 with related proteins.](image-url)
The positions of the initiation and stop codons of the open reading frame are indicated in Fig. 1A. This protein, which we have designated ACK-2 (with the original ACK now referred to as ACK-1), contains a tyrosine kinase domain, an SH3 domain, a Cdc42-binding domain (i.e. a CRIB motif), and a proline-rich carboxyl terminus that contains a number of minimal SH3-binding motifs (PXXP). Hydropathy analysis with Eisenberg’s method (31) indicates that ACK-2 does not contain membrane-spanning regions (Fig. 2B) and thereby represents a nonreceptor tyrosine kinase (i.e. like ACK-1).

Fig. 3A shows a sequence alignment between ACK-1 and ACK-2. The differences between these two tyrosine kinases lie within their amino- and carboxyl-terminal regions; the carboxyl termini of these two proteins are poorly aligned. ACK-2 has 56 fewer amino-terminal amino acid residues and 303 fewer carboxyl-terminal amino acids compared with ACK-1. In total, ACK-2 contains 344 fewer amino acids than ACK-1. This difference includes the proline-rich, 15-amino acid insert that immediately follows the Cdc42-binding domain in ACK-2. This region may allow ACK-2 to participate in a different signaling pathway (i.e. different from those utilizing ACK-1).

A BLAST search of the National Center of Biotechnology Information data base (32) with the ACK-2 sequence shows that the region between residues 132 and 378 (boxed in Fig. 2A), which represents the tyrosine kinase domain, shares similarity with a number of tyrosine kinases, including Tnk1, the EGF receptor, ErbB, Hek, focal adhesion kinase, and PYK-2 (Fig. 3B). The SH3 domain of ACK-2, residues 385–444 (underlined in Fig. 2A), shares homology with the SH3 domains of Grb2 and Vav (Fig. 3C). The CRIB domain of ACK-2, residues 454–477 (in boldface and italics in Fig. 2A), shares similarity with ACK-1, the PAKs, mixed lineage kinase 3, and MSE55.

ACK-2 Binds Activated Cdc42—To facilitate biochemical characterization, we have inserted the cDNA for ACK-2 into a pCDNA3 eukaryotic expression vector, such that ACK-2 can be expressed either as a HA-tagged or Myc-tagged fusion protein. Expression of the HA-ACK-2 in COS-7 cells yielded a 96-kDa protein (Fig. 4, left panel), as predicted from the amino acid sequence. Immunoprecipitation of the HA-ACK-2 with anti-HA antibody yielded an active tyrosine kinase, as assayed by phosphorylation of the tyrosine-containing polymer E4Y1 (10 μg/lane) was added as the substrate. The vector control sample was processed following the same procedures as those for the ACK-2 sample.

The binding specificity of the different GTP-binding proteins for ACK-2 was assessed by incubating COS-7 cell lysates with GST fusion proteins of Cdc42 or Rac1 (in different guanine nucleotide-bound states) and then precipitating the GST-binding proteins with glutathione-agarose. The ability of HA-ACK-2 to associate with the GST-binding proteins was then

![Image](image-url)
ACK-2 is activated by Cdc42 and associated with Cdc42 in vivo. Myc-tagged ACK-2 (2 μg/60-mm plate) was cotransfected with pcDNA3 (vector; 2 μg/60-mm plate), pcDNA3 HA-Cdc42, pcDNA3 HA-Cdc42(Q61L), or pKH3 HA-Cdc42(D57Y). After 48 h, the cells were serum starved overnight. The cells were lysed, and myc-ACK-2 was immunoprecipitated with anti-myc antibody. To determine the expression level of ACK-2 or Cdc42 in cells, equal amounts of cell lysates were loaded onto a 12% SDS-polyacrylamide gel, and the immunoblot was performed with anti-myc antibody or anti-HA antibody to detect the presence of Cdc42. The results in Fig. 5 show that ACK-2 was only able to associate with GTP-bound Cdc42 (left panel), not with guanine nucleotide-free Cdc42 (Fig. 5, NF), GDP-bound Cdc42, or any form of the Rac1 protein (Fig. 5, right panel). We also found that ACK-2 will effectively bind to a GTPase-defective Cdc42 mutant (e.g. Cdc42(Q61L)) but not to a dominant-negative mutant (Cdc42(T17N)) (Fig. 5, middle panel). These results suggest that ACK-2 serves as a highly specific target molecule for Cdc42.

Activation of ACK-2—Thus far, we have not detected a significant activation of ACK-2 by GTPγS-bound Cdc42 or by the GTPase-defective Cdc42(Q61L) protein in vitro. Fig. 6 shows the results of autophosphorylation and substrate phosphorylation (E2Y1) assays using ACK-2 in the presence of different guanine nucleotide-bound forms of Cdc42 or in the presence of a dominant-active (Q61L) or dominant-negative (T17N) mutant. These assays were performed by precipitating the HA-tagged ACK-2 from transfected cells and then adding the different GST-Cdc42 proteins to the resuspended pellets and assaying for (Cdc42)-stimulated tyrosine kinase activity. The inability of Cdc42 to significantly stimulate either ACK-2 autophosphorylation or its phosphorylation of an exogenous substrate under these assay conditions differs from what we previously observed for PAK-3 (9, 15). However, we have found that the co-expression of ACK-2 with wild type Cdc42 or with a dominant-active, GTPase-defective mutant (Cdc42(Q61L)) in COS-7 cells significantly enhanced ACK-2 tyrosine autophosphorylation (Fig. 7, top panel).

In addition, we have found that the dominant-active Cdc42(Q61L) can be co-immunoprecipitated with ACK-2 (Fig. 7, bottom panel), indicating that the activated form of Cdc42 will associate with ACK-2 in vivo. Conversely, co-expression of the dominant-negative Cdc42 mutant (Cdc42(D57Y)) (33) with ACK-2 resulted in a striking inhibition of ACK-2 autophosphorylation. These results suggest that Cdc42 mediates the activation of ACK-2 in cells, perhaps by providing for its proper cellular localization and/or by enabling ACK-2 to interact with other cellular factors that directly stimulate its activation. A similar role for activated Cdc42 has been proposed for the cellular regulation of the p70 S6 kinase (16).
To further determine which signal transduction pathways mediate the regulation of ACK-2, we have screened a number of growth factors, cytokines, or stress factors that activate either receptor tyrosine kinases, G-protein-coupled receptors, or stress-response pathways for their effects on ACK-2. These included fetal bovine serum, ultraviolet radiation, EGF, PDGF, bradykinin, IL-1, and TNF-α. In all cases, when we treated adherent cells with these factors, we observed no detectable effect on ACK-2 activity. In fact, under all conditions where cells were adherent (i.e., attached to culture plates), we found that ACK-2 was constitutively active. This activity was maintained even after 24 h of serum starvation. However, when cells were detached from the plates by treatment with trypsin, ACK-2 tyrosine phosphorylation was markedly decreased (Fig. 8A). Therefore, we examined whether the above factors could then influence ACK-2 activity under conditions where its basal tyrosine phosphorylation was low.

Various factors, including PDGF (20 ng/ml), IL-1 (100 units/ml), and TNF-α (300 units/ml), did not show a detectable effect on ACK-2 tyrosine phosphorylation, even when added to detached cells (Fig. 8B). However, the results presented in Fig. 8C show that when cells were first detached from plates and then treated with 10% fetal bovine serum, the tyrosine phosphorylation of ACK-2 was significantly enhanced after 10 min, and this enhancement was maintained through 1 h. The results presented in Fig. 8D show that the tyrosine phosphorylation of ACK-2 was increased by about 2-fold upon treatment of detached cells for 10 min with EGF or bradykinin. These findings suggest that under some conditions, ACK-2 may mediate signaling initiated by receptor tyrosine kinases or G-protein-coupled receptors.

Conclusions—In this report, we describe the cloning and characterization of a novel nonreceptor tyrosine kinase that shows a good deal of similarity to the ACK protein, which was first identified by Manser et al. (17) as a putative target/effector molecule for Cdc42. Thus, we have designated this new tyrosine kinase ACK-2. Both ACK-1 and ACK-2 contain the CRIB motif, as well as an SH3 domain and proline-rich regions. The major differences between these two protein tyrosine kinases exist within their amino- and carboxyl-terminal ends. At present we do not know the physiological implications of these differences, although the proline-rich domains differ between the two tyrosine kinases, suggesting that different SH3 domain-containing proteins participate in their signaling actions. The fact that ACK-2 is broadly distributed, as is Cdc42, suggests that this tyrosine kinase may play an important role in one or more of the functions of the Cdc42 protein. It is interesting that ACK-2 can be activated by both bradykinin and the EGF receptor, suggesting that it may participate in both receptor tyrosine kinase-signaling and in signaling pathways initiated by hepthelial/G-protein-coupled receptors. We also have found that the tyrosine kinase activity of ACK-2 is strongly activated by cell attachment, in a manner reminiscent of the Met tyrosine kinase (34) and the focal adhesion kinase (35, 36). Thus, ACK-2 may serve as a critical point of convergence for a number of cellular stimuli and probably requires activated Cdc42 to mark its appropriate cellular location for activation. Future studies will be directed at identifying the upstream and downstream signaling components that are associated with ACK-2 in a Cdc42-dependent manner.

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