Phosphorylation of WASP by the Cdc42-associated Kinase ACK1

DUAL HYDROXYAMINO ACID SPECIFICITY IN A TYROSINE KINASE

Noriko Yokoyama, Julie Lougheed, and W. Todd Miller

From the Department of Physiology and Biophysics, School of Medicine, Stony Brook University, New York 11794 and Exelixis Inc., South San Francisco, California 94083

ACK1 is a nonreceptor tyrosine kinase that associates specifically with Cdc42. Relatively few ACK1 substrates and interacting proteins have been identified. In this study, we demonstrated that ACK1 phosphorylates the Wiskott-Aldrich syndrome protein (WASP), a Cdc42 effector that plays an important role in the formation of new actin filaments. ACK1 and WASP interact in intact cells, and overexpression of ACK1 promotes WASP phosphorylation. Phosphorylation of WASP in vitro was enhanced by the addition of Cdc42 or phosphatidylinositol 4,5-biphosphate, presumably due to release of the autoinhibitory interactions in WASP. Surprisingly, when we mapped the sites of WASP phosphorylation, we found that ACK1 possesses significant serine kinase activity toward WASP (directed at Ser-242), as well as tyrosine kinase activity directed at Tyr-256. A serine peptide derived from the Ser-242 WASP phosphorylation site is also a substrate for ACK1. ACK1 expressed in bacteria retained its serine kinase activity, eliminating the possibility of contamination with a copurifying kinase. Serine phosphorylation of WASP enhanced the ability of WASP to stimulate actin polymerization in mammalian cell lysates. Thus, the tyrosine kinase ACK1 acts as a dual specificity kinase toward this substrate. In contrast to other dual specificity kinases that more closely resemble Ser/Thr kinases, ACK1 is a tyrosine kinase with an active site that can accommodate both types of hydroxyamino acids in substrates.

The nonreceptor tyrosine kinase ACK1 (activated Cdc42-associated kinase-1) is a specific effector kinase for the GTP-bound form of Cdc42. ACK1 is a member of a distinct family of nonreceptor tyrosine kinases that includes ACK1, ACK2, and TNK in mammals and homologs in Drosophila and Caenorhabditis elegans. The catalytic domain sequences of ACK family kinases are characteristic of tyrosine kinases, yet they are divergent from most other families of nonreceptor tyrosine kinases; they are most closely related to the epidermal growth factor receptor family kinases (3, 4). ACK kinases also have a unique tyrosine kinase domain, and overexpression of ACK1 promotes WASP phosphorylation. Thus, ACK kinases are activated by cell adhesion, and they play important roles in cell spreading and motility (5–7). Recent work has also focused on the involvement of ACK kinases in signaling pathways leading to clathrin-mediated receptor endocytosis (8, 9).

Relatively little is known about the physiological substrates of ACK kinases. Upon epidermal growth factor stimulation, ACK2 binds and phosphorylates sorting nexin 9 (SH3PX1), which promotes epidermal growth factor receptor degradation (9). In hematopoietic cells, ACK1 binds and phosphorylates the adaptor protein HSH2 (10). For both HSH2 and SH3PX1, ACK substrate recognition is governed by interactions with the noncatalytic regions of ACK; a proline-rich region of HSH2 binds to the SH3 domain of ACK1, whereas the SH3 domain of SH3PX1 mediates the interaction with the polyproline region of ACK2. The polyproline region of ACK kinases has also been shown to interact with Grb2 (11), Nck (8), Src (7), and Hck (12), although it is not known whether these proteins are physiological substrates for ACK. ACK1 has also been reported to phosphorylate and activate the guanine nucleotide exchange factors Dbl and Ras-GRF1 (13). Identification of additional ACK substrates would aid in investigations into the functional importance of ACK kinases.

In this report, we have identified an interaction between ACK1 and the Wiskott-Aldrich syndrome protein (WASP), a Cdc42 effector that plays an important role in the formation of new actin filaments. WASP family proteins contain a C-terminal VCA (verprolin, central, acidic) domain that binds to actin monomers and the Arp 2/3 actin nucleation complex (14, 15). In its unactivated state, the VCA domain is engaged in autoinhibitory interactions with the Cdc42-binding region of WASP (16). In the presence of Cdc42-GTP and PIP2, the autoinhibitory interactions are disrupted, and WASP stimulates the actin-nucleating activity of the Arp2/3 complex (17, 18). WASP is cooperatively activated by Cdc42 and PIP2, so WASP proteins act as integrating devices that respond maximally in the presence of both input signals (19). In Xenopus egg extracts, Toca-1 has recently been identified as an additional protein factor that is required for Cdc42- and PIP2-induced actin assembly (20). The activity of WASP is also regulated by phosphorylation at Tyr-256 by Hck and other kinases (21, 22). (N-WASP numbering is used throughout this report.) Binding of Cdc42 to WASP increases the accessibility of Tyr-256, promoting phosphorylation and increasing activity toward the Arp2/3 complex. ACK1 and WASP are present in many of the same signaling complexes. Both ACK1 and WASP contain CRIB domains that mediate interactions with Cdc42. We and others have demonstrated that both ACK1 and WASP interact with the Src family kinase Hck in intact cells (12, 22, 23). These findings led us to investigate whether WASP is a substrate for ACK1. Here, we demonstrated that ACK1 phosphorylates...
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WASP in vitro, and co-expression of ACK1 promotes WASP phosphorylation in intact cells. When we mapped the sites of WASP phosphorylation by tandem mass spectrometry, we found that ACK1 catalyzes phosphorylation of Ser-242 as well as Tyr-256 of WASP. We confirmed the intrinsic serine kinase activity of ACK1 using bacterially expressed enzyme. A serine peptide derived from the Ser-242 WASP phosphorylation site is a substrate for ACK1. Serine phosphorylation of WASP enhanced the ability of WASP to stimulate actin polymerization in cell lysates. Thus, the tyrosine kinase ACK1 acts as a dual specificity kinase toward WASP.

EXPERIMENTAL PROCEDURES

Materials—Monoclonal antibody against phosphotyrosine (4G10) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-ACK polyclonal antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) was obtained from Calbiochem. Ni-NTA affinity resin was from Qiagen, and TRITC-phalloidin was from Sigma. Plasmids encoding poly-histidine-tagged WASP (mini-N-WASP, 178–274-GSGSSGSGSG-392–501) and full-length rat N-WASP were gifts from Dr. Wendell A. Lim (University of California (UCSF), San Francisco, CA). A mammalian expression vector encoding a WASP fusion with green fluorescent protein was produced by subcloning N-WASP DNA into pEGFP-C3 (Clontech). Peptides were synthesized using Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry, purified by preparative reversed-phase high performance liquid chromatography, and characterized by matrix-assisted laser description/ionization time-of-flight mass spectrometry.

ACK1 was expressed and purified using the Sf9/baculovirus system, as described previously (12). This construct contains the ACK1 catalytic, SH3, and CRIB domains and poly-His and chitin-binding domain as described previously (12). This construct contains the ACK1 catalytic, SH3, and CRIB domains and poly-His and chitin-binding domain tags at the C terminus. To remove the tags, ACK1 was digested with thrombin, and untagged ACK1 was then refurplied with Mono Q-Sepharose or Superdex-200 chromatography. K158R ACK1 was produced by similar procedures.

ACK1 Activity Assay—ACK1 kinase activity was determined using the phosphocellulose paper assay, as described previously (12). Reaction mixtures contained 20 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 0.1 mM Na₃VO₄, 0.5 mM dithiothreitol, 0.25 mM ATP, varying concentrations of peptide substrate, and [γ³²P]ATP (200–400 cpm/pmol). Incorporation of ³²P into peptide was determined by liquid scintillation counting.

Purification of Mini-WASP—Mini-WASP was expressed in Escherichia coli BL21 cells. Protein expression was induced by the addition of 0.25 mM isopropyl-1-thio-β-D-galactopyranoside for 3.5 h. Cells (0.3 liters) were harvested and lysed in a French pressure cell in 20 mM Tris-HCl buffer (pH 8.0) containing 2 mM Na₃VO₄, 5 mM 2-mercaptoethanol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. The cell lysates were centrifuged at 40,000 × g for 30 min, and supernatants were applied to a Ni-NTA column (3 ml). The column was washed with buffer containing 15 mM imidazole, 0.3 M NaCl, 2 mM Na₃VO₄, 10% glycerol, 5 mM 2-mercaptoethanol, 20 mM Tris-HCl (pH 8.0) further washed with buffer containing 1 M NaCl, 2 mM Na₃VO₄, 5 mM 2-mercaptoethanol, 20 mM Tris-HCl (pH 8.0). Mini-WASP was eluted with buffer containing 100 mM imidazole, 5 mM 2-mercaptoethanol, 2 mM Na₃VO₄, 10% glycerol, and 20 mM Tris-HCl (pH 8.0). To produce the version of mini-WASP lacking tyrosines (YW-WASP), site-directed mutagenesis was carried out using the QuickChange mutagenesis system (Stratagene). Without further analysis with variable collision energy at 30, 40, and 50 eV. Several fragment ions (b₁₁, b₁₂, b₁₃, y₁₁, y₁₂, and y₁₃) with the phospho-moiety
were detected in the product ion spectrum at 30 eV of collision energy, indicating that the serine residue was phosphorylated. Phosphorylation of Tyr-256 was established by a similar procedure.

**Cell Culture and Transfection**—U937 cells were cultured in RPMI 1640 medium with 10% fetal calf serum and 1% antibiotic-antimycotic (penicillin, streptomycin, amphotericin, Invitrogen). COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 1% antibiotic-antimycotic at 37 °C. COS-7 cells were cultured to 70% confluence in 100-mm dishes. 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. 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. After the addition of 1 μl of protein A-agarose, incubations were continued 5 h or overnight at 4 °C. The resin was collected and washed twice with phosphate-buffered saline plus 0.5% Nonidet P-40. The precipitated proteins were analyzed by 10% SDS-PAGE and Western immunoblotting with the appropriate antibodies. Blots were visualized using horseradish peroxidase-conjugated second antibody with ECL (enhanced chemiluminescence, Amersham Biosciences).

**In Vitro Actin Polymerization Assay**—Ni-NTA beads were loaded with His-tagged mini-WASP (wild type or mutants). To phosphorylate immobilized mini-WASP, the beads were incubated with ACK1 that had been purified from Sf9 cells. The stoichiometry of phosphorylation under these conditions was 10–15% (data not shown).

U937 cells were lysed in a buffer containing 50 mM Tris, (pH 7.4), 130 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10 mM NaF, 2 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Lysates were clarified by centrifugation at 15,000 × g for 10 min, and 2 mM MgCl2 was added. Lysates (2 mg of protein) were incubated with 6 μl of immobilized mini-WASP beads for 2 h at 4 °C. Ni-NTA resin was used as control. The beads were washed with washing buffer plus 2 mM MgCl2, three times and once with 20 mM Tris-HCl buffer (pH 8.0) containing 15 mM imidazole, 0.5 M NaCl. Beads were fixed with 3.7% paraformaldehyde in phosphate-buffered saline buffer, washed with phosphate-buffered saline buffer, and stained with TRITC-phalloidin for 1 h. The beads were washed with phosphate-buffered saline three times and mounted on glass slides and dried. Images were obtained on a Zeiss Axiovert 200M wide-field fluorescence microscope. Images were captured using a Zeiss AxioCam with AxioVision software (version 4.3). Exposure times were kept constant for all experiments.

**RESULTS**

**ACK1 Phosphorylates WASP**—In our initial studies, we used a minimal fragment of N-WASP (mini-WASP) consisting of the control region linked to the VCA domain; this fragment retains the regulatory properties of full-length N-WASP (19). We expressed a construct consisting of the ACK1 kinase, SH3, and CRIB domains in Sf9 cells and then purified the enzyme as described previously (12). We produced mini-WASP in E. coli. Purified ACK1 phosphorylates mini-WASP in vitro (Fig. 1A). As described previously for Src kinases (21, 25), the addition of Cdc42 or PIP2 enhanced the phosphorylation of mini-WASP by ACK1, presumably by disrupting the autoinhibited conformation (Fig. 1A).

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In U937 monocytic cells, endogenous levels of ACK1 and WASP interact, as determined by co-immunoprecipitation (Fig. 1B). The two proteins also interact in ACK1-transfected COS-7 cells, as shown by co-immunoprecipitation using ACK1 or WASP antibodies (Fig. 1B). To test for ACK1-mediated phosphorylation of WASP, we carried out anti-WASP immunoprecipitation reactions from untransfected or ACK1-transfected COS-7 cells. Precipitated proteins were analyzed by SDS-PAGE and anti-phosphotyrosine immunoblotting. Tyrosine phosphorylation of WASP was increased by ACK expression (Fig. 1C), indicating that ACK1 can promote tyrosine phosphorylation of WASP in intact cells.

**ACK1 Acts as a Dual Specificity Kinase toward WASP**—To characterize the sites of phosphorylation on WASP, we treated bacterially expressed mini-WASP with purified ACK1 in the presence of [γ-32P]ATP and followed the reaction by autoradiography. Surprisingly, treatment of phosphorylated WASP with YOP, the Yersinia tyrosine-specific phosphatase, did not decrease phosphorylation of WASP (Fig. 2A). In contrast, treatment with the nonspecific (Ser/Thr/Tyr) acid phosphatase eliminated WASP phosphorylation (Fig. 2A), raising the possibility that WASP is phosphorylated on Ser/Thr residues. Treatment of tyrosine-phosphorylated p130Cas with YOP confirmed the activity of YOP under these conditions (Fig. 2B). There is only one tyrosine (equivalent to Tyr-256) in mini-WASP, and we mutated this tyrosine to phenylalanine. The YF-WASP mutant was phosphorylated by recombinant ACK1 (Fig. 2A), and phosphorylation was reversed by...
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![Diagram](https://via.placeholder.com/150)

**FIGURE 2.** ACK1 displays dual specificity. A, in vitro phosphorylation reactions were carried out on wild-type (WT) WASP or the mutant form lacking tyrosines (YF-WASP). Reactions were untreated (−) or treated with YOP tyrosine phosphate or acid phosphatase (AP). Detection was by autoradiography. B, treatment of phosphorylated Cas with YOP under similar conditions.

![Diagram](https://via.placeholder.com/150)

**FIGURE 3.** Phosphoamino acid analysis of WASP and ACK1. A, PAA of wild-type (WT) WASP phosphorylated by ACK1. pS, phospho-Ser; pT, phospho-Thr; pY, phospho-Tyr. B, PAA of an ACK1 autophosphorylation reaction. C, PAA of YF-WASP phosphorylated by untagged ACK1. D, PAA of WASP after reaction with kinase-inactive ACK1 (K158R). E, PAA of an autophosphorylation reaction with K158R.

![Diagram](https://via.placeholder.com/150)

**FIGURE 4.** GST-ACK1 reactions. A, phosphorylation of WT (WT) WASP or YF-WASP by GST-ACK1. Detection was by autoradiography. B, PAA of YF-WASP phosphorylated by GST-ACK1. pS, phospho-Ser; pT, phospho-Thr; pY, phospho-Tyr.

Treatment with acid phosphatase but not YOP (Fig. 2A). The results suggest that ACK1 has the capacity to phosphorylate Ser/Thr residues on WASP. In ACK1-transfected COS-7 cells, we detected serine phosphorylation of WASP by anti-WASP immunoprecipitation and Western blotting with phospho-Ser antibodies (Fig. 1C, pSer).

To analyze the phosphorylation in more detail, we carried out phosphoamino acid analysis (PAA) of ACK1-phosphorylated wild type and YF-WASP. As shown in Fig. 3A, ACK1 phosphorylates serine, threonine, and tyrosine residues of WASP. The signal for serine/threonine phosphorylation appeared to be stronger than that for tyrosine phosphorylation. We incubated pure ACK1 with [γ-32P]ATP in the absence of WASP to study ACK1 autophosphorylation. Phosphoamino acid analysis of this reaction also showed the presence of phospho-Ser, phospho-Thr, and phospho-Tyr (Fig. 3B, pS, pT, and pY). The ACK1 used in these experiments migrates as a single band on SDS-PAGE (12), but to reduce the possibility that a trace amount of Ser/Thr kinase was present as a contaminant, we cleaved off the C-terminal His and chitin-binding domain tags by thrombin treatment. The untagged ACK1 was then repurified by MonoQ anion exchange chromatography; untagged ACK1 eluted under different conditions from tagged ACK1. We treated YF-WASP with untagged ACK1 together with [γ-32P]ATP. PAA of this reaction showed predominantly phosphoserine (Fig. 3C).

We expressed a kinase-inactive variant of ACK1 (K158R) in Sf9 cells and purified the protein under identical conditions to those used for wild-type ACK1. We incubated K158R ACK1 with wild-type WASP and [γ-32P]ATP, but no phosphorylation of WASP was detected by PAA (Fig. 3D). Similarly, incubation of K158R ACK1 with [γ-32P]ATP in the absence of WASP failed to show a signal for ACK1 autophosphorylation by PAA (Fig. 3E). These results for kinase-inactive ACK1 confirmed that WASP phosphorylation was due to the intrinsic kinase activity of ACK1.

To eliminate the possibility that serine phosphorylation could be due to a contaminant in the ACK1 preparation, we expressed ACK1 in bacterial cells, which lack serine/threonine kinases. We subcloned the ACK1 fragment (kinase-SH3-CRIB) into the bacterial expression vector pGEX4T. This GST-ACK1 fusion protein was expressed in E. coli BL21 cells and purified on glutathione-agarose. Although the specific activity of the bacterial ACK1 was lower than the enzyme expressed in Sf9 cells, the bacterial enzyme was still able to phosphorylate wild-type mini-WASP or the YF mutant lacking all tyrosines (Fig. 4A). Phosphoamino acid analysis of the reaction between GST-ACK1 and the YF mutant form of WASP confirmed the serine kinase activity of the enzyme (Fig. 4B).

Identification of ACK1-catalyzed Serine and Tyrosine Phosphorylation Sites on WASP—Next, we analyzed the phosphorylation sites on mini-WASP by tandem mass spectrometry. ACK1-phosphorylated mini-WASP was digested with trypsin, and the fragments were analyzed by nano-liquid chromatography/electrospray mass spectrometry (nano-LC/ES/MSMS). A major phosphopeptide (NLFDMCGGипSEAQLK) was detected; the molecular mass of the peptide showed the 79.96 Da increase from the corresponding unphosphorylated peptide (Fig. 5A). This peptide corresponds to the sequence of mini-WASP from Asn-234 to Lys-247 (numbering as for full-length N-WASP). To directly detect phosphorylation of the single serine residue (Ser-242) within this sequence, the sample was further analyzed with variable collision energy at 30, 40, and 50 eV. Several fragment ions (b11, b12, b13, γ11, γ12, and γ13) with the phospho-moiety were detected in the product ion spectrum obtained at 30 eV of collision energy, confirming the phosphorylation of Ser-242 (Fig. 5A).

Mini-WASP contains only a single tyrosine residue (Tyr-256), and this site has previously been shown to be a target for phosphorylation by Src family nonreceptor tyrosine kinases. Phosphorylation at this site enhances the ability of WASP to promote actin polymerization. Mass spectrometric analysis under similar conditions confirmed the phosphorylation of this site (Fig. 5B). The product ions γy6, γy7, and γy8 from the...
FIGURE 5. Mapping phosphorylation sites. A, identification of Ser-242 site by LC-MSMS. B, identification of Tyr-256 site by LC-MSMS. C, phosphorylation of Ser-242 peptide by purified ACK1. The phosphocellulose paper assay was used.
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MS/MS experiment showed phosphorylation at Tyr-256. Thus, ACK1 behaves as a dual specificity kinase toward WASP, targeting both Ser-242 and Tyr-256. Although our PAA experiments showed the presence of phospho-Thr (Fig. 3A and Tyr-256. Although our PAA experiments showed the presence of phospho-Thr, we were unable to detect phospho-Thr by mass spectrometry.

We synthesized peptides based on the sequences of the WASP phosphorylation sites determined by mass spectrometry. The serine-containing peptide, incorporating the GISEAQLK motif surrounding Ser-242, was an efficient substrate for ACK1 in vitro (Fig. 5C). Peptide sequencing by LC/MS MS/MS confirmed phosphorylation of the serine residue in the synthetic peptide (data not shown). This experiment eliminated the possibility that a contaminant in the bacterially expressed WASP was able to catalyze serine phosphorylation. The ability of ACK1 to phosphorylate the Ser-242 peptide demonstrated that the three-dimensional structure of WASP is not essential for recognition as this short peptide is unlikely to adopt any stable structure. On the other hand, a peptide modeled on the Tyr-256 sequence of N-WASP was phosphorylated with a higher $k_{cat}/K_m$ than the Ser-242 peptide (data not shown). In intact WASP, serine and tyrosine phosphorylation are comparable (Fig. 3A); thus, maximal phosphorylation of Ser-242 may require the three-dimensional structure of WASP.

Serine Phosphorylation of WASP Stimulates Actin Polymerization—To analyze whether Ser-242 of WASP is phosphorylated in mammalian cells, we expressed WASP (wild type or S242A mutant) in COS-7 cells alone or in the presence of ACK1. As shown in Fig. 6, co-transfection of WASP with ACK1 and the ACK1 activator Cdc42 led to enhanced serine phosphorylation of wild-type WASP. In contrast, serine phosphorylation of the S242A WASP mutant was not increased by co-expression of ACK1 and Cdc42. These results suggested that Ser-242 is the major site of ACK1-catalyzed serine phosphorylation in these cells.

Phosphorylation of WASP at Tyr-256 (by Src kinases) or at Ser-483/484 in the VCA region (by CK2) has previously been shown to enhance actin polymerization activity (22, 26). We adopted a similar experimental strategy to test the importance of phosphorylation at Ser-242. These experiments were carried out by mixing immobilized WASP with U937 cell lysates under conditions that support actin polymerization. These lysates contain the Arp2/3 actin-nucleating complex, as well as other regulatory molecules needed for actin polymerization (22, 26). We immobilized untreated WASP or WASP that had been phosphorylated by ACK1. The beads were incubated with U937 cell lysates. After washing, the beads were fixed and stained with TRITC-phalloidin prior to fluorescence and phase-contrast microscopy. Wild-type WASP was able to stimulate actin polymerization in the presence of U937 cell lysates, and this activity was enhanced when ACK1-phosphorylated WASP was used in the assay (Fig. 7A). Next, we carried out experiments using YY-WASP to eliminate the contribution of tyrosine phosphorylation at Tyr-256. The actin polymerization activity of the YY-WASP mutant was enhanced after ACK1 phosphorylation (Fig. 7A). To specifically address the contribution of Ser-242, we expressed an S242A mutant. Phosphorylation of this mutant by ACK1 in vitro was reduced but not eliminated (Fig. 7B). Treatment of the S242A WASP mutant with ACK1 led to only a modest increase in actin polymerization activity (Fig. 7A). The double S242A/Y256F mutant gave levels of activity that were near background in the assay (Fig. 7A). A quantification of the actin polymerization data is presented in Fig. 7C. These results are consistent with a role for Ser-242 phosphorylation of WASP in regulating actin polymerization.

DISCUSSION

WASP has previously been shown to be regulated by tyrosine and serine phosphorylation. In several cases, the tyrosine phosphorylation site has been mapped to Tyr-256, within the conserved sequence IYDFI (22, 27, 28). Previously, we purified ACK1 and investigated the substrate specificity of ACK1 using representative synthetic peptides that are preferred by different classes of tyrosine kinases. The best sequence for ACK1 was the peptide selected by Abl tyrosine kinase from a peptide library (12), containing the sequence EAIYAAPF. Here, we have shown that ACK1 functions as a conventional tyrosine kinase, phosphorylating the IYDFI motif of WASP. The Ile residue at P-1 appears to be a determinant for ACK1 specificity. Mini-WASP has only one tyrosine residue, and at present, we do not know whether ACK1 phosphorylates other tyrosine sites on full-length WASP/N-WASP.

Surprisingly, we found that ACK1 kinase also phosphorylates Ser-242 in WASP. We mapped the phosphorylation site by tandem mass spectrometry, and we demonstrated that bacterially expressed ACK1 retains the ability to catalyze serine phosphorylation. Cory et al. (26) have identified two serine phosphorylation sites (serine 483 and 484) in the VCA domain of WASP. These phosphorylations were inhibited by the casein kinase 2 inhibitor DRB. Ser 483/484 phosphorylation increased affinity for the Arp2/3 complex and enhanced actin polymerization by WASP. We did not detect phosphorylation of WASP by ACK1 at Ser-483/484 in our studies. Furthermore, DRB had no effect on WASP phosphorylation by ACK1 (data not shown). Ser-242 is in the GTPase-binding domain, which binds to the output domain (VCA) in the autoinhibited state. Using U937 cell lysates, we demonstrated that serine phosphorylation of mini-WASP promotes actin polymerization. By analogy to the effects of Tyr-256 phosphorylation, we speculate that Ser-242 phosphorylation triggers a conformational change on WASP, releasing the VCA domain to activate the Arp 2/3 complex.

Several protein kinases have been classified as dual specificity kinases. The best known examples are the MEK1 and MEK2 kinases, which catalyze Thr/Tyr phosphorylation of ERK1 and ERK2, respectively (29). Although some other kinases are able to autophosphorylate on Ser/Thr/Tyr residues, relatively few are able to phosphorylate both tyrosine and serine/threonine residues on exogenous substrates (30). The Wee1 kinase phosphorylates Tyr-15 on CDK1 (31–33), whereas Myt1, a related kinase, phosphorylates both Thr-14 and Tyr-15 on CDK1 (34, 35). Wee1 phosphorylates a synthetic peptide modeled on Tyr-15 of CDK1 (31). Wee1, Myt1, and other dual specificity kinases contain catalytic domains that more closely resemble Ser/Thr kinases than Tyr

**FIGURE 6. Phosphorylation of WASP Ser-242 in COS-7 cells.** COS-7 cells were transfected with constructs encoding green fluorescent protein (GFP)-tagged WASP (wild type or S242A), ACK1, and Cdc42 as indicated above the figure. Immunoprecipitation (IP) reactions were carried out with anti-WASP antibody followed by Western blotting with anti-phosphoserine antibody. The blot was stripped and reprobed with anti-green fluorescent protein antibody, as indicated. The bottom two panels show expression of ACK1 and WASP in cell lysates. pSer, phospho-Ser.
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The structures of several kinases bound to substrate peptides have been solved (36–40). These structures have revealed the molecular basis of substrate recognition by serine and tyrosine kinases. The conformation of the $P + 1$ loop of the kinase is a key determinant in this specificity. Typically, antiparallel hydrogen bonds are formed between backbone atoms in the peptide substrate and the $P + 1$ loop of the kinase, placing the substrate phosphoacceptor site within hydrogen bonding distance of the catalytic base. Residues in the substrate are also positioned to form favorable interactions with the kinase. ACK1 is the first tyrosine kinase reported to have activity toward serine-containing peptides. (Replacement of Tyr with Ser/Thr in tyrosine kinase peptide substrates gives rise to inactive peptides (41).) As the structures of both phosphorylated and unphosphorylated ACK1 have been solved (42), we examined the structures to see whether there were any distinct features that might explain its dual specificity. Several unusual features are present in the ACK1 activation loop near the predicted substrate recognition site. ACK1 has atypical residues in this region, suggesting that it has divergent recognition properties from other tyrosine kinases (42). In particular, the residue predicted to interact with the $P + 1$ position in tyrosine substrates is a Phe rather than the typical Val, Ile, or Leu found in canonical tyrosine kinases, and this Phe protrudes from the surface of the protein. In addition, in the phosphorylated ACK1 activation loop, there is evidence for multiple conformations around residues 291–293. The electron density for the conserved proline, Pro–293, is somewhat weak, and no electron density for the residue Lys–291 was observed at all, suggesting that it is disordered. In unphosphorylated ACK1, although the activation loop has an active-like conformation, the $\phi$ and $\psi$ angles and conformation of the side chain of Val–292 differed from those observed in the phosphorylated protein, suggesting that the conformation of this residue is important in the activity of ACK1. Val–292 was in close proximity to the catalytic base (~7 Å), and the conformation of the residues in this area of the protein affected the binding of the substrate. It is possible that the flexibility observed in this region in ACK1 allows conformations to be obtained that will allow positioning of both serine and tyrosine within hydrogen bond distance of the catalytic base. Although flexibility within the activation loops of active tyrosine kinases is not unique (43), the flexibility of ACK1 appears to be particularly pronounced. In general, the residues immediately preceding the conserved proline are localized enough in individual kinase structures to be modeled. An exception to this rule is the C-terminal Src kinase, Csk (44), in which six activation loop residues preceding the residue equivalent to residue 291 in ACK1 are missing. Additionally, the $P + 1$ residues in the structure of active vascular endothelial growth factor receptor kinase are improperly positioned relative to typical active tyrosine kinases, implying that these residues adopt another conformation upon substrate binding (45). A structure of ACK1 bound to both a serine and a tyrosine peptide will be necessary to understand the molecular details of the dual specificity of ACK1.

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