ACK1 (activated Cdc42-associated kinase 1) is a non-receptor tyrosine kinase and the only tyrosine kinase known to interact with Cdc42. To characterize the enzymatic properties of ACK, we have expressed and purified active ACK using the baculovirus/Sf9 cell system. This ACK1 construct contains (from N to C terminus) the kinase catalytic domain, SH3 domain, and Cdc42-binding Cdc42/Rac interactive binding (CRIB) domain. We characterized the substrate specificity of ACK1 using synthetic peptides, and we show that the substrate specificity of the ACK1 catalytic domain most closely resembles that of Abl. Purified ACK1 undergoes autophosphorylation, and autophosphorylation enhances kinase activity. We identified Tyr284 in the activation loop of ACK1 as the primary autophosphorylation site using mass spectrometry. When expressed in COS-7 cells, the Y284F mutant ACK1 showed dramatically reduced levels of tyrosine phosphorylation. Although the SH3 and CRIB domains of purified ACK1 are able to bind ligands (a polyproline peptide and Cdc42, respectively), the addition of ligands did not stimulate tyrosine kinase activity. To characterize potential interacting partners for ACK1, we screened several SH2 and SH3 domains for their ability to bind to full-length ACK1 or to the catalytic-SH3-CRIB construct. ACK1 interacts most strongly with the SH3 domains of Src family kinases (Src or Hck) via its C-terminal proline-rich domain. Co-expression of Hck with kinase-inactive ACK1(K158R) in mammalian cells resulted in tyrosine phosphorylation of ACK1, suggesting that ACK1 is a substrate for Hck. Our data suggest that Hck is a novel binding partner for ACK1 that can regulate ACK1 activity by phosphorylation.

Members of the Rho family of small GTP-binding proteins couple extracellular signals to the regulation of cell morphology, adhesion, differentiation, and proliferation (1–5). A number of proteins have been identified as targets for the Rho family proteins Rac and/or Cdc42, including p21-activated kinases (6–8), Wiscott-Aldrich syndrome proteins (9–11), mixed lineage kinases (12), and IQGAP (13–16). The ACK family nonreceptor tyrosine kinases (ACK1 and ACK2) associate specifically with Cdc42 and act as Cdc42 effectors in several signaling pathways (6, 17, 18). ACK1 has been reported to phosphorylate Dbl, a guanine nucleotide exchange factor toward Rho family proteins, thereby promoting Dbl activity. In this way, ACK1 is thought to act as a mediator of EGF1 signals to Rho family GTP-binding proteins (19). ACK2 mediates cell adhesion signals initiated by integrin β1 in a Cdc42-dependent manner (20). ACKs interact directly with the clathrin heavy chain and participate in the regulation of receptor-mediated endocytosis (21, 22). However, the physiological functions as well as specific target molecules of ACKs are still incompletely understood.

The domain structure of ACK kinases consists of an N-terminal tyrosine kinase catalytic domain followed by an SH3 domain, a Cdc42/Rac interactive binding (CRIB) domain, and a proline-rich region (see Fig. 1A) (18). The position of the SH3 domain C-terminal to the catalytic domain is unique among families of cytoplasmic tyrosine kinases and in particular contrasts with the N-terminal SH3 domain observed in the Src, Csk, Abl, Frk, and Tec families. In Src family kinases, two intramolecular interactions tightly regulate enzymatic activity: (i) an interaction between the SH2 domain and the C-terminal tail and (ii) an interaction between the SH3 domain and a polypeptide type II helix in the SH2-kinase linker region (23–25). Exogenous ligands for the SH2 and SH3 domains can disrupt the autoinhibitory interactions, promote autophosphorylation at Tyr416, and stimulate Src kinase activity (25–27). The SH2 and SH3 domains of Src kinases also play an important role in substrate recognition (28, 29). The SH3 domains of Abl family kinases are likewise involved in autoinhibition and substrate binding. ACK2 has been shown to be activated by Cdc42 binding to the CRIB region (20, 30). However, the roles of the SH3 domain and proline-rich region of ACK in enzyme regulation have not been elucidated.

Apart from Cdc42 and clathrin, relatively few ACK-interacting proteins have been identified. The C-terminal proline-rich domain of ACK is involved in interaction with the Nck (22) and Grb2 (31) adaptor proteins and with sorting nexin protein 9 (SH3PX1) (32, 33). Co-immunoprecipitation studies indicate that ACK2, clathrin, and SH3PX1 form a complex; ACK2 cooperates with SH3PX1 to promote degradation of EGF receptor (32). HSH2, an adaptor protein found in hematopoietic cells, binds to the SH3 domain of ACK1 (34). Because of the importance of the SH3 domains in Src kinase substrate targeting, it is likely that additional substrates/effectors of ACK kinases will be found that bind to the SH3 domain.

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The abbreviations used are: EGF, epidermal growth factor; EGFR, EGF receptor; GST, glutathione S-transferase; CRIB, Cdc42/Rac interactive binding; HA, hemagglutinin; Ni-NTA, nickel-nitrilotriacetic acid; MOPS, 4-morpholinepropanesulfonic acid; LC, liquid chromatography; MS, mass spectrometry; HPLC, high pressure liquid chromatography; GMP-PCP, β,γ-methylene guanosine 5′-triphosphate.
The goal of these studies was to investigate several of these questions using purified polypeptide components. We report the first purification of active ACK from eucaryotic cells, using the Sf9/baculovirus expression system. We screened several synthetic peptides and show that the substrate specificity of ACK1 most closely resembles that of Abl. We also identified Tyr644 as the major autophosphorylation site of ACK1 and show that autophosphorylation enhances kinase activity. Neither an SH3 ligand nor Cdc42 activates ACK kinase on an Abl family kinase substrate. The bacterial expression vector pCR-BluntII-TOPO was used as an interacting partner of ACK. The Hck SH3 domain interacts with the proline-rich region of ACK1. Finally, we present evidence that ACK is phosphorylated by Hck.

**EXPERIMENTAL PROCEDURES**

**Materials**—Monoclonal antibodies against phosphotyrosine (4G10) and Hck were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-HA monoclonal antibody and anti-ACT and anti-Hck polyclonal antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A rabbit polyclonal antibody was raised against a peptide sequence in the kinase domain of ACK (KPDVLSQPEAMDDFT) by GeneMed Synthesis, Inc. (South San Francisco, CA). The antibody was partially purified by ammonium sulfate precipitation. pBACgus-9 Transfer plasmid and Bacvector-3000 DNA transfection kits were from Novagen (Madison, WI). Protein A-agarose was from Sigma. Glutathione-agarose and glutathione S-transferase (GST) antibody were purchased from Molecular Probes (Eugene, OR). Ni-NTA affinity resin was from Qiagen, and Affi-Gel-15 was from Bio-Rad. The mammalian expression plasmids pXJ-HA for wild type and K158R ACK1 and bacterial plasmids encoding GST fusions with the proline-rich region of ACK1 (amino acids 584–642 and 670–738) were gifts from Dr. Edward Manser (Glaxo-IMCB Group, Institute of Molecular and Cell Biology, Singapore). The mammalian expression plasmid encoding polyhistidine tag; Novagen) and expressed in Sf9 cells in the Sf9/baculovirus expression system. We screened several peptide substrates, and the peaks of activity were pooled, concentrated, and applied to a Mono Q fast protein liquid chromatography column. The column was washed with buffer containing 0.5 mM idazole, 0.5 mM NaCl, 2 mM Na2VO4, 10% glycerol, 5 mM 2-mercaptoethanol, 20 mM Tris-HCl (pH 8.0), and 1 mM ACK1 was eluted with buffer containing 150 mM imidazole, 0.5 mM NaCl, 2 mM Na2VO4, 10% glycerol, and 20 mM Tris-HCl (pH 8.0). The peaks of activity were pooled, concentrated, and applied to a Mono Q fast protein liquid chromatography column. The column was washed with buffer containing 0.05–0.6 mM NaCl, and the proteins were eluted with a linear gradient of 0.05–0.6 mM NaCl in the same buffer. The Y284F mutant version of the kinase-SH3-CRIB ACK1 construct was purified by the same column steps.

Full-length ACK1 was partially purified by similar column steps. To confirm that the kinase activity present was due to full-length ACK1 and not to co-purifying kinases, we carried out a parallel purification of kinase-inactive (K158R) ACK1. The K158R mutant sample had ~7% of the activity of wild type ACK1.

**ACK1 Kinase Assay Using Synthetic Peptide Substrates**—ACK1 kinase activity was determined using the phosphocellulose paper assay. Reaction mixtures contained 20 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 0.1 mM Na2VO4, 0.5 mM diethiothreitol, 0.25 mM ATP, varying concentrations of peptide substrate, and [γ-32P]ATP (200–400 cpm/pmol). The reactions were terminated by the addition of 50% acetic acid, and the samples were spotted on p81 phosphocellulose paper (36). Incorporation of 32P into peptide was determined by liquid scintillation counting. For each peptide substrate, the value of K for enzyme activity was determined using a range of peptide concentrations (0.05–0.25) and 0.25 mM [γ-32P]ATP. Kinetic parameters were calculated by fitting data to the Michaelis-Menten equation. Initial studies on several peptides (Src, EGFR, IRS-1, SH3, and IR peptides) established that their Michaelis constants were in the millimolar range (>2 mM). Thus, it was not possible to determine accurate Michaelis constants by using initial rate kinetics. The complete time course for the phosphorylation of each of these peptides was measured by using peptide concentrations less than the Michaelis constant. We analyzed the data graphically as described (37) to determine K for enzyme activity.

**Determine the effects of autophosphorylation on enzyme activity, ACK1 was autophosphorylated with 0.5 mM ATP for 1 h at 30 °C. Autophosphorylated enzyme was then assayed with a range of peptide concentrations (0.25–2.0 μM) and 0.25 mM [γ-32P]ATP. Kinetic parameters were calculated by fitting data to the Michaelis-Menten equation.

**Autophosphorylation of ACK1**—Purified ACK1 was incubated with 0.25 mM [γ-32P]ATP (400–700 cpm/pmol) in kinase buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 0.5 mM diethiothreitol, and 0.1 mM Na2VO4, at 30 °C. In some experiments, ACK1 was preincubated with 0.25 mM unlabeled ATP in kinase buffer for 40 min, followed by incubation with [γ-32P]ATP for 40 min at 30 °C. The reactions were stopped by the addition of SDS sample buffer and analyzed by SDS-PAGE and autoradiography. To measure 32P in ACK1, the procedure was repeated by incubating with [γ-32P]ATP. ACK1 was incubated with 0.5 mM unlabeled ATP in kinase reaction buffer for 40 min at 37 °C. The activity was then monitored using the phosphocellulose paper assay.

**Peptide Binding Assays—Synthetic peptides (1.1 mM) were coupled to Affi-gel 15 (Bio-Rad) in 0.1 M MOPS buffer (pH 7.5). After treatment with ethanolamine to unreacted sites, the gel was manually washed with 0.1 M MOPS buffer and suspended in 20 mM Tris-HCl (pH 8.0) buffer as a 50% slurry. As a control, Affi-gel 15 was blocked with 1% ethanolamine and washed in the same way. The cell lysates (1 mg of protein) from ACK1-overexpressing Sf9 cells were incubated with 6 μl of polyproline peptide-coupled Affi-gel or control gel at 4 °C overnight. The gels were centrifuged, washed with phosphate-buffered saline, and washed with phosphate-buffered saline containing 0.5% Nonidet P-40 four times. The bound proteins were eluted with SDS sample buffer and subjected to SDS-PAGE. The proteins were analyzed by Western blotting with ACK antibody.

**Mass Spectrometry**—Matrix-assisted laser desorption/ionization
time of flight mass spectrometry was carried out on an API QSTAR Pulsar LC/MS/MS system (Applied Biosystems/MDS Sciex, Foster City, CA) equipped with a Protana nanospray source (Protana Engineering A/S, Starmosegardsvej, Denmark) and an UltiMate Capillary HPLC System (LC Packings, San Francisco, CA). ACK1 or autophosphorylated ACK1 (4 μg) was subjected to SDS-PAGE, and the proteins were stained with Coomassie Brilliant Blue. ACK1 bands were excised from the gel, cut into small pieces, and transferred to siliconized microcentrifuge tubes. After washing with 50% methanol and 5% acetic acid, the gel fragments were dehydrated by the addition of acetonitrile. The proteins were reduced and alkylated by the addition of dithiothreitol and iodoacetamide and digested with trypsin in 50 mM ammonium bicarbonate overnight at 37°C. The tryptic peptides were extracted by treatment with 50% (v/v) acetonitrile and 5% (v/v) formic acid in water.

**Cell Culture and Transfection**—Cos-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 1% antibiotic-antimycotic (penicillin/streptomycin/amphotericin; Invitrogen) at 37°C. Cos-7 cells were cultured to 70% confluence in 100-mm dishes. The transfections were performed in OPTI medium (Invitrogen) using 10 μg of plasmid DNA and TransIT polyamine transfection reagent (Mirus) according to the manufacturer’s instructions. The cells were harvested 40 h after transfection.

**Immunoprecipitation and Western Blotting**—Cos-7 cells were harvested, washed twice with ice-cold phosphate-buffered saline, and lysed in buffer containing 50 mM Tris-HCl (pH 7.2), 5 mM EDTA, 2 mM Na3VO4, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The postnuclear lysate was precleared with protein A-agarose and used for immunoprecipitation with the appropriate antibody. For experiments using Sf9 cells, the lysates were centrifuged at 40,000 × g for 30 min, and the supernatants were used for immunoprecipitations. After addition of 10 μl of protein A-agarose, the incubations were continued 5 h or overnight at 4°C. The resin was collected and washed four times with phosphate-buffered saline plus 0.5% Nonidet P-40. The precipitated proteins were analyzed on 8% SDS-polyacrylamide gels and transferred to Immobilon membrane (Millipore, Bedford, MA) in the presence of 0.1% SDS. The membranes were blocked using 5% milk in Tris-buffered saline plus 0.1% Tween 20 and then probed with the appropriate antibodies. The blots were visualized using horseradish peroxidase-conjugated secondary antibody with ECL (Amersham Biosciences).

**Pull-down Experiments Using GST Fusion Proteins**—The following SH3 and SH2 domains were expressed in Escherichia coli NB42 cells as GST fusion proteins: Src SH2, Src SH2/SH3, SH2 domain-containing protein phosphatase 2 tandem SH2, Nck SH2, Nck SH3, Hck SH2, Hck SH3, Grb2 SH3, and Crk SH3. The fusion proteins were purified on glutathione-agarose, as described previously (38).

**Lysates from Sf9 cells expressing full-length ACK1 or ACK1 kinase-SH3-CRIB (0.33–1 mg of protein) were incubated with glutathione-agarose containing immobilized GST fusion proteins overnight. After gels were washed with phosphate-buffered saline containing 0.5% Nonidet P-40, the bound proteins were eluted with SDS-PAGE sample buffer. The samples were subjected to SDS-PAGE and analyzed by Western blotting with anti-Ack or anti-GST antibody.**

**Biochemical Properties of ACK1**

**Fig. 1. Purification of ACK1 kinase-SH3-CRIB construct.** A, domain structure of ACK1 showing the kinase-SH3-CRIB domains that were purified in this study. B, left panel, proteins from each step of the purification were separated by SDS-PAGE and visualized with Coomassie Brilliant Blue. MW, molecular weight. Lane 1, crude Sf9 cell lysate. Lane 2, pooled fractions after Source-Q chromatography. Lane 3, pooled fractions after Ni-NTA chromatography. Lane 4, pooled fractions after Mono Q chromatography. Right panel, purified ACK1 was analyzed by Western blotting using anti-phosphotyrosine (P-Tyr) and anti-Ack antibodies.
SDS, 1% Nonidet P-40, 0.25% deoxycholate, 5 mM β-mercaptoethanol, and 2 mM imidazole four times. The bound proteins were eluted by SDS sample buffer and analyzed by SDS-PAGE and Western immunoblotting with anti-GST and anti-ACK antibodies.

Pull-down Experiments with ACK Proline-rich Region—GST fusion proteins containing the ACK1 proline-rich regions (584–642 and 670–738) were immobilized on glutathione-agarose. The purified SH3 domain of Hck was incubated with the proline-rich regions (or glutathione-agarose as a control) at 4°C overnight. The gels were washed with 10 mM Tris-HCl (pH 7.5), and bound SH3 domain was analyzed by SDS-PAGE with silver staining.

In Vitro Phosphorylation of Full-length ACK by Hck—Plasmids encoding wild type, K158R, or Y284F ACK1 were transfected into Cos-7 cells. ACKs were isolated by immunoprecipitation with ACK antibody. The immunoprecipitated proteins were incubated in the presence or absence of purified Hck (0.24 µg) with 0.25 mM [γ-32P]ATP (400 cpm/pmol) in kinase buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 0.5 mM dithiothreitol, and 0.1 mM Na₂VO₄ at 30°C for 30 min. The reactions were terminated with SDS sample buffer and subjected to SDS-PAGE. Phosphorylation of ACK was detected by autoradiography.

**RESULTS**

Purification of ACK1 and Characterization of Substrate Specificity—Our first objective in this study was to obtain a sample of purified ACK for enzymatic studies. We produced full-length ACK1 by infection of Sf9 cells with a recombinant baculovirus vector. This version of ACK1 was tagged at the N terminus with a hexahistidine tag. The reactions were terminated with SDS sample buffer and subjected to SDS-PAGE. Phosphorylation of ACK was detected by autoradiography.
stable consisted of the kinase catalytic domain, SH3 domain, and CRIB domain (Fig. 1A). This version of ACK1 possesses CBD and His tags at the C terminus. We purified the kinase-SH3-CRIB construct to homogeneity by chromatography on Source-Q, Ni-NTA, and Mono Q columns (Fig. 1B).

Kinase activity was monitored during the purification by the phosphocellulose paper binding assay, using the Src peptide as a substrate. The purified ACK1 migrates with the expected molecular mass (~60.4 kDa). ACK1 reacted with a rabbit polyclonal antibody raised against a peptide sequence in the ACK1 catalytic domain, as well as with anti-phosphotyrosine antibody, suggesting that the purified protein is autophosphorylated or phosphorylated by an endogenous Sf9 cell kinase (Fig. 1B).

The peptide/protein substrate specificity for ACK kinases has not previously been investigated. We carried out experiments using six peptide substrates containing recognition motifs for different subfamilies of tyrosine kinases. Two of the peptides contain motifs that are preferred by nonreceptor tyrosine kinases (Src and Abl), and two peptides contain motifs that are preferred by receptor tyrosine kinases (EGF receptor and insulin receptor). Another substrate is derived from the sequence of IRS-1 and has been shown to be an excellent substrate for receptor tyrosine kinases (EGF receptor and insulin receptor). Finally, we included a substrate that possesses an SH3 domain-binding polyproline sequence. For Src family kinases, the addition of an SH3 domain ligand to a substrate increases its phosphorylation by activating down-regulated kinase and by decreasing substrate $K_m$ (41). The purified ACK1 kinase-SH3-CRIB construct clearly preferred the Abl substrate from this group of peptides (Fig. 2A). We carried out kinetic measurements with saturating concentrations of ATP and varying concentrations of Abl peptide. These experiments yielded a $K_m$ for the Abl peptide of 507 μM and a $k_{cat}$ of 0.249 min$^{-1}$ ($k_{cat}/K_m = 4.8 \times 10^{-4}$ min$^{-1}$ μM$^{-1}$) (Table I). The next best substrate for ACK1, the Src-specific peptide, was phosphorylated with a $k_{cat}/K_m$ of $2.2 \times 10^{-5}$ min$^{-1}$ μM$^{-1}$, ~20 times lower than the Abl substrate (Table I). Phosphorylation of the EGFR- and insulin receptor-specific peptides was barely detectable above background. Despite the presence of a functional SH3 domain in this ACK1 construct (see below), the SH3-binding substrate was not phosphorylated appreciably by ACK1 (Fig. 2A).

Peptides containing either a longer spacer length between the substrate sequence and the SH3 ligand or with these sequences reversed were also poorly phosphorylated (data not shown). We carried out a binding experiment to confirm that the SH3-binding substrate used in these experiments was able to bind to ACK1. ACK1 bound to the immobilized SH3-binding substrate but not to a control peptide with mutations in the critical proline residues (Fig. 2A). This is in contrast to results from Src family kinases and suggests that the SH3 domain of ACK1 may not play a direct role in substrate targeting.

The preference of ACK1 for the Abl-specific peptide prompted us to test whether ACK1 was inhibited by Gleevec (STI-571), a specific Abl inhibitor that is an effective therapeutic agent against chronic myelogenous leukemia (42). Alignment of the sequences of Abl and ACK1 showed that Thr$^{315}$ of Abl, an important determinant for STI-571 binding (43), is conserved in ACK1. Other Abl residues that make contact with STI-571 are not conserved in ACK1. We tested various concentrations of STI-571 and found that this compound is a relatively poor inhibitor of ACK1, with an IC$_{50}$ value of 235 μM (Fig. 2B). In contrast, we showed using similar methods that the Abl catalytic domain is inhibited with a $K_i$ of 37 nM (43).

**Autophosphorylation of ACK1**—For many tyrosine kinases, autophosphorylation within the activation loop (in the catalytic domain) produces a conformational change that increases enzymatic activity. Because we detected tyrosine phosphorylation in purified ACK1 (Fig. 1B), we investigated whether ACK1 activity is controlled by autophosphorylation. ACK1 was incubated with [$\gamma$-$^{32}$P]ATP in kinase buffer, and the reaction mixtures were analyzed by SDS-PAGE and autoradiography. ACK1 was autophosphorylated in a time-dependent manner, and preincubation of ACK1 with unlabeled ATP reduced the level of autophosphorylation (Fig. 3A). We investigated the effect of autophosphorylation by preincubating ACK1 with ATP and then measuring phosphorylation of the Abl synthetic peptide substrate. In these experiments, autophosphorylation activated ACK1 kinase 3-fold as compared with unphosphorylated ACK1 (Fig. 3B). We note that the ACK1 used in these experiments contained a basal level of phosphorylation after purification from Sf9 cells (Fig. 1B), so we consider the activation observed here to be a lower limit to the amount of kinase activation upon autophosphorylation.

To further characterize the effect of autophosphorylation on ACK1 activity, we measured kinetic parameters using the autophosphorylated enzyme. In the presence of saturating concentrations of ATP and varying concentrations of Abl peptide substrate, we determined that the $K_m$ for the peptide was 0.68 mM. The value for $k_{cat}$ was increased to 0.97 min$^{-1}$ (versus 0.25 min$^{-1}$ for unphosphorylated ACK1), and the value for $k_{cat}/K_m$ (14.03 $\times$ 10$^{-4}$ min$^{-1}$ μM$^{-1}$) was increased ~3-fold with respect to the unphosphorylated enzyme ($k_{cat}/K_m = 4.8 \times 10^{-4}$ min$^{-1}$ μM$^{-1}$).
autophosphorylated peptide by sequence analysis by LC/MS/MS. Based on a sequence alignment of the ACK kinase catalytic domain with the Src catalytic domain, Tyr\textsuperscript{284} of ACK1 is predicted to be in the kinase activation loop. The sequence surrounding Tyr\textsuperscript{284} of ACK1 is similar to the sequence surrounding Tyr\textsuperscript{416} of Src, the major autophosphorylation site.

To investigate the importance of Tyr\textsuperscript{284} phosphorylation in intact cells, we expressed full-length wild type ACK1 and a Tyr\textsuperscript{284}F mutant in COS-7 cells. The expression of the mutant was similar to that of wild type (Fig. 4B). The Y284F mutation dramatically reduced tyrosine phosphorylation of ACK1 (Fig. 4B). This suggests that Tyr\textsuperscript{284} is the major autophosphorylation site on ACK1.

**Inability of SH3 or CRIB Domain Ligands to Activate ACK1**—It is not clear whether the SH3 domains of ACK kinases play a role in regulating enzyme activity. For Src and Abl family nonreceptor tyrosine kinases, the SH3 domains bind to proline-rich type II helices in the linker region between the SH2 domain and the catalytic domain. These intramolecular interactions, together with interactions involving the SH2 domains, stabilize inactive conformations of the kinases. Exogenous ligands for the SH3 domains of Src or Abl family kinases disrupt these intramolecular interactions and potently stimulate enzymatic activity (25–27). One significant difference between these kinases and ACK1 is that in ACK1 the SH3 domain is C-terminal to the catalytic domain, and it is unlikely that the kinase would assemble into a similar inactive structure. To investigate the role of the ACK1 SH3 domain in regulating kinase activity, ACK1 was preincubated with varying concentrations of a polyproline peptide (DFPLGPPPPLPPRATPSR), and kinase activity was measured using 0.5 mM Abl peptide as substrate. As shown in Fig. 5A, the polyproline peptide did not activate ACK1. To confirm that the SH3 domain of purified ACK1 is functional and that the polyproline peptide can bind to the ACK1 SH3 domain, we carried out pull-down experiments with the immobilized peptide. The kinase-SH3-CRIB construct was then determined using the phosphocellulose binding assay.

**Interaction of ACK1 with Hck**—The C terminus of ACK1 contains a proline-rich domain with the potential to interact with cellular proteins containing SH3 domains. This region of ACK2 has previously been shown to interact with the SH3 domains of Src (44), Grb2 (31), and Nck (22). To screen for potential binding partners of ACK1, we tested the ability of ACK1 to bind to a variety of immobilized SH2 and SH3 domains (the SH2 domains of Src, SH2 domain-containing protein phosphatase 2, Nck, and Hck; the SH2/SH3 domains of Src; and the SH3 domains of Hck, Nck, Crk, and Grb2). These GST fusion proteins were immobilized on glutathione-agarose and incubated with lysates from SF9 cells overexpressing full-length ACK1. Bound proteins were analyzed by SDS-PAGE and Western blotting using anti-ACK antibody. In agreement with previous reports, ACK1 interacted with the Src SH2, Src SH2/SH3, and Nck SH2 domains (Fig. 7A). ACK1 did not interact with the SH2 domain-containing protein phosphatase 2 SH2 domain. We observed a particularly strong interaction between ACK1 and the SH3 domain of Hck (Fig. 7, A and B). We compared ACK1 binding to four different SH3 domains and found that the Hck SH3 domain bound most strongly, followed by the Grb2 SH3 domain. Nck SH3 gave weaker binding,
Fig. 4. Identification of Tyr284 autophosphorylation site. A, identification of ACK1 autophosphorylation sites by LC/nanoelectrospray mass spectrometry. The purified ACK1 kinase-SH3-CRIB construct (4 μg) was incubated without (Sample 1, top panel) or with (Sample 2, bottom panel) 0.25 mM ATP for 2 h at 30 °C. The reactions were stopped by addition of SDS sample buffer and analyzed by SDS-PAGE. The ACK1 bands were stained with Coomassie Brilliant Blue, excised from the gel, and subjected to in-gel trypsin digestion. The samples were analyzed by LC/nanoelectrospray mass spectrometry as described under “Experimental Procedures.” The only peptide that was present in Sample 1 in the unphosphorylated form and in Sample 2 in the phosphorylated form is shown. The identity of this phosphorylated peptide containing Tyr284 was verified by LC/MS/MS sequencing. B, wild type ACK1 or a Y284F mutant were expressed in COS-7 cells. The cells were lysed, and the proteins were immunoprecipitated with polyclonal anti-ACK antibody or rabbit IgG as control. In the top and middle panels, immunoprecipitated proteins were analyzed with anti-phosphotyrosine and ACK antibodies, respectively. The bottom panel shows expression of wild type and Y284F ACK1 in COS-7 cell lysates.
whereas the Crk SH3 domain did not bind significantly in this experiment (Fig. 7B). We compared the ability of full-length ACK1 and the kinase-SH3-CRIB construct to bind to the Hck SH3 domain. Lysates from Sf9 cells expressing the two forms of ACK1 (full-length and kinase-SH3-CRIB constructs) were incubated with immobilized GST-HckSH3 domain, and the bound proteins were analyzed by SDS-PAGE and anti-ACK Western blotting. As shown in Fig. 7C, only full-length ACK1 was able to interact with the Hck SH3 domain. These data suggest that the Hck SH3 domain interacts with the C-terminal proline-rich domain of ACK1. To confirm this, we carried out binding experiments with the proline-rich regions of ACK1 (residues 584–642 and 670–738). We expressed these regions as GST fusions and immobilized them on glutathione-agarose. Purified Hck SH3 domain (un-tagged) bound to the proline-rich regions but not to GST (Fig. 7D).

The Hck SH2 domain also interacted with ACK1 (Fig. 7A). To test whether the binding site for the Hck SH2 domain falls in the kinase-SH3-CRIB domains of ACK1, we carried out a binding experiment in which the kinase-SH3-CRIB construct was immobilized on Ni-NTA resin. GST-HckSH2 domain bound to this construct but not to control resin (Fig. 7E). We also tested the possibility that the binding site for the Hck SH2 domain is Tyr^284, the major autophosphorylation site on ACK1. However, Hck SH2 domain still bound to the Y284F mutant form of the kinase-SH3-CRIB construct, suggesting that the binding site is elsewhere (Fig. 7E).

Many proteins that bind to the SH3 domains of Src family tyrosine kinases are excellent substrates. We investigated whether ACK1 is a substrate for Hck. We co-expressed Hck with a kinase-inactive mutant of ACK1 (K158R) in COS-7 cells. We immunoprecipitated the inactive ACK1 and analyzed the results by anti-phosphotyrosine and anti-ACK immunoblotting. K158R ACK1 showed no tyrosine phosphorylation (Fig. 8A). In contrast, co-expression of Hck dramatically enhanced the tyrosine phosphorylation of K158R ACK1, even though co-expression of Hck consistently lowered the level of total ACK1 expression (Fig. 8A). We also carried out in vitro phosphorylation assays using purified Hck plus full-length, kinase-inactive K158R ACK1 that had been immunoprecipitated from COS-7 cells. In agreement with the studies in cells, Hck was able to phosphorylate K158R ACK1 in vitro (Fig. 8B). Hck was
also able to phosphorylate immunoprecipitated Y284F ACK1, suggesting that additional sites exist that are targets for Hck phosphorylation (Fig. 8B).

**DISCUSSION**

The Cdc42-associated ACK kinases form a distinct subfamily of nonreceptor tyrosine kinases. The catalytic domains of ACK kinases are most closely related to the JAK, Syk, and EGF receptor families (45, 46). We report here the first purification of an ACK family member, ACK1. The availability of the purified ACK1 enzyme has enabled us to compare several of the biochemical properties of this class of kinases with other tyrosine kinase families. First, we note that the specific activity of the purified ACK1 is rather low as compared with other baculovirus-expressed nonreceptor tyrosine kinases. For example, the $k_{cat}$ for ACK1 with its preferred substrate was 0.24 min$^{-1}$.
and increases access of ATP and peptide/protein substrates to the active site. Phosphorylation of Tyr^{412} in Abl plays a similar role in regulating kinase activity. We have mapped the major autophosphorylation site of ACK1 as Tyr^{284}, and mutation of Tyr^{284} decreases phosphorylation dramatically when ACK1 is expressed in mammalian cells (Fig. 4B). We show that auto-phosphorylation causes a modest increase in enzyme activity, as measured toward synthetic peptide substrate (Fig. 3B). This increase is driven by an increase in enzyme turnover number rather than by a decrease in $K_m$ for peptide substrate. However, the purified form of ACK1 contains some phosphorylation after isolation from Sf9 cells (Fig. 1), so we postulate that the difference between the down-regulated and autophosphorylated forms of ACK1 is greater in vivo. We obtained evidence for additional sites of tyrosine phosphorylation on ACK1 (for example, the Hck SH2 domain binds to the Y284F mutant); however, we have not yet mapped these sites.

The ACK1 family kinases are unique among nonreceptor tyrosine kinases in that they possess SH3 domains that are C-terminal to the catalytic domains. For Src and Abi family kinases, the SH3 domains have dual roles: they are essential in maintaining the autoinhibited state, and once the enzymes are activated they are also involved in substrate recognition. It has not been clear whether the SH3 domains of ACK kinases play analogous roles. The availability of purified ACK1 has allowed us to investigate these questions. We found that ACK1 was not activated by the addition of a polyproline peptide, despite the ability of this peptide to bind to the ACK1 SH3 domain. This contrasts with Src and Abi family kinases and suggests that the C-terminal SH3 domain of ACK1 is not involved in autoinhibition. The kinase-SH3-CRIB construct we studied lacks the C-terminal proline-rich regions, so it is possible that it cannot adopt an autoinhibited state. However, full-length ACK1 was also not activated by polyproline peptide in vitro, arguing against this possibility. We cannot exclude the possibility that these in vitro experiments do not faithfully reproduce the way that the SH3 domain of ACK1 acts in the cell.

Src kinases preferentially phosphorylate peptide substrates containing SH3 ligands (41), but ACK1 showed no activity toward these peptides, although ACK1 is able to bind to the SH3-binding substrate (Fig. 2A). This suggests that the SH3 domain of ACK1, perhaps because of its position relative to the catalytic domain, may not play a direct role in substrate recognition. In contrast, the C-terminal proline-rich region of ACK is important in directing phosphorylation of the sorting nexin SH3PX1.

In agreement with previous results on ACK2, we found that co-expression of Cdc42 activated ACK1 in mammalian cells. However, the addition of recombinant Cdc42 to purified ACK1 did not stimulate kinase activity. (This result is similar to that of an in vitro experiment previously carried out on ACK2 (17).) As described above, it is possible that the ACK1 sample we purified was already in the active conformation; ACK1 was partially phosphorylated after purification from Sf9 cells (Fig. 1). Full-length ACK1 expressed in Sf9 cells also did not respond to the addition of recombinant activated Cdc42. The discrepancy between the in vitro results and the findings in intact cells may be due to the presence of other co-stimulatory molecules that are necessary for Cdc42 activation of ACK1. Alternatively, Cdc42 may act to alter the subcellular localization of ACK1, indirectly stimulating its activity.

ACK2 has previously been shown to interact with the SH3 domain of Src (44). To test the possibility that the C-terminal polyproline region might target ACK1 to other binding partners, we screened several SH3-containing proteins. We confirmed an interaction between Src and ACK1 and found a
robust interaction with the SH3 domain of the Src family kinase Hck. Binding of polyproline ligands to the SH3 domain of Hck has previously been demonstrated to potently activate the catalytic domain (25). Hck is expressed predominantly in granulocytic and monocyteic cells (50, 51), whereas ACK is broadly expressed in many cell types (17). Hck has been implicated in a wide variety of signaling pathways in hematopoietic cells, including phagocytosis and integrin-mediated signaling (52–57). However, at present relatively few substrates or effectors of Hck have been identified. ACKs interact with clathrin and participate in regulation of receptor-mediated endocytosis (21, 22), and ACK2 also mediates cell adhesion signals initiated by integrin β1 in a Cdc42-dependent manner (20). It is possible that some of these effects are regulated by Hck in hematopoietic cells. One connection could be through the hematopoietic cell adaptor protein HSH2, which binds ACK through its N-terminal proline-rich region and also binds the tyrosine kinase c-Fes (34). HSH2 is postulated to regulate cytokine signaling and cytoskeletal reorganization downstream of tyrosine kinases. In this study, we showed that co-expression of Hck in mammalian expression plasmids for HA-tagged Cdc42. We thank Mike Feigin for assistance with ACK1 purification.

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