The activation mechanism of ACK1 (activated Cdc42-associated tyrosine kinase 1)

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INTRODUCTION

ACK [activated Cdc42 (cell division cycle 42)-associated tyrosine kinase; also called TNK2 (tyrosine kinase, non-receptor, 2)] is activated in response to multiple cellular signals, including cell adhesion, growth factor receptors and heterotrimeric G-protein-coupled receptor signalling. However, the molecular mechanism underlying activation of ACK remains largely unclear. In the present study, we demonstrated that interaction of the SH3 (Src homology 3) domain with the EBD [EGFR (epidermal growth factor receptor)-binding domain] in ACK1 forms an auto-inhibition of the kinase activity. Release of this auto-inhibition is a key step for activation of ACK1. Mutation of the SH3 domain caused activation of ACK1, independent of cell adhesion, suggesting that cell adhesion-mediated activation of ACK1 is through releasing the auto-inhibition. A region at the N-terminus of ACK1 (Leu10–Leu14) is essential for cell adhesion-mediated activation. In the activation of ACK1 by EGFR signalling, Grb2 (growth-factor-receptor-bound protein 2) mediates the interaction of ACK1 with EGFR through binding to the EBD and activates ACK1 by releasing the auto-inhibition. Furthermore, we found that mutation of Ser445 to proline caused constitutive activation of ACK1. Taken together, our studies have revealed a novel molecular mechanism underlying activation of ACK1.

Key words: activated cell division cycle 42-associated tyrosine kinase (ACK1), auto-inhibition, epidermal growth factor receptor (EGFR); epidermal growth factor receptor (EGFR) binding, growth-factor-receptor-bound protein 2 (Grb2), tyrosine phosphorylation.

ACK [activated Cdc42 (cell division cycle 42)-associated tyrosine kinase] [also called TNK2 (tyrosine kinase, non-receptor, 2)] was initially identified as a downstream effector of the GTPase Cdc42 [1,2]. ACK is a member of the type VIII tyrosine kinase family and possesses multiple functional domains, including the SAM (sterile α-motif) domain, the tyrosine kinase domain, the SH3 (Src homology 3) domain, the Cdc42-binding domain [PBD (p21-binding domain) or CRIB (Cdc42/Rac-interacting binding domain)], the SNX9 (sorting nexin 9)-binding domain, the CBD (clathrin-binding domain), the WWBD (WW-binding domain), the EBD [EGFR (epidermal growth factor receptor)-binding domain] and the Uba (ubiquitin association) domain [1–8]. Biochemical studies have shown that ACK functions in the regulation of ligand-induced EGFR degradation and integrin-mediated cell adhesion and migration [7–11]. ACK is amplified and overexpressed in multiple cancers, and associated with tumour progression through promoting cell growth and migration [11–14]. ACK is activated by multiple cellular signals. It has been shown that ACK is activated by cell adhesion and multiple receptor signals, including EGFR, PDGFR (platelet-derived growth factor receptor), insulin receptor, the Gas-6 (growth-arrest-specific protein 6) receptor Mer and M3 muscarinic receptor [2,3,9,12,15–17]. In cell adhesion signalling, activated ACK interacts with and phosphorylates p130Cas for assembly of the p130Cas–Crk–Elmo–Dock180 complex that serves as a guanine-nucleotide-exchange factor for Rac GTPase and promotes cell migration [18]. In EGF signalling, ACK1 interacts with activated EGFR through the EBD that is conserved among ACK1 and Mig-6 (mitogen-inducible gene 6), and facilitates lysosomal-mediated degradation of EGFR [7]. ACK has a conserved clathrin-binding motif and directly interacts with clathrin [5,6]. Overexpression of ACK impairs clathrin-mediated endocytosis [5]. In Caenorhabditis elegans, Ark-1 genetically interacts with UNC101, the homologue of the mammalian clathrin-associated protein AP47, and SLI-1, the homologue of mammalian Cbl, an E3 ubiquitin ligase for ubiquitination of EGFR, and negatively regulates EGFR signalling [19]. In Gas-6 receptor signalling, ACK phosphorylates and down-regulates the tumour suppressor WWOX, thus promoting prostate tumour cell growth [12]. ACK may also mediate ErbB2 signalling in prostate cancer cells through phosphorylation and activation of androgen receptor [13]. However, how receptor or cell adhesion signalling activates ACK is poorly understood.

Cdc42 activates ACK in vivo, but not in vitro [2]. We observed that treatment of adherent cells with growth factors, such as EGF or PDGF, yielded little activation of ACK, since ACK was already activated by cell adhesion [2]. The kinase domain of ACK has a pre-organized activated conformation even in the absence of autophosphorylation, suggesting that ACK kinase is constitutively active [20]. In addition to its tyrosine kinase activity, ACK also phosphorylates serine residues in WASP (Wiskott–Aldrich

Abbreviations used: ACK, activated Cdc42-associated tyrosine kinase; CBD, clathrin-binding domain; Cdc42, cell division cycle 42; CRIB, Cdc42/Rac-interacting binding domain; EGF, epidermal growth factor; EGFR, EGFR receptor; EBD, EGFR-binding domain; GFP, green fluorescent protein; Grb2, growth-factor-receptor-bound protein 2; GST, glutathione transferase; HA, haemagglutinin; HEK, human embryonic kidney; KBR, kinase-binding region; Mig-6, mitogen-inducible gene 6; Nedd4-1, neural-precursor-cell-expressed developmentally down-regulated 4-1; PAK, p21-activated kinase; PD, p21-binding domain; PDGFR, platelet-derived growth factor receptor; pY, phospho-tyrosine; RNAi, RNA interference; SAM, sterile α-motif; SH3, Src homology 3; SBR, SH3-binding region; SNX9, sorting nexin 9; Uba/UBA, ubiquitin association; WWBD, WW-binding domain.

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syndrome protein) in vitro, indicating that ACK has dual kinase activity [21]. Although ACK is well characterized, few cellular substrates have been identified. Currently, three proteins, SNX9 (also called SH3PX1), p130Cas and androgen receptor, are reported to be putative substrates of ACK [4,13,17].

Given that ACK’s kinase domain is in a constitutively active conformation, how its kinase activity is regulated in response to receptor or cell adhesion signalling becomes an intriguing question. It was noticed that the EBD of Mig-6, which is homologous with the EBD of ACK1, directly interacts with the kinase domain of EGFR that structurally resembles the ACK1 kinase domain [22]. The in vitro binding assay demonstrated that the EBD of ACK1 binds to its own kinase domain [23]. The kinase-binding defective mutations of the EBD caused activation of ACK1 [23]. Thus it has been proposed that the EBD of ACK1 might fold back to bind to its own kinase region to form an auto-inhibitory structure [22,23]. However, this model cannot explain how receptor signals activate ACK1, and why activation of EGFR is required for interaction with ACK1. Our previous studies observed that mutation of the SH3 domain of ACK eliminated the binding to clathrin, and proposed that the proline-rich region, which is localized within the EBD, might bind to its own SH3 domain to expose the clathrin-binding site [5]. As the SH3 domain is adjacent to the kinase domain, the interaction between the SH3 domain and the proline-rich region within the EBD may also regulate the kinase activity of ACK. On the basis of this hypothesis, in the present study we investigated the auto-inhibition of ACK1 kinase formed by the interaction between the SH3 domain and the EBD, and found that EGFR and cell adhesion activate ACK1 through release of SH3–EBD-mediated auto-inhibition in cells.

MATERIALS AND METHODS

Materials

Anti-EGFR (1005), anti-Grb2 (growth-factor-receptor-bound protein 2) and anti-ACK (A11) antibodies were purchased from Santa Cruz Biotechnology; anti-pY (phospho-tyrosine) antibody (4G10) was from Millipore; anti-HA (haemagglutinin), anti-clathrin LC (light chain) (CON.1) and anti-GFP (green fluorescent protein) antibodies were from Covance; anti-phospho-ACK, anti-phospho-EGFR-pY1045 and anti-phospho-EGFR-pY1068 antibodies were from Cell Signaling Technology; anti-clathrin HC (heavy chain) antibody was from Pharmingen; and anti-Myc antibody was from the Iowa University Hybridoma Bank. RNAi (RNA interference) oligonucleotides and EGF were purchased from Invitrogen. Anti-actin, glutathione–agarose and Protein A beads were purchased from Sigma. HEK (human embryonic kidney)-293 and human non-small cell lung cancer A549 cells were purchased from A.T.C.C.

Cell culture, transfection and cell lysate preparation

The cells were cultured in DMEM (Dulbecco’s modified Eagle’s medium) plus 10% (v/v) FBS (fetal bovine serum). For transfection, cells were cultured to 50–80% confluence. The transfection was performed with a Lipofectamine™ 2000 transfection kit (Invitrogen). The cell lysates were prepared by adding mammalian cell lysis buffer (40 mM Heps, pH 7.4, 100 mM NaCl, 1% Triton X-100, 25 mM glycerol phosphate, 1 mM sodium orthovanadate, 1 mM EDTA, 10 μg/ml aprotinin and 10 μg/ml leupeptin) or RIPA buffer (40 mM Heps, pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 100 mM NaCl, 1 mM EDTA, 25 mM 2-glycerophosphate, 1 mM sodium orthovanadate, 10 μg/ml leupeptin and 10 μg/ml aprotinin) [for GST (glutathione transferase)–ACK1Uba pull-down assays] to the cells followed by rocking the cells at 4°C for 30 min. The cell lysates were collected into 1.5 ml Eppendorf tubes and cleared by centrifugation at 15000 g for 10 min at 4°C in a microcentrifuge.

Construction of cDNAs and ACK mutants

ACK1 mutants

All of the ACK1 mutants were made by PCR-based site-directed mutagenesis or truncation using mouse Trk2 as a template. The truncation mutants ΔN9, ΔN14, ΔN34, ΔN52 and ΔN88 were generated by deletion of 9, 14, 34, 52 and 88 amino acid residues from the N-terminus of ACK1. N53, N100, N729, N816 and N879 contained 53, 100, 729, 816 and 879 amino acid residues of the N-terminus of ACK1.

Grb2 and its mutants

The cDNA of human Grb2 and its mutants were subcloned into pcDNA3-HA, an HA-tagged mammalian expression vector, or pEGFP-C1, a GFP-tagged mammalian expression vector. The point mutations were: W36K, the N-terminal SH3 domain defective mutant; and W193A, the C-terminal SH3 domain-defective mutant. The truncation mutants were: Grb2-SH2, containing amino acid residues 60–155; Grb2-SH3-N, containing amino acid residues 1–59; and Grb2-SH3-C, containing amino acid residues 156–217.

Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were performed as described previously [7]. Briefly, the cell lysates were incubated with 20 μl of Protein A beads plus 1–4 μg of primary antibody for 2–3 h at 4°C with rotation. The Protein A beads were then washed with mammalian cell lysis buffer three times, resuspended in 30 μl of SDS/PAGE sample buffer, and used for gel electrophoresis. The proteins on the gel were transferred on to a PVDF membrane and immunoblotted using a Western Lightning ECL detection kit (PerkinElmer). The protein bands were visualized by a Fujifilm Las-400 imaging system.

Quantification of the immunoblotting bands was performed using Kodak Molecular Imaging software. The tyrosine phosphorylation level of each ACK1 construct was calculated as the ratio of density of the phosphotyrosine band to the density of the Myc-tagged ACK1 band. The tyrosine phosphorylation level of wild-type ACK1 or the substrate phosphorylation level by wild-type ACK1 was normalized to 1.00 in order to eliminate differences in the basal level of immunoblots from separate experiments.

GST-fusion protein pull-down assay

The GST-fusion protein pull-down assay was performed as described previously [7]. Briefly, the GST-fusion proteins were immobilized on glutathione-conjugated agarose beads. The GST-fusion protein beads (5–10 μg of protein) were incubated with the cell lysates for 2–3 h at 4°C with rotation, subsequently washed with mammalian cell lysis buffer, and resuspended in SDS/PAGE sample buffer for gel electrophoresis and immunoblotting.

Cell adhesion assay

The cell adhesion assay for activation of ACK1 was performed as described previously [9]. Briefly, non-coated culture plates
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Figure 1 The N-terminal region is required for cell adhesion-mediated activation of ACK1

(A) Schematic representation of the N-terminal truncation mutants of ACK1. PRD1, proline-rich domain 1; TK, tyrosine kinase. (B–D) Myc-tagged ACK1 or the indicated truncation mutant was co-transfected with or without Cdc42-Q61L into HEK-293 cells for 48 h. The tyrosine phosphorylation of ACK1 was detected by immunoblotting with an anti-phospho-ACK1 antibody. The expression of Myc-ACK1, the mutants or Cdc42-Q61L was detected by immunoblotting with anti-ACK1 and anti-Cdc42 antibodies. Immunoblotting (B) and quantification of immunoblotting from three independent experiments (C and D). The tyrosine phosphorylation level of each ACK1 construct was calculated as the ratio of the density of the pY band to the density of the Myc-tagged ACK1 band. Results in (C and D) are means ± S.D.

RESULTS

Cell adhesion-induced activation of ACK is dependent on an N-terminal region containing Leu<sup>10</sup>-Leu<sup>14</sup> (L<sub>10</sub>LELL<sub>14</sub>) and through release of the SH3-domain-mediated inhibition

Our previous studies have shown that ACK is activated by cell adhesion, which results in a high level of basal auto-phosphorylation of ACK in adherent cells [2,9]. To determine the molecular basis of this activation, we made a series of N-terminal truncation mutants of ACK1, as shown in Figure 1(A), expressed the truncation mutants in HEK-293 cells, and examined tyrosine phosphorylation. As shown in Figures 1(B) and 1(C), wild-type ACK1 had a basal tyrosine phosphorylation, and co-expressing with the GTPase-defective (constitutively active) mutant of Cdc42, Cdc42-Q61L, enhanced the phosphorylation 2-fold. Deletion of the first nine amino acid residues had little effect on tyrosine phosphorylation. However, further deletion of residues 10–14 (L<sub>10</sub>LELL<sub>14</sub>) diminished the basal tyrosine phosphorylation of ACK1, and enlarged the activation effect of Cdc42 to ∼15-fold (Figure 1D). The enlargement of the activation effect of Cdc42

were incubated with fibronectin (10 μg/ml) for 12 h at 4°C followed by blocking with 2 % BSA for 2 h at 37°C. The control plates (used for cells in suspension), incubated with PBS (the solvent for fibronectin) instead of fibronectin, were set up in parallel with the fibronectin-coated plates. The cells, which were transfected with ACK1 or its mutants for 48 h, were detached from the plates by incubation in 2 % (w/v) EGTA and washed three times with PBS. The suspended cells were incubated on the control (suspension) or the fibronectin-coated plates for 30 min. Both suspended and adhered cells were harvested and lysed. The tyrosine phosphorylation of ACK1 and its mutants was detected by immunoblotting with an anti-phospho-ACK antibody (Cell Signaling Technology).

RNAi knockdown

All of the RNAi oligonucleotide sequences were: control, 5′-GGAAUCUCAUUGCAUAUC-3′; and human Grb2, 5′-UUGAUAGUCUUUGGAAGAUCG-3′. The RNAi oligonucleotide sequences were transfected into HEK-293 or A549 cells using Lipofectamine<sup>TM</sup> 2000 (Invitrogen) following the protocol provided by the manufacturer. Briefly, the cells were seeded 1 day before transfection at 60 % confluence. For a 35 mm dish, 250 pmol of RNAi oligonucleotides were incubated with 5 ml of Lipofectamine<sup>TM</sup> 2000 for 10 min at 22°C. The cells were used for further analysis 48–72 h after transfection.

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Figure 2  Cell adhesion-induced and N-terminal-mediated activation of ACK is through release of the SH3 domain-mediated inhibition

(A and B) ACK1- or its mutant-transfected cells were plated either on BSA-blocked (Suspension) or fibronectin (FN)-coated dishes for 30 min. The tyrosine phosphorylation of ACK1 was detected by immunoblotting with an anti-phospho-ACK1 antibody. The expression of ACK1 was determined by immunoblotting with an anti-Myc antibody. Immunoblotting (A) and quantification of immunoblotting from two independent experiments (B). S, suspension. (C and D) Myc-tagged ACK1 or the indicated truncation mutant was co-transfected with or without Cdc42-Q61L into HEK-293 cells for 48 h. The tyrosine-phosphorylated ACK1 and Myc-tagged ACK1 were detected by immunoblotting with anti-phospho-ACK1 and anti-Myc antibodies. Immunoblotting (C) and quantification of immunoblotting from two independent experiments (D). WT, wild-type. Results in (B and D) are means ± S.D. (E) Myc-tagged ACK1 or its mutants were co-transfected with HA-tagged SNX9 with or without Cdc42-Q61L into HEK-293 cells for 48 h. The tyrosine phosphorylation of SNX9 was detected by immunoblotting the anti-HA-immunoprecipitated SNX9, and the expression level of HA–SNX9 or Myc–ACK1 was detected by immunoblotting the cell lysates with anti-HA or anti-Myc antibodies. IP, immunoprecipitation.

by truncation of the first 14 amino acid residues is due to loss of the basal tyrosine phosphorylation, since the truncation did not increase the effect of Cdc42 on the tyrosine phosphorylation of ACK1 (Figure 1C). Further truncation of 52 amino acid residues at the N-terminus had a similar effect to the tyrosine phosphorylation of ACK1 and the activation by Cdc42 to that produced by the truncation mutant ACKΔN14 (Figures 1C and 1D). Deletion of the first 68 or 88 amino acid residues dramatically reduced the auto-phosphorylation of ACK1 and the activation by Cdc42 (Figures 1C and 1D), probably due to disruption of the conformation of the kinase domain.

Because the basal tyrosine phosphorylation of ACK is caused by cell adhesion [2,9], it is likely that the N-terminal truncation mutants lose cell adhesion-dependent activation. To test this hypothesis, we performed cell adhesion-mediated activation assays for ACK1. As shown in Figures 2(A) and 2(B), suspension of the cells dramatically reduced the tyrosine phosphorylation of ACK1 (Figure 2A, lane 1). Re-adhesion of the cells on fibronectin-coated plates restored tyrosine phosphorylation, indicating that the fibronectin-mediated cell adhesion activates ACK1 (Figure 2A, lane 2). As expected, truncation of the first 34 amino acid residues of ACK1 eliminated the activation by fibronectin-mediated cell adhesion (Figure 2A, lane 3 and 4). Note that we used ACKΔN34, instead of ACKΔN14, in the experiment, because ACKΔN34 is ectopically expressed better than ACKΔN14, whereas it has a similar effect to ACKΔN14 on basal tyrosine phosphorylation and activation by Cdc42. Binding to Cdc42 is not required for cell adhesion-mediated activation of ACK1, because the Cdc42-binding defective mutant ACK1-2H2A, in which the conserved Cdc42-binding motif HXXH in the PBD was mutated into AXXA, had the same activation by fibronectin as the wild-type ACK1 (Figure 2A, lanes 7 and 8). Interestingly, the SH3-domain-defective mutant of ACK1, ACK1-2W2A (the conserved WW residues in the SH3 domain were mutated into AA residues), was constitutively active, independent of the fibronectin-mediated cell adhesion (Figure 2A, lanes 5 and 6, and Figure 2B), suggesting that the SH3 domain mediates inhibition of ACK kinase activity, and that this inhibition can be released by the fibronectin-mediated cell adhesion. Furthermore, mutation of the SH3 domain restored the auto-phosphorylation in the N-terminal truncation mutant ACKΔN34 (Figures 2C and 2D), confirming that the SH3 domain plays a role in inhibition of the kinase activity. To examine the kinase activity of ACK1 affected by mutation of the SH3 domain, we co-expressed ACK1, the SH3 domain mutant 2W2A and the N-terminal truncation mutant Δ34 with the ACK substrate SNX9 [4] and detected the tyrosine phosphorylation of SNX9 by ACK1. As shown in Figure 2(E), the SH3 domain mutant 2W2A phosphorylated SNX9 independently of Cdc42 (lanes 3 and 4), suggesting that the kinase activity of ACK1 is activated by mutation of the SH3 domain. Taken together, the data suggest that cell adhesion signalling activates ACK1 through interaction with the N-terminus that releases the SH3 domain-mediated inhibition of the kinase activity.
Interaction of the SH3 domain with the EBD confers auto-inhibition of ACK

How does the SH3 domain mediate the inhibition? We hypothesize that intramolecular interaction of the SH3 domain with the EBD forms an auto-inhibitory structure, since the EBD of ACK1 contains a proline-rich region [7]. To determine the interaction between the SH3 domain and the EBD, we employed the GST–EBD pull-down assay. The ACK1 truncation mutant ACK1-N729, in which the EBD is deleted (Figure 3A), was expressed in HEK-293 cells for the pull-down assay. As shown in Figure 3(B), the EBD specifically precipitated ACK1-N729 (lane 1), whereas no binding to the SH3 domain-defective mutant ACK1-N729-2W2A was observed (lane 2). To confirm the data, we also used ACK2-N551, which is more than 90% identical with ACK1 and does not have the EBD, for the EBD pull down (Figure 3C). Consistent with the data in Figure 3(B), the EBD precipitated ACK2-N551, whereas it could not bind to the SH3 domain-defective mutant ACK2-N551-2W2A (Figure 3C, lanes 1 and 2). These results indicate that the SH3 domain interacts with the EBD, which may form an auto-inhibition structure.

However, the SH3 domain-mediated inhibition on autophosphorylation of ACK1 can be produced through interaction with an SH3-binding protein to impede the kinase activity, instead of producing auto-inhibition. To exclude this possibility, we constructed the truncation mutant ACK1[N35-478], in which the first 34 amino acids and the C-terminus after residue 478 were deleted (Figure 3A). Autophosphorylation of ACK1[N35-478] should not be affected by cell adhesion and by auto-inhibition, but can still be affected by the SH3 domain-interactive protein.

To control the effect produced by the SH3 domain, we also made ACK1[N35-478]2W2A, the SH3 domain-defective mutant of ACK1[N35-478]. As shown in Figure 3(D), truncation of the C-terminus after residue 478 restored the autophosphorylation of ACK1 (lane 1) and diminished the activation by Cdc42-Q61L (lane 2), suggesting that the SH3 domain-mediated inhibition of autophosphorylation is eliminated by the C-terminal truncation. Mutation of the SH3 domain had an insignificant effect on the autophosphorylation (lanes 3 and 4), suggesting that the SH3 domain-interactive protein, if it exists, is not involved in inhibition of ACK autophosphorylation. In summary, our data support the model in which the SH3 domain interacts with the EBD to produce auto-inhibition in ACK1.

ACK1-L487F is not an active mutant

Previous studies have shown that mutation of Leu487 to a phenylalanine residue causes constitutively active kinase activity [24]. To determine whether the activation mechanism of the mutation is related to the SH3 domain-mediated auto-inhibition, we re-examined the activation of the mutant. Surprisingly, we observed no activation of ACK1 by mutation of Leu487 to phenylalanine (Figure 4A, lanes 1 and 3, and Figure 4B). Leu487 was first proposed as a residue in ACK that interacts with Leu67 of Cdc42, the activator of both ACK and PAK (p21-activated kinase), in a similar way to Leu107 in PAK [25]. Mutation of Leu107 to a phenylalanine residue in PAK caused constitutive activation in kinase activity [26]. Thus mutation of Leu487 to phenylalanine in ACK was expected to produce a similar activation effect.

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to that in PAK [25]. However, we carefully re-examined the crystal structure in both the ACK–Cdc42 (1CF4) and the PAK–Cdc42 complexes (1E0A) deposited in the PDB and found that Leu\(^{485}\), not Leu\(^{487}\), is the leucine residue similar to Leu\(^{107}\) in PAK that interacts with Leu\(^{67}\) of Cdc42 (Supplementary Figure S1 at http://www.BiochemJ.org/bj/445/bj4450255add.htm). Thus we conclude that L487F is not an active mutant of ACK1.

We further sequenced the original ACK1-L487F mutant that showed the activation [24], and found that an additional mutation located at the C-terminal end of the SH3 domain, which replaces Ser\(^{445}\) with a proline residue, was the cause for constitutive activation of ACK1 (Figures 4A and 4B). Interestingly, when Ser\(^{445}\) was mutated into an alanine residue, ACK1 was not active, as determined by in vivo phosphorylation of Nedd4-1 (neural-precursor-cell-expressed developmentally down-regulated 4-1), one of the substrates of ACK1 (Figures 4C and 4D), suggesting that the activation caused by mutation of Ser\(^{445}\) to proline is due to conformational changes. Because Ser\(^{445}\) is located at the C-terminal end of the SH3 domain, mutation of Ser\(^{445}\) to proline may significantly alter the structure of the SH3 domain and disrupt the interaction between the SH3 domain and the EBD.

The molecular mechanism by which EGFR activates ACK1

Previous studies have shown that EGF activates ACK1 [2,15]. We also confirmed the activation of ACK1 by EGF stimulation (Supplementary Figure S2 at http://www.BiochemJ.org/bj/445/bj4450255add.htm). However, how ACK1 is activated by EGFR signalling remains elusive. We noticed that the EBD of ACK1 contains multiple proline-rich regions (Figure 5A). It is possible that the EBD interacts with EGFR through Grb2, which is similar to the interaction with Axl [27]. Our hypothesis is that Grb2 competes with the SH3 domain of ACK to bind to the EBD to release the auto-inhibition in response to EGFR stimulation.

To test the hypothesis, we first determined binding of the EBD to Grb2. We made a series of truncation constructs of the EBD to separate the proline-rich regions (Figure 5A). It is possible that the EBD interacts with EGFR through Grb2, which is similar to the interaction with Axl [27]. Our hypothesis is that Grb2 competes with the SH3 domain of ACK to bind to the EBD to release the auto-inhibition in response to EGFR stimulation.

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activated EGFR [7], suggesting that the tyrosine phosphorylation of EGFR is essential for the binding. Given that the proline-rich region of the EBD interacts with Grb2, and Grb2 is bound only to the activated EGFR (Figure 6A), we speculated that Grb2 mediates binding of ACK1 to EGFR. To test this, we depleted endogenous Grb2 in lung cancer A549 cells by RNAi and examined the effect of the knockdown on binding of ACK1 to EGFR. As shown in Figures 6(B) and 6(C), by either co-immunoprecipitation or GST–EBD pull-down assay, knockdown of Grb2 eliminated the interaction of ACK1 with EGFR upon EGF stimulation (Figure 6B, second panel from the top, lane 4; and Figure 6C, top panel, lane 4), indicating that Grb2 mediates the binding of ACK1 to activated EGFR.

If EGFR signalling activates ACK1 through Grb2-mediated release of auto-inhibition of ACK1, then binding to Grb2 should activate the tyrosine kinase activity of ACK1. To test this idea, we co-transfected GFP-tagged Grb2 with wild-type ACK1 and the N-terminal truncation mutant ACK1-ΔN34, which is not activated by cell adhesion, in HEK-293 cells and examined the activation of ACK1 or ACK1-ΔN34. As shown in Figure 7, co-expression with Grb2 enhanced tyrosine phosphorylation of wild-type ACK1 2-fold and the N-terminal truncation mutant ACK1-ΔN34 11-fold, indicating that Grb2 binding activates ACK1, and supporting the hypothesis that EGFR activates ACK1 through Grb2-mediated release of auto-inhibition of ACK1.

**DISCUSSION**

The auto-inhibition model of ACK was first proposed as the EBD directly binds to the kinase domain, on the basis of studies on the complex structure of the ErbB-binding region of Mig-6, which is homologous with the EBD of ACK, and the EGFR kinase domain, which is homologous with the ACK kinase domain [22]. This auto-inhibition model was further supported by a recent study analysing the activating mutations of ACK, in which the EBD mutant ACK1-F820A is activated by disruption of the interaction of the EBD with the ACK kinase domain using the GST-fusion protein pull-down assay [23]. However, our data showed that the EBD did not bind to the kinase domain in ACK1-N729, which is defective in the SH3 domain (Figures 3B and 3C). Our previous studies have shown that ACK or the EBD interacts only with activated EGFR, suggesting that tyrosine phosphorylation of EGFR is essential for the binding [7]. These results imply that the EBD cannot directly interact with the kinase domain of full-length ACK1.
ACK or EGFR. Furthermore, our data suggest a model different from the EBD-kinase auto-inhibition model proposed previously by Zhang et al. [22] and Prieto-Echagüe et al. [23]. In our model, the SH3 domain interacts with the EBD to form an auto-inhibition of ACK kinase activity, as depicted in Figure 8. The mechanism underlying cell adhesion- and EGFR-induced activation of ACK releases this auto-inhibition. The factor, such as a cell adhesion signalling component, Cdc42 or Grb2, that can disrupt interaction of the SH3 domain with the EBD, is the activator of ACK.

How can we explain the discrepancy between our ACK activation model and the one proposed in [22] and [23]? Our explanation is that the EBD contains both the SH3 domain- and the EGFR or ACK kinase domain-binding regions, and the binding of both are required for formation of auto-inhibition of ACK and for interaction with EGFR. The SBR (SH3-binding region) is located between Gly786 and Gly814 of mouse ACK1 (Figure 5A), and the KBR (kinase-binding region) is located between Gly814 and Leu891 [22]. However, kinase binding is dependent on SH3 binding, because the EBD could not bind to the SH3 domain-defective mutant of ACK1 (Figure 3), inactivated EGFR [7], or activated EGFR upon Grb2 depletion (Figure 6). We speculate that the KBR is covered or its conformation is regulated by the SBR. The interaction of the SBR with the SH3 domain enables the KBR accessible for the kinase domain or the conformation of the KBR to fit to the kinase domain. This co-operation between SH3 binding and kinase binding can explain why the EBD does not bind to the kinase domain of ACK that is defective in the SH3 domain (Figure 3) and why ACK interacts only with activated EGFR, and EBD-ANC binds to Grb2, but not to EGFR [7] (Figure 5). This model can also explain why the mutations in the SH3 domain or the EBD cause activation of ACK [28].

In the present study, we have identified a region at the N-terminus containing L10LELL14 that determines the cell adhesion-associated activation of ACK1 (Figures 1 and 2). This region is required for cell adhesion signalling to activate ACK1, probably through disrupting the SH3-EBD-formed auto-inhibition, as the N-terminus deletion mutant ACK1ΔN34 is no longer activated by cell adhesion, whereas mutation of the SH3 domain restored the phosphorylation (Figure 2). How exactly this region regulates cell adhesion-mediated activation of ACK is not known. The SAM domain (at the N-terminus) of ACK1 functions in dimerization and subcellular localization, and regulates tyrosine phosphorylation of ACK1 [3,29]. Thus the role of the region L10LELL14 in regulating cell adhesion-mediated activation of ACK1 may be through the dimerization and the subcellular localization, which result in release of the auto-inhibition. However, the exact role of the region in regulation of cell adhesion-mediated activation of ACK needs further investigation.

A recent report argues that ACK1 does not have auto-inhibition [30]. This report observed no activation of ACK1 upon mutation of the SH3 domain or truncation of the EBD. We speculate that the discrepancy may be produced from cell adhesion-associated activation of ACK1 that is determined by the N-terminal region (Figure 1). The mutants used in the report contain the N-terminal region that may release the SH3/kinase/EBD-mediated auto-inhibition upon cell adhesion, which may compromise the activation effect of the mutations.

The results of the present study indicate that L1487F is not a constitutively active mutation, and is not similar to L107F in PAK1. Comparing the structure of the complex of Cdc42 and the CRIB domain of ACK with that of Cdc42 and the PBD of PAK1, we found that Leu485, similar to Leu107 of PAK, is the residue contacting Leu97 of Cdc42 (Supplementary Figure S1). Leu107 in PAK1 plays a key role in auto-inhibition of PAK1 kinase activity by binding to the kinase domain [26]. Cdc42 competes for the binding, thus releasing Leu107-mediated auto-inhibition. It has been proposed that ACK has a similar auto-inhibition mediated by a leucine residue that is similar to Leu107 of PAK1 [25]. If this is correct, the L485F mutant should be a constitutively active mutant. Currently, our model for ACK activation is the release of the SH3/kinase domain/EBD-formed auto-inhibition, which is different from that...
of PAK1. Cdc42-mediated activation of ACK should also be through this mechanism. Thus, testing whether L485F is an active mutant will provide confirmation of the activation mechanism of ACK.

Activation of ACK by EGF stimulation was observed more than a decade ago [2,15]. Our results suggest that Grb2 is the activator for ACK1 in EGFR signalling. Grb2 couples the interaction of ACK1 with EGFR to release auto-inhibition of ACK1. Grb2 has been observed to mediate interaction of ACK1 with a number of receptor tyrosine kinases, such as Axl, Mer, PDGFR and LTK [27]. It has been shown that Gas-6, a ligand of Axl, activates ACK1 [12], suggesting that receptor tyrosine kinases may utilize the same mechanism for activation of ACK1.

We observed that the maximal association of endogenous ACK1 with activated EGFR occurs 30–60 min after EGF stimulation, and lags behind the interaction of Cbl with EGFR [7]. The observation suggests that interaction of ACK1 with EGFR may take place on endosomes or late endosomes after dissociation of Grb2 with other interactive proteins, such as SOS (Son of sevenless), that bind to the EGFR signalling complex in the early activation phase. We have shown that ACK1 regulates the trafficking of EGFR to lysosomes for degradation [7,8]. We suspect that ACK1 interacts with EGFR on endosomes to organize an endosomal EGFR signalling complex. Genetic and cellular studies indicate that ACK1 promotes cancer cell migration and expression of ACK1 is associated with tumour progression [11]. A previous study suggests that EGFR endosomal signalling promotes cell migration [31]. inhibition of p38, a known protein kinase required for EGFR degradation, or overexpression of EGFR-Y1045F, a ubiquitination- and degradation-defective EGFR mutant, impairs EGF-promoted cell migration [31]. ACK1 may mediate EGFR endosomal signalling-dependent cell migration through interaction with and activation by EGF. Thus how ACK1 is activated by EGF endosomal signalling through the release of auto-inhibition will be a focus in our future studies, and is critical for understanding the role of ACK1 in mediating EGF-promoted cancer cell migration.

AUTHOR CONTRIBUTION
Qiong Lin designed the experiments, and performed the experiments in Figures 1–5 and Supplementary Figure S2. Jian Wang performed the experiments in Figures 5 and 6. Chandra Childress performed the experiments in Figure 7. Wannian Yang designed the Supplementary Figure S2. Jian Wang performed the experiments in Figures 5 and 6. We thank Dr Takaya Satoh (Department of Biochemistry and Molecular Biology, Kobe University, Kobe, Japan) for generously providing the constitutively active human ACK1 (ACK1 (to W.Y.)). This work was partially supported by the American Cancer Society [grant number ACS-RSG 110602 (to W.Y.)].

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SUPPLEMENTARY ONLINE DATA

The activation mechanism of ACK1 (activated Cdc42-associated tyrosine kinase 1)

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Figure S1 Leu487 of ACK1 is not the residue that interacts with Leu67 of Cdc42

(A) Alignment of the PBD of ACK1 with that of PAK1. (B) Structure of the Cdc42–ACK1–PBD complex (PDB code 1CF4) and the Cdc42–PAK–PBD complex (PDB code 1E0A). Cdc42 is in magenta, and ACK1 and PAK are in blue. The residues addressed are circled and in yellow. Note that Leu485, not Leu487, of ACK1 contacts Leu67 of Cdc42.

Figure S2 EGF activates ACK1

HEK-293 cells were transfected with Myc-tagged ACK1 or ACK1–ΔN34 for 36 h, followed by 12 h of serum starvation. The cells were stimulated with EGF (50 ng/ml) for the times indicated. Tyrosine phosphorylation of ACK1 or the truncation mutant was detected by immunoblotting with anti-phospho-ACK1 (top panel) or anti-pY (4G10) (the middle panel) antibodies. Expression of ACK1 or its mutant was determined by immunoblotting with an anti-Myc antibody (bottom panel).

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