ACK1 is a multidomain non-receptor tyrosine kinase that is an effector of the Cdc42 GTPase. Members of the ACK family have a unique domain ordering and are the only tyrosine kinases known to interact with Cdc42. In contrast with many protein kinases, ACK1 has only a modest increase in activity upon phosphorylation. We have solved the crystal structures of the human ACK1 kinase domain in both the unphosphorylated and phosphorylated states. Comparison of these structures reveals that ACK1 adopts an activated conformation independent of phosphorylation. Furthermore, the unphosphorylated activation loop is structured, and its conformation resembles that seen in activated tyrosine kinases. In addition to the apo structure, complexes are also presented with a non-hydrolyzable nucleotide analog (adenosine 5'-(β,γ-methylenetriphosphate)) and with the natural product debromohymenialdisine, a general inhibitor of many protein kinases. Analysis of these structures reveals a typical kinase fold, a pre-organization into the activated conformation, and an unusual substrate-binding clef.

ACK1 (activated Cdc42-associated kinase-1) is a member of a small family of non-receptor tyrosine kinases that bind Cdc42 in its GTP-bound form. Members of the ACK family have been identified in many species, including human (TNK1) (1), cow (ACK2) (2), Drosophila (DAck, DPR2) (3, 4), and Caenorhabditis elegans (ARK1) (5). ACK1 is a multidomain protein, and all family members contain an SH33 domain and a C-terminal proline-rich region. ACK family members are largely expressed in brain and skeletal tissue (2), and multiple lines of evidence suggest that they are involved in the regulation of cell adhesion and growth (6), receptor degradation (7), and axonal guidance (3). ACK1 has been implicated as a mediator of epidermal growth factor signaling to Rho family GTP-binding proteins through phosphorylation and activation of guanosine exchange factors such as Dbl, suggesting a role in regulation of the cytoskeleton (8, 9). ACK1 has also been reported to bind to adaptor proteins, suggesting that it is involved in several different signaling pathways. The SH3 domain of ACK1 binds to the proline-rich region of HSH2, an adaptor protein in hematopoietic cells, leading to its phosphorylation (10), whereas the C-terminal proline-rich domain of ACK1 interacts with the adaptor proteins Nck (11) and Grb2 (12), which are involved in cytoskeletal rearrangement (13) and Ras activation (14), respectively. In addition, both DaCK and ACK2 interact with the sorting nexin SH3PX1 through their C-terminal domains, leading to its phosphorylation (3, 7), and this ACK2 interaction with SH3PX1 together with clathrin promotes degradation of EGFR (7).

The domain architecture of the ACK family is unique compared with other non-receptor tyrosine kinases, and ACK family members are the only tyrosine kinases known to interact with Cdc42 (15). Members of the ACK family are specific for Cdc42 and do not bind the closely related Rac and Rho GTPases (2, 15). The 114-kDa human ACK1 protein consists of an N-terminal tyrosine kinase domain; an SH3 domain; and a C-terminal CRIB motif, which is required for Cdc42 binding (15). In addition, several proline-rich sequences and clathrin-binding motifs are located in the C-terminal region (11). All ACK family members share a similar domain structure, although DaCK and TNK1 do not contain the CRIB domain.

PAK serine/threonine kinases also bind to Cdc42 through a CRIB motif, and this interaction promotes kinase activity (16). Structural studies have revealed that the molecular basis for this activation in PAK1 involves release of an intramolecular autoinhibitory module upon Cdc42 binding (17). ACK kinases appear functionally similar to PAK kinases in cell-based experiments, in which Cdc42 binding promotes ACK1 activation (8, 18, 19). However, as the sequence characteristics and domain organization around the CRIB motif are dissimilar in ACK and PAK1, the molecular details of ACK activation by Cdc42 are likely to be different. Additionally, unlike members of the PAK family, Cdc42 is unable to promote the activation of ACKs in vitro (2, 19), suggesting that other cellular components may be involved. The ACKs are also the only tyrosine kinases with an SH3 domain C-terminal to the kinase domain. The Src family tyrosine kinases, including Src and Hck, and the Abl tyrosine kinase have SH3 domains (located N-terminal to the kinase domain) that serve as autoinhibitory modules of the unphosphorylated kinase. These kinases can be activated by binding of a protein or peptide to the SH3 domain (20–23); however, an SH3 ligand does not increase ACK1 catalytic activity in vitro (19), suggesting that the SH3 domain does not directly regulate the enzymatic activity of ACK1. The SH3 domain might alternatively play a role in protein localization or in substrate re-
amplified twice to produce the P 3 viral stock to be used for large-scale production of protease to His 6-ACK1. After overnight incubation at room temperature, the mixture was adjusted to a final NaCl concentration of 500 mM and protease remained bound to the resin. The fractions containing cleaved ACK1 were combined and buffer-exchanged into 50 mM Tris (pH 7.7), 500 mM NaCl, and 5 mM β-mercaptoethanol. For the final step, ACK1 was purified by size exclusion on a Superdex 75 column (Amersham, Buckinghamshire, UK). Crystallization—All crystals were grown at 18 °C in sitting drop by vapor diffusion using 1.5 μL of protein solution and 1.5 μL of reservoir solution. Crystals of apo-ACK1K were obtained by mixing protein at 4 μg/ml in 50 mM Tris HCl (pH 8.0), 300 mM NaCl, 5 mM β-mercaptoethanol, and 1 mM tris(2-carboxyethyl)phosphine with a reservoir solution of 200 mM MgCl2, 200 mM ammonium sulfate, and 40% ethylene glycol (pH 7.5). Addition of apo-ACK1K microcrystals to the reservoir solution before mixing with the protein solution aided in the growth of single crystals. The seeds were prepared by vortexing apo crystals in a seed bead kit (Hampton Research). Crystals of ACK1K with AMP-PCP were grown by mixing 4 mg/ml ACK1K in 2 mM AMP-PCP, 15 mM Tris (pH 7.7), 300 mM NaCl, 1.5 mM β-mercaptoethanol, and 1 mM tris(2-carboxyethyl)phosphine, and 5 mM MgCl2, with a reservoir solution of 25% polyethylene glycol 4000, 100 mM Tris HCl (pH 8.5), and 100 mM LiSO4. Crystals typically grew to dimensions of ~100 × 200 × 50 μm in 1–4 days and were transferred to cryoprotectant solutions consisting of the reservoir solution supplemented with 200 mM NaCl and 15% ethylene glycol before being flash-cooled by immersion in liquid nitrogen.

The ACK1K debromohymenialdisine complex was obtained from apo crystals soaked in solutions containing the complex. In this case, apo crystals were grown by mixing 3 mg/ml ACK1K in 20 mM Tris HCl (pH 7.6), 300 mM NaCl, and 1 mM dithiotreitol with a reservoir solution of 25–25% polyethylene glycol 2000, 100 mM Tris (pH 7.5), and 200 mM MgCl2. These apo crystals were soaked overnight at 18 °C in the reservoir solution supplemented with 3% (v/v) 10 mM debromohymenialdisine (in dimethyl sulfoxide) and 300 mM NaCl before being cryoprotected as described above.

Data Collection and Processing—X-ray data were collected at the Stanford Synchrotron Radiation Laboratory on beamlines 7-1 and 11-1 and a single crystal of ACK1K was selected for cryoprotection, respectively, and at the Advanced Light Source on beamline 5.0.1 on a single crystal of the debromohymenialdisine complex. The apo and debromohymenialdisine complex diffraction data were integrated with MOSFLM (26) and scaled with SCALA (27), whereas the AMP-PCP complex data set was processed with d*TREK as implemented in CrystallClear (Rigaku/MSC, The Woodlands, TX). The apo and debromohymenialdisine complex data sets belong to space group P21, with two molecules/asymmetric unit, whereas the AMP-PCP complex belongs to space group C2, also with two molecules/asymmetric unit. There is no obvious crystal packing relationship between the P2 and C2 crystal forms.

Structure Solution and Refinement—All structures were solved by molecular replacement using either EPMR (29) or MOLREP (30) as implemented in CCP4 Version 4.2.2 (27). For the apo structure, a polyserine model (conserved alanines and glycines intact) of the C-terminal domain of the Hck kinase domain (residues 343–518, with a total correlation coefficient of 0.279 and an R-factor of 0.587. The electron density map showed clear electron density for a single crystal of the debromohymenialdisine complex. The apo and debromohymenialdisine complex diffraction data were integrated with MOSFLM (26) and scaled with SCALA (27), whereas the AMP-PCP complex data set was processed with d*TREK as implemented in CrystallClear (Rigaku/MSC, The Woodlands, TX). The apo and debromohymenialdisine complex data sets belong to space group P21, with two molecules/asymmetric unit, whereas the AMP-PCP complex belongs to space group C2, also with two molecules/asymmetric unit. There is no obvious crystal packing relationship between the P2 and C2 crystal forms.
Crystal Structures of ACK

Table I

| Structure          | Data collection and refinement statistics for the ACK1K structures |
|--------------------|-------------------------------------------------------------------|
| ACK1K structure    | Apo                                                                |
| Space group        | P2₁                                                               |
| Unit cell (a, b, c (Å)); β (°) | 81.2, 43.4, 84.5; 111.8                                           |
| Resolution (Å)     | 30 to 2.0                                                         |
| R cryst (Å)        | 0.678 (0.530)*                                                   |
| Completeness (%)   | 100.0 (100.0)                                                     |
| Multiplicity       | 3.8 (3.8)                                                         |
| Average Iot(I)     | 11.8 (2.8)                                                        |
| R-factor           | 0.199 (0.248)                                                     |
| r.m.s.d. bonds (Å) | 0.020                                                             |
| r.m.s.d. angles    | 1.705°                                                            |
|                   | AMP-PCP                                                           |
|                   | 200.4, 42.3, 70.9; 96.3                                          |
|                   | 39 to 2.8                                                         |
|                   | 0.658 (0.319)                                                     |
|                   | 98.3 (97.8)                                                       |
|                   | 3.6 (3.6)                                                         |
|                   | 12.9 (3.7)                                                        |
|                   | 0.222 (0.239)                                                     |
|                   | 0.019                                                             |
|                   | 1.97°                                                             |
|                   | Debromohymenialdisine                                            |
|                   | P2₁                                                               |
|                   | 81.6, 42.9, 85.1; 112                                            |
|                   | 47 to 2.1                                                         |
|                   | 0.059 (0.373)                                                     |
|                   | 99.7 (99.7)                                                       |
|                   | 3.5 (3.4)                                                         |
|                   | 14.6 (2.6)                                                        |
|                   | 0.2025 (0.247)                                                    |
|                   | 0.248 (0.325)                                                     |

* Values in parentheses indicate the highest resolution shell.

** R free was calculated with a randomly chosen 5% of the data.

390–395 in molecule A and residues 391–395 in molecule B) were not modeled. The atomic coordinates have been deposited in the Protein Data Bank (code 1U46).

For both the AMP-PCP and debromohymenialdisine complexes, two molecules were found by molecular replacement using a single apo monomer as a search model, with all non-protein atoms removed. The solutions were subsequently rigid body-refined using maximum likelihood protocols in REFMAC. At this stage, electron density was seen for two molecules of ligand in each data set. The protein models were iteratively improved by manual building in QUANTA, followed by refinement in REFMAC. All ligands were placed into the Fc – F map using QUANTAX-LIGAND. For the debromohymenialdisine complex, the initial solvent structure was built with ARP WATERS. For the AMP-PCP complex, which was at lower resolution, waters were added by hand in QUANTA. During the last stages of refinement of the AMP-PCP complex, TLS restraints (34) were added, with each lobe defined as a TLS group, which resulted in a decrease in Rfree of 2%. The final ACK1K-AMP-PCP model contains 523 residues and 10 water molecules, whereas the final ACK1K-debromohymenialdisine model contains 520 residues and 157 water molecules. The atomic coordinates have been deposited in the Protein Data Bank (codes 1U54 and 1U4D for the AMP-PCP and debromohymenialdisine complexes, respectively). All figures were prepared with the program PyMOL (DeLano Scientific, San Carlos, CA).

RESULTS AND DISCUSSION

Overview of the Apo Structure—The structure of unphosphorylated ACK1K was determined from 2.0-Å resolution diffraction data, collected from crystals that were determined to be long to the monoclinic space group P2₁ (Table I). The asymmetric unit of these crystals contains two molecules of ACK1K (molecules A and B). The two molecules are very similar, with a global Cα r.m.s.d. of 0.8 Å. The structure is consistent with the typical bilobate kinase fold: a smaller, mostly β-sheet N-terminal lobe and a larger, mostly α-helical C-terminal lobe are connected by a short peptide linker region (residues 206–210). The nucleotide-binding pocket is formed by a cleft between the two lobes. The N-terminal lobe of ACK1K consists of six β-strands (β1–β6) and one α-helix (αC), whereas the C-terminal lobe contains seven short β-strands (β7–β13) and seven α-helices (αD–αJ and αEF) (Fig. 1).

The most closely related kinase for which a structure is available is the EGFR kinase domain (EGFRK) (Protein Data Bank code 1M14) (36). The kinase domains of ACK1 and EGFR are 39% identical in sequence, and the r.m.s.d. is 1.1 Å when 237 of 252 possible Cα pairs are superimposed. Comparison of the ACK1K structure with EGFRK and other active kinase structures, including phosphorylated LCK (lymphocyte-specific kinase; Protein Data Bank code 3LCK) (37), suggests that ACK1K is in an active conformation. Examination of the area surrounding the ATP-binding site reveals that the relative positions of residues known to be critical for catalytic activity correspond to those seen in active kinases. These include the catalytic residue Asp8252 and the residues involved in coordinating magnesium, Asn257 (catalytic loop) and Asp270 (activation loop). Additionally, the helix αC is positioned such that the side chain of Glu177 is within hydrogen-bonding distance of the Lys158 side chain, facilitating formation of the conserved salt bridge indicative of an active kinase. This salt bridge is formed in molecule B, whereas the lysine side chain is partially disordered in molecule A. The N- and C-terminal lobes of the two molecules have a slightly different juxtaposition, with molecule B in a more “closed” conformation by ~2°. It is presumed that this slight difference in conformation is simply the result of the crystalline environment and represents the lack of crystallographic symmetry between the two protein molecules. Residues 134–137 of the nucleotide-binding loop are disordered in both molecules.

The activation loop, which modulates catalytic activity in many protein kinases by alternating between inactive and active states in response to the phosphorylation state (38), is well ordered in unphosphorylated ACK1K and adopts an “active” conformation generally observed only in phosphorylated protein kinases. Several unusual features of the ACK1K activation loop appear to stabilize the active conformation (discussed below). This active conformation is in direct contrast to several tyrosine kinases that have unphosphorylated autoinhibitory activation loop structures. In IRK (insulin receptor kinase) and the muscle-specific kinase MuSK, the conserved sequence Asp-Phe-Gly (which initiates the activation loop) occupies the ATP-binding pocket, blocking the catalytic function of the Asp residue and preventing ATP bind-
rylation was observed in molecule B. Importantly, Tyr284 is the only tyrosine in the activation loop and is the primary site of autophosphorylation in ACK1K (19). Examination of crystal structures of ACK1K, crystals were grown in the presence of a non-hydrolyzable ATP analog (AMP-PCP). The protein preparation used in the experiment contained a small amount of +1 phosphorylated protein as indicated by mass spectroscopy (data not shown). The +1 phosphorylated protein was most likely produced during fermentation and was co-purified with the +0 form of the protein. Crystals were obtained that belong to space group C2 and that diffract to 2.8 Å (Table I). The asymmetric unit contains two molecules of ACK1K (molecules A and B). In molecule A, clear electron density was observed for a phosphorylated tyrosine in the activation loop (Tyr284), whereas no evidence of phosphorylation of AMP-PCP and forms bridging interactions with the backbone carbonyl oxygen of Asn257 (catalytic loop). Contrary to some protein kinase-nucleotide complexes. For example, in the LCK-nucleotide analog structure, a serine side chain forms hydrogen bonds with the ribose 2'-hydroxyl (44). ACK1K also has a serine at this position (Ser212); however, the side chain of this serine points away from the ribose and is positioned to form a hydrogen bond with the backbone amide of Asp215. It is possible that an alternative conformation of the serine side chain that forms a hydrogen bond with the ribose is present but could not be detected at this resolution. The phosphates are anchored by hydrogen bonds formed between the β-phosphate oxygens and the side chain of the conserved salt bridge lysine (Lys158). Electron density supporting the presence of two magnesium ions in the ATP-binding pocket was present. One modeled magnesium ion coordinates oxygens on the β- and γ-phosphates of AMP-PCP and forms bridging interactions with the side chain oxygens of the highly conserved Asp270 (activation loop). The other magnesium ion coordinates oxygens on the α- and γ-phosphates of AMP-PCP and interacts with the side chain oxygen of Asn207 (catalytic loop). Contrary to some protein kinase-nucleotide complexes in which the nucleotide-binding loop interacts with the ligand (42), in the ACK1K-AMP-PCP complex, the nucleotide-binding loop remains disordered and forms no direct interactions with the nucleotide analog.

**Activation Loop Conformation**—The activation state of most protein kinases is controlled in part by phosphorylation of residues in the activation loop. Generally, phosphorylation of one to three sites in the activation loop results in a conformational change that can relieve autoinhibition and that properly orients residues for catalysis (38, 45). In contrast, our structures indicate that the phosphorylation state of ACK1K confers no substantial conformational difference and does not affect the orientation or conformation of the catalytic residues. We also examined the local structures of both the phosphorylated and unphosphorylated activation loops to determine what structural differences are present and to better understand the factors that stabilize the loops.

There are two independent molecules present in the asymmetric unit of the unphosphorylated apo-ACK1K structure, which allowed multiple observations of the activation loop structure. In both molecules, the loop is well ordered and has a defined structure. In molecule A, 25 of 31 activation loop residues are observable in well defined electron density, whereas the entire loop is visible in molecule B (Fig. 3c). Crystal lattice significantly ordered upon AMP-PCP binding, and only one additional residue is observed in the electron density for the AMP-PCP complex compared with the apoenzyme.

In the ACK1K-AMP-PCP complex structure, the nucleotide makes interactions with the protein analogous to those described in previous structures of active protein kinases (42, 43). The interactions between AMP-PCP and ACK1K are similar in the phosphorylated and unphosphorylated molecules, although the N- and C-terminal lobes of the unphosphorylated molecule are slightly more closed (by ~2°), which has the effect of shortening some of the contact distances. At the resolution of this structure (2.8 Å), the definitive presence of a hydrogen bond is hard to determine. For the purpose of this discussion, we considered hydrogen bond donors and acceptors that were within 3.5 Å and in good electron density to form a hydrogen bond. The adenine moiety forms two conserved hydrogen bonds with the backbone of the linker residues Glu206 and Ala208 (Fig. 2). One hydrogen bond is observed between the ribose 3'-hydroxyl and the backbone carbonyl oxygen of Arg207 (3.4 Å; catalytic loop) in the unphosphorylated molecule, whereas these atoms are too far apart to form a hydrogen bond in the phosphorylated molecule (4.8 Å). No hydrogen bonds are observed between the ribose 2'-hydroxyl and the protein as has been reported in some protein kinase-nucleotide complexes. For example, in the LCK-nucleotide analog structure, a serine side chain forms hydrogen bonds with the ribose 2'-hydroxyl (44). ACK1K also has a serine at this position (Ser212); however, the side chain of this serine points away from the ribose and is positioned to form a hydrogen bond with the backbone amide of Asp215. It is possible that an alternative conformation of the serine side chain that forms a hydrogen bond with the ribose is present but could not be detected at this resolution. The phosphates are anchored by hydrogen bonds formed between the β-phosphate oxygens and the side chain of the conserved salt bridge lysine (Lys158). Electron density supporting the presence of two magnesium ions in the ATP-binding pocket was present. One modeled magnesium ion coordinates oxygens on the β- and γ-phosphates of AMP-PCP and forms bridging interactions with the side chain oxygens of the highly conserved Asp270 (activation loop). The other magnesium ion coordinates oxygens on the α- and γ-phosphates of AMP-PCP and interacts with the side chain oxygen of Asn207 (catalytic loop). Contrary to some protein kinase-nucleotide complexes in which the nucleotide-binding loop interacts with the ligand (42), in the ACK1K-AMP-PCP complex, the nucleotide-binding loop remains disordered and forms no direct interactions with the nucleotide analog.

**Activation Loop Conformation**—The activation state of most protein kinases is controlled in part by phosphorylation of residues in the activation loop. Generally, phosphorylation of one to three sites in the activation loop results in a conformational change that can relieve autoinhibition and that properly orients residues for catalysis (38, 45). In contrast, our structures indicate that the phosphorylation state of ACK1K confers no substantial conformational difference and does not affect the orientation or conformation of the catalytic residues. We also examined the local structures of both the phosphorylated and unphosphorylated activation loops to determine what structural differences are present and to better understand the factors that stabilize the loops.

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The phosphorylated activation loop of IRK3P, respectively. The unphosphorylated loop in IRK3P (residues involved in these interactions and the corresponding residues that differ from those found in other kinase activation loops. The unphosphorylated loop is structured and adopts a global conformation similar to that of the phosphorylated loop. Residues that shift in position upon phosphorylation are shown in both loops. Residues that could not be well placed in electron density are omitted.

In the structure of the AMP-PCP complex, all residues but one in the phosphorylated activation loop of molecule A are visible in the electron density, and the entire activation loop of the unphosphorylated molecule B could be modeled. The molecule B activation loop makes similar crystal contacts with the apo molecule B activation loop, and electron density for an anion was also observed in phosphate-binding site of this molecule. Overall, the structures of the unphosphorylated activation loops are extremely similar. The similarity between the conformations of the unphosphorylated activation loops observed in multiple crystalline environments supports the position that, rather than being unduly enhanced by lattice packing constraints, this active conformation is also found in solution.

In the structure of the AMP-PCP complex, all residues but one in the phosphorylated activation loop of molecule A are visible in the electron density, and the entire activation loop of the unphosphorylated molecule B could be modeled. The molecule B activation loop makes similar crystal contacts with the apo molecule B activation loop, and electron density for an anion was also observed in phosphate-binding site of this molecule. Overall, the structures of all of the unphosphorylated activation loops are extremely similar and, in contrast to many protein kinases, closely resemble the structure of the phosphorylated activation loop. However, although the main chain conformation of the activation loop in ACK1 appears pre-organized without phosphorylation, there are some differences in side chain conformations (Fig. 3, a and b). Upon phosphorylation, Tyr284 and several arginine residues shift in position to facilitate interactions with the anionic phosphate group. Additionally, the electron density is somewhat diffuse for Tyr284 in the unphosphorylated activation loop lacking the chloride ion, suggesting that it has more mobility than in the phosphorylated protein.

In each of these structures, the activation loop does not occlude either the nucleotide- or substrate-binding site, and its conformation resembles that seen in active tyrosine kinases such as the phosphorylated insulin receptor tyrosine kinase IRK3P (Fig. 3, c and d) (43). Like IRK3P, the ACK1K activation loop structure is stabilized by antiparallel main chain hydrogen bonds. In ACK1K, these are formed between loop residues 275–276 and residues 248–249 of the C-terminal lobe as well as between loop residues 283–285 and residues 306–308. Within the interior of the activation loop, between these short structural elements, the aliphatic portion of the Arg275 side chain packs against the face of Tyr284. These hydrogen-bonding interactions and the packing of a hydrophobic aliphatic side chain against the face of the tyrosine are conserved among known tyrosine kinase activation loops that have been observed in the active conformation. However, the phosphate of Tyr284 in ACK1K is chelated by three arginine side chains, compared with just one in both IRK3P and phosphorylated LCK (37), placing it in a slightly different position.

Several key interactions in the ACK1K activation loop differ from other kinases and stabilize the maintenance of this active conformation when in the unphosphorylated state (Fig. 3, c and d). In ACK1K, the backbone of residues 276–288 is held closer to the ATP-binding site than in IRK3P, facilitating the packing of the Met276 side chain with Arg251, which packs against Tyr284. On the opposite strand of the loop, the side chain of Met274 extends outside of the loop and packs between two conserved phenylalanines, Phe248 and Phe271. The presence of methionine at this position is unique in human protein-tyrosine kinases; smaller residues (Ala, Ser, or Thr) are found in most kinases. The unusual nature of this methionine combined with the strong conservation of a large hydrophobic residue at this position in the ACK1 family supports its importance in the stabilization of the unphosphorylated loop structure. The location of this methionine immediately prior to the loop residues involved in hydrogen-bonding interactions with the C-terminal lobe suggests that it may help anchor these interactions.

Only two other known protein-tyrosine kinase structures, those of CSK (46) and EGFRRK (36), have an activation loop in the active conformation despite being in the unphosphorylated state. As the CSK activation loop lacks any tyrosine that could be phosphorylated and is shorter than the ACK1K activation loop by 11 residues, EGFRRK is the more relevant of the two. In EGFRRK, the unusually large number of acidic residues in the activation loop has been suggested to contribute to the stabilization of the active conformation (36). The ACK1K activation loop does not have this concentration of acidic residues and is likely to be stabilized by different factors. The structure of EGFRRK also suggests that an arginine from the C-terminal lobe is involved in stabilizing the structure of the unphosphorylated activation loop. In the EGFRRK structure, the side chain of this arginine (Arg508) forms a single hydrogen bond with an activation loop main chain carbonyl oxygen, anchoring the loop against the C-terminal lobe. An analogous arginine (Arg347) is found in ACK1K, which forms hydrogen bonds with the main chain carbonyl oxygens of Pro277a and Asp284a within the activation loop (Fig. 3c), supporting the importance of this arginine in stabilization of the active loop structure.

Role of Tyr284 Phosphorylation—ACK1 contains a single tyrosine (Tyr284) in its activation loop, and mutation of this tyrosine to phenylalanine reduces levels of tyrosine phosphorylation in vivo. However, phosphorylation of this tyrosine

![Image: Comparison of activation loops in several structures. Residues 271–290 of the ACK1K activation loop (a–c) and the corresponding residues in IRK3P (d) are shown. a and b, comparison of the unphosphorylated activation loop of the apo-ACK1K structure and the phosphorylated activation loop of the AMP-PCP complex, respectively. The unphosphorylated loop is structured and adopts a global conformation similar to that of the phosphorylated loop. Residues that shift in position upon phosphorylation are shown in both loops. Residues that could not be well placed in electron density are shown in orange. c and d, comparison of the phosphorylated activation loop of ACK1K and the phosphorylated activation loop of IRK3P, respectively.](image-url)
results in only a modest 3-fold increase in activity in vitro (19). This increase in activity is low compared with kinases that are autoinhibited by their unphosphorylated activation loop. For example, the insulin-like growth factor-1 receptor kinase exhibits a >120-fold increase in catalytic efficiency when fully phosphorylated (47). The current structural results suggest that the activation loop of ACK1 is not autoinhibitory, that phosphorylation is not necessary for reorganization of the catalytic machinery, and that the binding of ATP and substrate should be largely unaffected by the phosphorylation state. However, phosphorylation may be necessary to establish an effective electrostatic environment for catalysis. This role for activation loop phosphorylation has been proposed for the protein kinases v-Fps, protein kinase A, and others (48, 49), wherein 2–5-fold increases in phosphoryl transfer rates have been attributed to distal effects from changes in activation loop electrostatics. It is possible that electrostatics play a similar role in ACK1. Indeed, ACK1K has an unusually electropositive environment around the activation loop tyrosine; four arginines and one lysine are within 10 Å of Tyr284, and three of these arginines directly chelate the Tyr(P) phosphate. Typically, just one basic residue is found in other protein kinases anchoring this phosphorylated residue. In contrast, EGFR, which has no apparent difference in activity when its phosphorylated tyrosine is mutated to phenylalanine (50), contains many glutamic acid residues in the activation loop, one of which sits above the activation loop tyrosine in the structure of the kinase domain (36).

Description of the Substrate-binding Site—Although the physiological substrates of ACK1 are not known, the substrate specificity of ACK1 against a panel of peptide substrates for different subfamilies of tyrosine kinases has been reported (19). Comparison of the predicted peptide-binding site of ACK1 with IRK3P in complex with an IRK substrate peptide lends insight to the origin of the reported substrate preferences (Protein Data Bank code 1IR3) (43). ACK1 has a phenylalanine (Phe294) at the P+1 substrate-binding position. This phenylalanine is unique in human tyrosine kinases and is conserved in mammalian ACKs. The majority of tyrosine kinases have Ile, Val, or Leu at this position. The superposition of ACK1 with the structure of IRK3P bound to a substrate peptide shows that although the valine in IRK3P is in van der Waals contact with the peptide substrate P+1 methionine, the corresponding phenylalanine in ACK1 would sterically clash with this methionine.

Comparison of the predicted peptide-binding site of ACK1 with IRK3P bound to an IRK peptide substrate (green) is shown. ACK1 contains a phenylalanine (Phe284) at the predicted P+1 binding site. Although the valine at this position in IRK makes van der Waals contacts with the substrate P+1 methionine, the phenylalanine in ACK1 would sterically clash with this methionine.
makes contacts with the ligand, sequestering it from solvent. The asymmetric unit contains two molecules (A and B), and the inhibitor binds similarly in both. In each molecule, the inhibitor makes at least four direct hydrogen bonds with the protein and a number of water-mediated hydrogen bonds. All of the direct hydrogen bonds interact with key elements of the ATP-binding site, which explains its ability to inhibit many protein kinases. Two hydrogen bonds are formed with the backbone of linker residues 206 and 208. Another hydrogen bond is formed between the oxygen of the dihydroimidazoline moiety and the conserved salt bridge lysine (Lys156). The amino group of the dihydroimidazoline forms a hydrogen bond with the side chain of Asp270. Asp270 participates in the catalytic mechanism by orienting the γ-phosphate of ATP to transfer to the substrate and is part of the highly conserved Asp-Phe-Gly motif that initiates the activation loop. The same amino group also forms a hydrogen bond with the backbone carbonyl oxygen of Asp134 in the nucleotide-binding loop in molecule A (data not shown) and likely contributes to the structural stabilization of this loop. In molecule B, the nucleotide-binding loop is not held in as closely, and instead of a direct hydrogen bond, a water molecule bridges the loop and the inhibitor. Conclusions—ACK1 is a multidomain non-receptor tyrosine kinase that has only a modest increase in activity upon phosphorylation in vitro (19). We have solved the structures of both the phosphorylated and unphosphorylated states of ACK1 and determined that they are extremely similar. Phosphorylation of the activation loop is not required to position the activation loop and the catalytic machinery in an active conformation. Several uncommon hydrophobic residues in the activation loop appear to stabilize the active conformation in the absence of phosphorylation. In particular, an uncommon methionine in the activation loop (Met274) extends outside the loop and packs between two conserved phenylalanines. Many interactions found in typical hydrophobic residues in the activation loop appear to stabilize the unphosphorylated and unphosphorylated states of ACK1K and define residues in this region creates atypical structural features. This may explain why ACK1 does not detectably phosphorylate many peptide substrates common to other tyrosine kinases (19). The unique domain structure of full-length ACK1, together with the active conformation of the unphosphorylated kinase domain and the unusual peptide substrate-binding region, suggests that the biological mechanism and method of substrate recruitment in ACK1, when identified, may be divergent from those of other tyrosine kinases.