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Activity of the nonreceptor tyrosine kinase Ack1 is regulated by tyrosine phosphorylation of its Mig6 homology region

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Abstract

Ack1 is a proto-oncogenic tyrosine kinase with homology to the tumour suppressor Mig6, an inhibitor of the epidermal growth factor receptor (EGFR). The residues critical for binding of Mig6 to EGFR are conserved within the Mig6 homology region (MHR) of Ack1. We tested whether intramolecular interactions between the Ack1 MHR and kinase domain (KD) are regulated by phosphorylation. We identified two Src phosphorylation sites within the MHR (Y859, Y860). Addition of Src-phosphorylated MHR to the Ack1 KD enhanced enzymatic activity. Co-expression of Src in cells led to increased Ack1 activity; mutation of Y859/Y860 blocked this increase. Collectively, the data suggest that phosphorylation of the Ack1 MHR regulates its kinase activity. Phosphorylation of Y859/Y860 occurs in cancers of the brain, breast, colon, and prostate, where genomic amplification or somatic mutations of Ack1 play a role in disease progression. Our findings suggest that MHR phosphorylation could contribute to Ack1 dysregulation in tumours.

Keywords

Ack1; activated Cdc42-associated kinase; phosphorylation; protein tyrosine kinase

Activated Cdc42-associated kinase (Ack1) is a nonreceptor tyrosine kinase (NRTK) that belongs to the Ack family of kinases. Ack1 is activated in response to growth factors and integrin-mediated cell adhesion [1] and is recruited to activated receptor tyrosine kinases (RTKs) such as platelet-derived growth factor receptor (PDGFR) [2,3], insulin receptor, Mer RTK [4], and epidermal growth factor receptor (EGFR) [2]. Ack1 is linked to numerous disorders, including cancer and immune diseases. Aberrant expression and dysregulation of Ack1 have been documented in prostate, breast, ovarian, and pancreatic cancers [4–7].

Ack1 has several characteristics that distinguish it from other NRTKs (Fig. 1A). First, Ack1 lacks an SH2 domain, and it is the only NRTK to have an SH3 domain C-terminal to the kinase domain (KD). Furthermore, Ack1 contains an N-terminal sterile alpha motif (SAM)

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Author contributions

YK and WTM conceived, designed, performed the experiments, analyzed the data, and wrote the manuscript.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

[2] that is thought to participate in membrane localization and dimerization [8]. Ack1 is the only tyrosine kinase to contain a Cdc42/Rac-interactive domain (CRIB) through which it binds and inhibits active GTP-bound Cdc42 [9,10]. The C-terminal region of Ack1 contains additional noncatalytic domains such as the clathrin-binding domain (CBD) [11] and the ubiquitin-associated domain (UBA) [12].

Notably, Ack1 contains a region highly homologous to Mig6, a feedback inhibitor of EGFR [13–15]. Two regions of Mig6 (segments 1 and 2) are responsible for the Mig6–EGFR interaction [15,16], which leads to the downregulation of the receptor by clathrin-mediated endocytosis. The key residues for Mig6-EGFR binding are conserved in the Mig6 homology region (MHR) of Ack1. Ack1 is the only protein in the protein database known to have homology to Mig6; the two proteins are thought to be evolutionarily linked. Both Ack1 and Mig6 share distinctive features (Fig. 1A): Like Ack1, Mig6 also contains a CRIB domain and binds to the small GTPase Cdc42. Similar to the CBD of Ack1, Mig6 possesses an endocytic domain, which has analogous functions in the endocytosis of EGFR [17]. The proline-rich sequences that are binding sites for proteins with SH3 domains are also shared between Mig6 and Ack1. Furthermore, both Mig6 and Ack1 bind to EGFR [2,12,16,18].

While the role of the MHR in Ack1 function has not been well characterized, structural and biochemical studies of Mig6 itself shed light on the molecular basis of EGFR binding. In normal EGFR signalling, binding of EGF to an EGFR monomer causes the formation of an asymmetric dimer, in which the C-lobe of one kinase (activator) contacts and induces conformational changes in the N-lobe of another (receiver) [19]. Mig6-mediated inhibition of EGFR is two-fold: allosterically, segment 1 of Mig6 blocks the formation of the activator-receiver dimer by binding to the C lobe of the EGFR kinase domain, preventing the formation of the asymmetric dimer [20]. Second, segment 2 acts as a competitive inhibitor by occupying the substrate-binding site [16]. Both segments are crucial for the complete inhibition of EGFR. Segment 1 alone cannot suppress kinase activity; the conformation of the segment-1-binding surface in the C-lobe does not change upon the activation of the kinase [19]. Rather, segment 1 serves as an anchor to facilitate the binding of segment 2 to the substrate-binding site of activated EGFR [20].

These segments from Mig6 are highly conserved within the MHR of Ack1 (Fig. 1B), suggesting that the Ack1 MHR sequences might play a similar regulatory role. The C-lobe of the Ack1 kinase domain has a segment-1-binding surface, similar to that of EGFR [20]. A phenylalanine residue (F821) that plays a key role in the Mig6–EGFR interaction is conserved and has an analogous role in the Ack1 kinase domain–MHR interaction [21]. Mutation of this residue to alanine results in a nearly 80-fold increase in kinase activity. Moreover, mutation of the corresponding residue in the segment-1-binding region of Ack1 KD (E346K), has a similar functional effect, suggesting that the engagement of MHR segment 1 could be inhibitory. Activation of Ack1 involves autophosphorylation at Y284 [22]. Unlike other NRTKs, the unphosphorylated activation loop of Ack1 KD does not block the substrate-binding site, suggesting that other intramolecular interactions regulate the activity of Ack1. Several cancer-associated frameshift mutations that lack the MHR strengthen the hypothesis that the C-terminal MHR domain has an autoinhibitory role; Ack1 P632fs and Q831fs frameshift mutants lacking MHR and UBA domains were found to have

increased expression and autophosphorylation [23]. In addition, missense mutations in the SH3 and MHR domains identified in various human cancers have been reported to enhance catalytic activity and autophosphorylation of Ack1 [2,21,24]. Therefore, the activity of Ack1 is thought to be suppressed by intramolecular interactions between the catalytic domain and the C-terminal region SH3 domain and the MHR [25,26].

The phosphorylation status of two tyrosines in Mig6 (Y394 and Y395 of segment 2) has been found to affect the inhibition of EGFR [27]. Phosphorylation of Y394 enhances the binding of segment 2 to the substrate-binding site and facilitates the phosphorylation of Y395 by EGFR. This priming phosphorylation is catalysed by Src, and the resulting phosphorylation on both Y394 and Y395 enables tight inhibition of EGFR. The co-crystal structure of EGFR bound to a Mig6 segment 2 peptide has revealed that segment 2 can interact directly with the activation loop and the active site [27].

Src is known to phosphorylate Ack1 at Y284 located on the activation loop [28], as well as additional residues in the C-terminus. The SH2 and SH3 domains of Src bind to residue Y518 and 623–652 of Ack1, respectively, and this interaction promotes the phosphorylation of Ack1 at Y284 in the activation loop. The SH3 domains of other Src family kinases (Hck, Fyn, Lyn and Yes1) have also been shown to interact with Ack1 through its C-terminal proline-rich domain [22]. Ack1 is a substrate for Hck, which can regulate Ack1 activity by phosphorylation. Therefore, an important unanswered question is whether Src (or another Src family kinase) regulates Ack1 by phosphorylating the enzyme at additional sites, including the MHR.

Here, we investigate the relationship between the MHR domain and the regulation of Ack1 by phosphorylation. We used peptides derived from the MHR domain and tested them as substrates for Src and Ack1. We expressed the entire MHR as a fusion protein and analysed its phosphorylation by Src using mass spectrometry. These experiments led to the identification of two tandem Src phosphorylation sites within the MHR (Y859, Y860). Mutation of these residues in Ack1 (Y859 and Y860) to phenylalanine reduced the Src-mediated enhancement of its kinase activity, consistent with a role for MHR phosphorylation in the regulation of Ack1.

Materials and methods

Reagents and antibodies

Nickel-nitriloacetic acid (Ni-NTA) resin was purchased from Qiagen (Germantown, MD, USA). Glutathione-agarose resin was obtained from Thermo Scientific (Waltham, MA, USA). Bovine serum albumin, leupeptin, aprotinin, PMSF, sodium orthovanadate, and EZview red anti-FLAG M2 affinity gel were obtained from MilliporeSigma (Burlington, MA, USA). Trypsin–EDTA solution was from Corning Inc. (Corning, NY, USA). Mouse monoclonal anti-phosphotyrosine antibody (clone 4G10) was obtained from MilliporeSigma; rabbit polyclonal anti-GST was from Invitrogen (A-5800, Waltham, MA, USA). Horseradish peroxidase-linked anti-FLAG mouse antibodies were obtained from MilliporeSigma (F1804). The secondary antibodies were horseradish peroxidase-linked secondary antibodies (donkey anti-rabbit IgG and sheep anti-mouse IgG horseradish) from

Cytiva (Marlborough, MA, USA). The P81 paper was from Whatman (Maidstone, UK) and [γ^{32}P]ATP was from Perkin Elmer (Waltham, MA, USA).

Peptides

All peptides were purchased from Genemed Synthesis Inc. (San Antonio, TX, USA) and purified by semipreparative HPLC on a Vydac C18 column, lyophilized, and resuspended in Tris–HCl buffer pH 8.0, as previously described [29].

Cell culture

HEK293T cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Cellgro, Mediatech, Inc, Manassas, VA, USA) supplemented with 10% fetal bovine serum (Sigma) and antibiotic-antimycotic solution (Corning). The medium was changed every 3 days. Sf9 insect cells were maintained in Sf-900 medium (Gibco) supplemented with 5% fetal bovine serum and 1000 IU·mL^{-1} streptomycin, 25 ng·mL^{-1} amphotericin B (Cellgro, Mediatech, Inc.).

Cloning and site-directed mutagenesis

The p3XFLAG-CMV7.1 plasmid was purchased from Sigma. The full-length Ack1 was subcloned as previously described [21]. Site-directed mutagenesis was performed using the Quik-Change XL II kit (Agilent, 200523, Santa Clara, CA, USA). The mutations were verified by DNA sequencing. Plasmid pUSE was obtained from MilliporeSigma and pGEX4T.1 was purchased from GE Healthcare [21].

Cell transfection and Western blotting

HEK293T cells (from American Type Culture Collection, Manassas, VA, USA) were plated in 100-mm diameter dishes. Cells were maintained in DMEM (Corning) supplemented with 10% fetal bovine serum (Sigma) and 1000 IU·mL^{-1} penicillin and streptomycin. After 24 h, the cells were transfected using 3 μg DNA with TransIT reagent (Mirus, Marietta, GA, USA) at a ratio of 3 μL TransIT per μg of DNA. Cells were harvested after 48 h, washed with phosphate-buffered saline, and lysed with modified RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40) supplemented with protease inhibitors leupeptin (10 μg·mL^{-1}), aprotinin (10 μg·mL^{-1}), PMSF (200 μM), and the phosphatase inhibitor Na_3VO_4 (1 mM).

Protein expression and purification

A recombinant baculovirus encoding the Ack1 kinase domain [21] was used to infect 600 mL of Sf9 cells at 1.8 × 10^6 cells·mL^{-1}. The infected cells were harvested after 3 days and lysed in a French pressure cell in buffer containing 20 mM Tris–HCl (pH 8.0), 5 mM β-mercaptoethanol, 10 μg·mL^{-1} leupeptin, 10 μg·mL^{-1} aprotinin, 1 mM PMSF, and 2 mM Na_3VO_4. The cell lysates were cleared by centrifugation at 30 000 ***g*** for 30 min, filtered with a 0.8 μm filter, and applied to a 4 mL Ni-NTA column. The column was first washed with 75 mL buffer A (20 mM Tris–HCl buffer (pH 8.0), 20 mM imidazole, 0.5 M NaCl, 10% glycerol, 5 mM β-mercaptoethanol, 2 mM $Na_3VO)_4$. A second wash was done with 50 mL buffer B (20 mM Tris–HCl (pH 8.0) and 1 M NaCl and a third wash was carried out with 50 mL of 20

mM Tris–HCl buffer (pH 8.0)). His-tagged proteins were eluted with a buffer containing 20 mM Tris–HCl (pH 8.0), 100 mM imidazole, 5 mM β-mercaptoethanol, and 10% glycerol. The fractions containing the Ack1 kinase domain were pooled, concentrated, and supplemented with 20% glycerol, and stored at −80 °C. Human c-Src containing SH3 and SH2 domains (residues 82–533) [30] was a gift from Dr Markus A. Seeliger (Stony Brook University). The MHR domain of Ack1 was expressed as a glutathione S-transferase (GST)-tagged fusion protein in *Escherichia coli* BL21(DE3) cells (1 L) as described previously [21]. Phospho-MHR was purified from the phosphorylation reaction with Src using a Superose 12 10/300 GL column equilibrated with 50 mM Tris–HCl, pH 8; 0.1 M NaCl and 10% glycerol.

Immunoprecipitation (IP) Kinase assay

IP-kinase assays were performed as previously described [21] with the following changes: 1 mg of total cell lysates was incubated with 30 μL of anti-Flag M2 affinity resin on a rotator at 4 °C overnight, then washed three times with Tris-buffered saline (TBS). A fraction of each sample was eluted with Laemmli buffer and analysed by 8% SDS/PAGE followed by anti-Flag Western blotting. The remaining sample was used for the phosphocellulose paper-binding assay [31]. A synthetic peptide derived from WASP (Table 1) was used as a substrate at a 0.8 mM concentration [32].

Phosphocellulose paper-binding assay

Kinase activity was measured using phosphocellulose paper-binding assays with [γ^{32}P]ATP. The reactions contained 20 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 0.25 mM ATP and 20–50 cpm·pmol^{-1} of [γ^{32}P]ATP. The peptides used in this assay are listed in Table 1. The phosphopeptides were tested at a concentration of 20, 50, and 100 μM as modulators; at 0.75 mM as substrates. Reactions were carried out at 30 °C for 15 min with 1 μM Ack KD-SH3 or Src SH3-SH2-KD.

Peptide identification by liquid chromatography-electrospray ionization tandem mass spectrometry (LC–MS/MS)

Phosphopeptides were analysed by LC–MS/MS using orbital trap (Q-Exactive HF; ThermoFisher Scientific, Waltham, MA, USA) and Q-TOF instruments (5600Plus; Applied Biosystems/MDS Analytical Technologies SCIEX, Toronto, Canada) as previously described [33]. The data were collected using ANALYST QS (Version 1.1; Applied Biosystems/MDS Analytical Technologies SCIEX). Phosphotyrosines were identified at > 92% confidence from product ion spectra data.

Analysis of evolutionarily conserved residues

Sequence conservation of MHR was determined using the ConSurf server [34,35]. The Ack1 sequence from UniProt (Q09712) was used to find homologs with the HHMER algorithm with three iterations against UniProt. The minimum and maximum sequence identities were set to 35% and 95%, respectively. Redundant sequences of high homology were filtered out and an alignment of 150 sequences was generated using the MAFFT-L-INS-i algorithm. ConSurf calculates a conservation score for each residue using an empirical Bayesian method and normalizes the scores to a mean of 0 and a standard deviation of 1. The scores

are then grouped into 9 grades, where 1 indicates the least conserved (highly evolving) sites, and 9 corresponds to the most evolutionarily conserved positions. The conservation grades were mapped onto the Ack1 MHR (Q07912, 733–876) using the ConSurf colour code, with cyan to maroon gradient corresponding to the variable (1) to conserved (9) positions, respectively. ConSurf additionally combines evolutionary conservation findings with solvent accessibility predictions and categorizes residues as putatively functional and structural. If a highly conserved residue is buried or exposed, it is classified as either structural or functional, respectively.

Results

Alignment of Mig6 and the Ack1 MHR

Both segment 1 (residues 804–833) and segment 2 (residues 842–877) of the Ack1 MHR show significant homology to their corresponding segments in Mig6. We generated a pairwise sequence alignment with EMBOSS Water using the Smith–Waterman algorithm (UniProt Q07912 and Q9UJM3 [36]) (Fig. 1B). Ack1 and Mig6 are 80.6% homologous and 53.7% identical within the 132 residues they share. The alignment shows less conserved positions in light blue and highlights identical residues in dark blue. Segment 2 of Mig6 is highly conserved within the MHR of Ack1 with near 75% sequence identity. Tandem tyrosines in Mig6 (Y394, Y395) that regulate the inhibition of EGFR are also conserved (Ack1 Y859 and Y860), as are three additional tyrosines (Ack1 Y827, Y868, and Y872).

Src phosphorylation of MHR

Considering the known role of Src in Mig6 phosphorylation and regulation, and the strict conservation of five tyrosines within the Ack1 MHR, we tested whether these sites are phosphorylation sites. First, we confirmed that full-length Ack1 can be phosphorylated by Src in mammalian cells. We co-expressed FLAG-tagged Ack1 and a constitutively active form of Src (Y527F) in HEK293T cells. We carried out immunoprecipitation reactions with anti-FLAG antibody followed by Western blotting with anti-phosphotyrosine antibody. Co-expression of Src greatly enhanced Ack1 tyrosine phosphorylation (Fig. 2A).

Next, we designed synthetic peptides derived from segment 1 and segment 2 of the Ack1 MHR, designated MHR1 and MHR2, respectively (Table 1). These peptides encompass all the tyrosines present in MHR: Y827 (MHR1); Y859, Y860, Y868, and Y872 (MHR2). Given that both Mig6 (Y395) and Ack1 (Y284) are known substrates of Src [27,28], we tested these MHR peptides as *in vitro* substrates for a purified Src construct consisting of the SH3, SH2, and catalytic domains using the phosphocellulose paper-binding assay, as described previously [31,37]. Src phosphorylated both MHR1 and MHR2 peptides (Fig. 2B). We also tested the peptides as substrates for Ack1 under identical conditions. A purified Ack1 construct comprising the catalytic and the SH3 domains [32] showed no activity toward the MHR1 and MHR2 peptides.

To test a larger MHR construct as a substrate for Src and identify phosphorylation sites, we expressed the entire domain as a GST fusion protein in *E. coli*. We ran an *in vitro* phosphorylation reaction with purified Src and analysed the aliquots at various time points

on SDS/PAGE. Immunoblotting with phosphotyrosine antibodies revealed that GST-MHR was phosphorylated by Src (Fig. 2C). We then analysed the reaction mixture containing phospho-MHR by subjecting it to LC–MS/MS and identified the residues phosphorylated by Src as Y827, Y859, and Y860 (Figs 2D and S1).

To investigate the potential effects of these phosphorylations on the catalytic activity of Ack1, we produced synthetic phospho-peptides based on these sites, designated as pY827, pY859, and pY860 (Table 1). For the pY859 peptide, the only site available for phosphorylation is Y860, and for the pY860 peptide, it is Y859. The pY827 peptide contains no phosphorylation sites. In Mig6, the corresponding tandem tyrosines show a unique pattern of phosphorylation: phosphorylation of the second tyrosine (Y395) by Src primes phosphorylation of the first (Y394) by EGFR. We tested whether the pY859 and pY860 peptides were substrates for Src or Ack1. Although the MHR2 peptide (containing unphosphorylated Y859–Y860) is not phosphorylated by Ack1 (see above), the phosphopeptides were Ack1 substrates (Fig. 3A). Interestingly, Ack1 strongly preferred the pY860 peptide as a substrate, suggesting that phosphorylation of Y859 is dependent on prior phosphorylation of Y860 (Fig. 3A). Src, however, preferentially phosphorylated Y860 of MHR (Fig. 3B), mirroring its phosphorylation of Y395 of Mig6 [27].

Next, we tested whether the pY827, pY859, and pY860 peptides affect Ack1 kinase *in vitro*. The substrate for these experiments was the WASP peptide (Table 1), the most efficient Ack1 substrate reported to date [32]. We immunoprecipitated full-length Ack1 from transfected HEK293T cells and carried out IP-kinase reactions with WASP peptide and [γ^{32}P]-ATP. The synthetically phosphorylated peptides had no effect on Ack1 kinase activity (Fig. 3C). We obtained similar results in an experiment using the purified Ack1 kinase-SH3 domain construct (Fig. 3D).

Effects of phosphorylated MHR on Ack1 kinase activity

Phosphorylation of Mig6 on Y394 by active EGFR is essential for its inhibitory effects on the catalytic domain [27,38–41]. In contrast to the interaction dynamics observed with EGFR, Mig6 binds to inactive c-Abl in mammary epithelial cells and is phosphorylated by c-Abl on Y394-Y395 [42]. Strikingly, binding and subsequent phosphorylation of Mig6 activate c-Abl, indicating that Mig6-mediated tyrosine kinase regulation could be context dependent.

To gain insights into the functional outcomes of the homologous phosphotyrosines, Y859–Y860, we evaluated whether Src-phosphorylated MHR would have an inhibitory or stimulatory effect on the kinase activity of Ack1. We carried out an *in vitro* reaction with GST-MHR and Src, then purified phospho-MHR (pMHR) by gel filtration chromatography. Unphosphorylated MHR had no effect on purified Ack1 kinase activity against a synthetic peptide substrate (Fig. 4, MHR). Incubation of pMHR led to a concentration-dependent increase in Ack1 kinase domain activity, as measured using [γ^{32}P]ATP and a peptide substrate (Fig. 4).

Ack1 is a substrate for Src

Several high-throughput proteomic studies identified that Ack1 residues Y827, Y859, and Y860 of Ack1 are phosphorylated in various contexts: in BCR-Abl transformed cell lines [43], downstream of insulin signalling [44], in EGF-treated HEK293T cells, and in human mammary epithelial cells. Phospho-Y827 of Ack1 was also identified in HeLa cells stimulated with EGF [45] and in human mammary epithelial cells overexpressing HER2 (an EGFR family member). It is possible that Ack1 could be a substrate for EGFR or insulin receptors themselves, or another tyrosine kinase downstream of these signalling pathways. To date, the effector enzyme in cells remains unknown. Therefore, determining the effects of these phosphorylation sites on Ack1 activity and their biological relevance is important for our understanding of the function of the MHR domain.

We tested whether the effects of the MHR on the catalytic activity would also be evident in the context of the full-length protein in intact cells. We generated nonphosphorylatable (Y to F) mutants of full-length Ack1 (Y827F, Y859F, and Y860F). To test whether loss of hydroxyl sidechains would interfere with the putative inhibitory interactions between the MHR and the kinase domain, we transiently expressed the full-length wild-type or nonphosphorylatable forms of Ack1 in HEK293T cells and co-transfected with a constitutively active form of Src. We measured Ack1 activity by immunoprecipitating Ack1, incubating with WASP peptide and [γ^{32}P]ATP, and carrying out *in vitro* phosphorylation assays (Fig. 5). In cells expressing Ack1 alone, the Tyr-to-Phe mutations caused a ≥ 2-fold increase in Ack1 catalytic activity, relative to WT Ack1 (Fig. 5, grey bars). Co-expression with Src enhanced the catalytic activity of WT Ack1. However, co-transfection of Src with the nonphosphorylatable mutant-expressing cells (Y859F and Y860F) did not produce a significant increase in catalytic activity. A similar result was observed when Y394–Y395 in Mig6 was mutated to phenylalanine, impairing its ability to bind EGFR [46]. The activity of Y827F was higher than that of WT Ack1. This is consistent with studies of Mig6; the equivalent tyrosine in Mig6 makes contact with a Val residue in the C-lobe. Alanine substitution of this residue in the MHR of Ack1 was found to increase autophosphorylation [21]. Interestingly, the segment 1 phosphorylation site mutant Y827F retained the ability to be activated by Src; the phosphorylation sites on Y859 and Y860 were intact in this mutant. This agrees with our hypothesis that segment 1 serves as a hinge that orients segment 2 toward the activation loop of Ack1. Overall, our observations are consistent with the hypothesis that the autoinhibitory interactions of MHR can be modulated by phosphorylation.

Analysis of the evolutionarily conserved residues within the MHR

We used the ConSurf server to determine the evolutionarily conserved regions or residues within the MHR of Ack1 based on the multiple sequence alignment of 150 homologs (Fig. S2) [34,35,47,48]. ConSurf categorized Y827, Y859, and Y860 as conserved residues with respective conservation scores of 9, 9, and 8 (Fig. 6). Y860 was replaced with H892 in TNK2 of the channel catfish (*Ictalurus punctatus*) included in the alignment. Interestingly, in zebrafish (*Danio rerio*), whose genome is the most similar to that of catfish, the YY in MHR is conserved. Another homolog of Ack1 studied in *Drosophila melanogaster*, DAck1, also possesses the YY motif.

ConSurf predicted Y827 of segment 1 to be exposed whereas the tandem tyrosines in segment 2, Y859-Y860, were expected to be buried residues. Other residues in segment 2, especially 857–866, are considerably more conserved compared to segment 1 (Fig. S3). Except for prolines N-terminal to segment 1, residues outside the EGFR-binding region (segment 1 + segment 2) are variable within the MHR. Moreover, the other two tyrosines in segment 2 (Y868 and Y872) were found to be moderately conserved. Y872 had a conservation score of 3, indicating that this position is variable and rapidly evolving (Fig. S3). These findings lend strength to the idea that the tandem tyrosines in the Ack1 MHR (Y859, Y860) play an important regulatory role, analogous to their function in Mig6.

Discussion

The purpose of this study was to test if tyrosine phosphorylation of the MHR is a potential regulatory mechanism for Ack1 activity. We hypothesized that the autoinhibition of Ack1 KD by its C terminal MHR domain might be influenced by the phosphorylation of tandem tyrosines. We identified Src phosphorylation sites within the MHR (Fig. 2). There is precedent in the literature for phosphorylated Mig6 serving as either a kinase inhibitor (for EGFR) or activator (for c-Abl). We found that phosphorylated MHR enhanced Ack1 kinase activity (Fig. 4). Moreover, mutation of these residues to Phe diminished the Src-mediated activation of full-length Ack1 in HEK cells (Fig. 5). Thus, we postulate that phosphorylation of the MHR releases the domain from an autoinhibitory interaction (Fig. 7).

The kinase domains of EGFR and Ack1 share ~ 40% sequence homology and have closely related structures. Since there is no crystal structure of full-length Ack1 or the KD bound to MHR-mimicking peptides, we utilized the crystal structure of the EGFR kinase domain in complex with Mig6 peptides to interpret our study. We superimposed the Ack1 kinase domain onto the EGFR kinase domain using the crystal structure of the EGFR kinase domain bound to the Mig6 EGFR-binding domain (segment 1 + segment 2; Fig. 8). The root-mean-square-deviation between the 260 atom pairs aligned was 1.95 Å. Both the substrate binding cleft and the activation loop of Ack1 are surrounded by positively charged residues (Fig. 8C,D) [26]. The superimposed model suggests that three arginines near the substrate-binding site (R251, R290, and R305) might be H-bonding with MHR, which could explain the increase in catalytic activity observed for the Y859F and Y860F mutants (Fig. 5).

Most residues involved in the interaction between EGFR KD and Mig6 segment 2 are conserved in Ack1 KD and MHR, including the tandem tyrosines and their interacting partners. However, a key lysine residue, K879, of EGFR is replaced by A295 in Ack1 (Fig. 8A). In EGFR, this lysine coordinates pY395 of Mig6, stabilizing Mig6 as a substrate, and facilitates the positioning of Y394 toward the active site of EGFR. Interestingly, c-Abl also has a lysine at this position, suggesting the involvement of other residues in defining the nature (activating vs. suppressing) of Mig6's regulatory role. The presence of A295 at this position for Ack1 could explain why the exogenously added phospho-MHR activates the enzyme. This model assumes that the Ack1 KD adopts a conformation similar to that of EGFR KD in the crystal structures of EGFR KD-Mig6, which is unknown and a limitation for our study at present.

The unphosphorylated MHR1 and MHR2 peptides were not substrates for the isolated KD of Ack1. A fundamental difference between the EGFR–Mig6 and Ack1–MHR interactions is that Mig6 is a separate protein that regulates EGFR through association and dissociation, while MHR is a domain on Ack1 itself that presumably binds *via* intramolecular interactions. In full-length Ack1, the affinity of the MHR for the kinase domain would be increased by the entropic effect of being present on the same polypeptide. This effect would be lost in experiments with exogenously added MHR or MHR-derived peptides. Hence, the affinities of the isolated MHR domain or peptides for Ack1 KD might be expected to be much less than those of Mig6 for EGFR KD. However, and similarly to EGFR, a 'primed' peptide containing pY860 is phosphorylated at Y859 by Ack1 (Fig. 3A).

Besides its role in blocking dimerization, segment 1 is proposed to aid in positioning segment 2 near the active site. Segment 1 of the Ack1 MHR may play a similar role. Ack1 dimerizes *via* its N-terminal SAM domain [8], rather than *via* its kinase domain. Hence, segment 1 of MHR is more likely to serve as a hinge to orient segment 2. This hypothesis agrees with a previous study reporting that a cancer-related mutation in the segment-1-binding surface of the C-lobe, E346K, resulted in the 6-fold activation of the kinase [27].

Our studies used Src to phosphorylate the MHR of Ack1, based on the prior work with Mig6. We do not exclude the possibility that other kinases in addition to Src might also phosphorylate MHR. Hck phosphorylates Ack1, although the phosphosites are yet to be identified [22]. The kinases of the Src family Fyn, Lyn, and Yes1 all interact with the proline-rich C-terminal region of Ack1 and could potentially phosphorylate the MHR. EGFR is also a candidate; it is suggested to prefer substrates that contain the YpY motif [27]. Ack1 interacts only with catalytically active EGFR and cannot bind kinase-impaired EGFR, paralleling Mig6 [12]. Given the sequence identity between segment 2 of MHR and Mig6, Y859-Y860 of Ack1 MHR are likely to be recognized by EGFR. Moreover, the MHR of Ack1 could be a substrate for other RTKs that are known to interact with Ack1, including MER, PDGFR, AXL, ALK, or LTK, modulating autoinhibition.

More work is required to determine the cellular functions of these phosphorylations. Ack1 is involved in the internalization and degradation of EGFR, a process facilitated by its binding through the MHR [12,49,50]. It is currently unknown whether the phosphorylation of Y859–Y860 affects the binding of Ack1 to EGFR and, concomitantly, the turnover of EGFR. This could have implications for EGFR-driven cancers such as glioblastoma, nonsmall-cell lung cancer, and colorectal cancer.

A substantial number of phosphoproteomic studies have reported the presence of Ack1 pY859 and pY860 in tumour tissues isolated from brain, breast, colon and stomach cancer, leukaemias and lymphomas, lung cancer, ovarian cancer, pancreatic, and prostate cancer. Genomic amplification, overexpression, or mutation of Ack1 has been well-studied to play a role in the progression of these cancers. Dysregulation of signalling pathways resulting in phosphorylation of Y859–Y860 might be an additional mechanism through which Ack1 exerts its tumorigenic effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data accessibility

The data that support the findings of this study are available in Figs 1–8 and S1–S3 of this article.

Abbreviations

CBD	clathrin-binding domain
CRIB	Cdc42/Rac interactive binding
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
GST	glutathione S-transferase
HEK	human embryonic kidney
KD	kinase domain
LC–MS/MS	liquid chromatography tandem mass spectrometry
MHR	Mig6 homology region
Ni-NTA	Nickel-Nitrilotriacetic acid
NRTK	nonreceptor tyrosines kinase
pMHR	phosphorylated MHR
RTK	receptor tyrosines kinase
SAM	sterile alpha motif
SH2	Src Homology 2
SH3	Src Homology 3
UBA	ubiquitin-associated domain
WASP	Wiskott–Aldrich syndrome protein

References

1. Yang W, Lin Q, Guan JL, Cerione RA. Activation of the Cdc42-associated tyrosine kinase-2 (ACK-2) by cell adhesion via integrin beta1. J Biol Chem. 1999;274:8524–30. [PubMed: 10085085]
2. Galisteo ML, Yang Y, Urena J, Schlessinger J. Activation of the nonreceptor protein tyrosine kinase Ack by multiple extracellular stimuli. Proc Natl Acad Sci USA. 2006;103:9796–801. [PubMed: 16777958]
3. Zhang J, Chen T, Mao Q, Lin J, Jia J, Li S, et al. PDGFR-beta-activated ACK1-AKT signaling promotes glioma tumorigenesis. Int J Cancer. 2015;136:1769–80. [PubMed: 25257795]
4. Mahajan NP, Whang YE, Mohler JL, Earp HS. Activated tyrosine kinase Ack1 promotes prostate tumorigenesis: role of Ack1 in polyubiquitination of tumor suppressor Wwox. Cancer Res. 2005;65:10514–23. [PubMed: 16288044]
5. Mahajan K, Coppola D, Challa S, Fang B, Chen YA, Zhu W, et al. Ack1 mediated AKT/PKB tyrosine 176 phosphorylation regulates its activation. PLoS ONE. 2010;5:e9646. [PubMed: 20333297]
6. van der Horst EH, Degenhardt YY, Strelow A, Slavin A, Chinn L, Orf J, et al. Metastatic properties and genomic amplification of the tyrosine kinase gene ACK1. Proc Natl Acad Sci USA. 2005;102:15901–6. [PubMed: 16247015]
7. Mahajan K, Coppola D, Chen YA, Zhu W, Lawrence HR, Lawrence NJ, et al. Ack1 tyrosine kinase activation correlates with pancreatic cancer progression. Am J Pathol. 2012;180:1386–93. [PubMed: 22322295]
8. Prieto-Echague V, Gucwa A, Brown DA, Miller WT. Regulation of Ack1 localization and activity by the amino-terminal SAM domain. BMC Biochem. 2010;11:42. [PubMed: 20979614]
9. Manser E, Leung T, Salihuddin H, Tan L, Lim L. A non-receptor tyrosine kinase that inhibits the GTPase activity of p21cdc42. Nature. 1993;363:364–7. [PubMed: 8497321]
10. Burbelo PD, Drechsel D, Hall A. A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. J Biol Chem. 1995;270:29071–4. [PubMed: 7493928]
11. Teo M, Tan L, Lim L, Manser E. The tyrosine kinase ACK1 associates with clathrin-coated vesicles through a binding motif shared by arrestin and other adaptors. J Biol Chem. 2001;276:18392–8. [PubMed: 11278436]
12. Shen F, Lin Q, Gu Y, Childress C, Yang WN. Activated Cdc42-associated kinase 1 is a component of EGF receptor signaling complex and regulates EGF receptor degradation. Mol Biol Cell. 2007;18:732–42. [PubMed: 17182860]
13. Fiorentino L, Pertica C, Fiorini M, Talora C, Crescenzi M, Castellani L, et al. Inhibition of ErbB-2 mitogenic and transforming activity by RALT, a mitogen-induced signal transducer which binds to the ErbB-2 kinase domain. Mol Cell Biol. 2000;20:7735–50. [PubMed: 11003669]
14. Xu D, Makkinje A, Kyriakis JM. Gene 33 is an endogenous inhibitor of epidermal growth factor (EGF) receptor signaling and mediates dexamethasone-induced suppression of EGF function. J Biol Chem. 2005;280:2924–33. [PubMed: 15556944]
15. Anastasi S, Fiorentino L, Fiorini M, Fraioli R, Sala G, Castellani L, et al. Feedback inhibition by RALT controls signal output by the ErbB network. Oncogene. 2003;22:4221–34. [PubMed: 12833145]
16. Anastasi S, Baietti MF, Frosi Y, Alema S, Segatto O. The evolutionarily conserved EBR module of RALT/MIG6 mediates suppression of the EGFR catalytic activity. Oncogene. 2007;26:7833–46. [PubMed: 17599051]
17. Frosi Y, Anastasi S, Ballarò C, Varsano G, Castellani L, Maspero E, et al. A two-tiered mechanism of EGFR inhibition by RALT/MIG6 via kinase suppression and receptor degradation. J Cell Biol. 2010;189:557–71. [PubMed: 20421427]
18. Hackel PO, Gishizky M, Ullrich A. Mig-6 is a negative regulator of the epidermal growth factor receptor signal. Biol Chem. 2001;382:1649–62. [PubMed: 11843178]
19. Zhang X, Gureasko J, Shen K, Cole PA, Kuriyan J. An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. Cell. 2006;125:1137–49. [PubMed: 16777603]

20. Zhang X, Pickin KA, Bose R, Jura N, Cole PA, Kuriyan J. Inhibition of the EGF receptor by binding of MIG6 to an activating kinase domain interface. Nature. 2007;450:741–4. [PubMed: 18046415]
21. Prieto-Echague V, Gucwa A, Craddock BP, Brown DA, Miller WT. Cancer-associated mutations activate the nonreceptor tyrosine kinase Ack1. J Biol Chem. 2010;285:10605–15. [PubMed: 20110370]
22. Yokoyama N, Miller WT. Biochemical properties of the Cdc42-associated tyrosine kinase ACK1. Substrate specificity, authphosphorylation, and interaction with Hck. J Biol Chem. 2003;278:47713–23. [PubMed: 14506255]
23. Maxson JE, Abel ML, Wang J, Deng X, Reckel S, Luty SB, et al. Identification and characterization of tyrosine kinase nonreceptor 2 mutations in leukemia through integration of kinase inhibitor screening and genomic analysis. Cancer Res. 2016;76:127–38. [PubMed: 26677978]
24. Chua BT, Lim SJ, Tham SC, Poh WJ, Ullrich A. Somatic mutation in the ACK1 ubiquitin association domain enhances oncogenic signaling through EGFR regulation in renal cancer derived cells. Mol Oncol. 2010;4:323–34. [PubMed: 20359967]
25. Lin Q, Wang J, Childress C, Yang W. The activation mechanism of ACK1 (activated Cdc42-associated tyrosine kinase 1). Biochem J. 2012;445:255–64. [PubMed: 22553920]
26. Gajiwala KS, Maegley K, Ferre R, He YA, Yu X. Ack1: activation and regulation by allostery. PLoS ONE. 2013;8:e53994. [PubMed: 23342057]
27. Park E, Kim N, Ficarro SB, Zhang Y, Lee BI, Cho A, et al. Structure and mechanism of activity-based inhibition of the EGF receptor by Mig6. Nat Struct Mol Biol. 2015;22:703–11. [PubMed: 26280531]
28. Chan W, Sit ST, Manser E. The Cdc42-associated kinase ACK1 is not autoinhibited but requires Src for activation. Biochem J. 2011;435:355–64. [PubMed: 21309750]
29. Chan PM, Keller PR, Connors RW, Leopold WR, Miller WT. Amino-terminal sequence determinants for substrate recognition by platelet-derived growth factor receptor tyrosine kinase. FEBS Lett. 1996;394:121–5. [PubMed: 8843147]
30. Seeliger MA, Young M, Henderson MN, Pellicena P, King DS, Falick AM, et al. High yield bacterial expression of active c-Abl and c-Src tyrosine kinases. Protein Sci. 2005;14:3135–9. [PubMed: 16260764]
31. Casnellie JE. Assay of protein kinases using peptides with basic residues for phosphocellulose binding. Methods Enzymol. 1991;200:115–20. [PubMed: 1956315]
32. Yokoyama N, Miller WT. Purification and enzyme activity of ACK1. Methods Enzymol. 2006;406:250–60. [PubMed: 16472662]
33. Petti F, Thelemann A, Kahler J, McCormack S, Castaldo L, Hunt T, et al. Temporal quantitation of mutant Kit tyrosine kinase signaling attenuated by a novel thiophene kinase inhibitor OSI-930. Mol Cancer Ther. 2005;4:1186–97. [PubMed: 16093434]
34. Ashkenazy H, Erez E, Martz E, Pupko T, Ben-Tal N. ConSurf 2010: calculating evolutionary conservation in sequence and structure of proteins and nucleic acids. Nucleic Acids Res. 2010;38:W529–33. [PubMed: 20478830]
35. Ashkenazy H, Abadi S, Martz E, Chay O, Mayrose I, Pupko T, et al. ConSurf 2016: an improved methodology to estimate and visualize evolutionary conservation in macromolecules. Nucleic Acids Res. 2016;44:W344–50. [PubMed: 27166375]
36. Consortium UniProt. UniProt: the universal protein knowledgebase in 2021. Nucleic Acids Res. 2020;49:D480–9.
37. Garcia P, Shoelson SE, George ST, Hinds DA, Goldberg AR, Miller WT. Phosphorylation of synthetic peptides containing Tyr-Met-X-Met motifs by nonreceptor tyrosine kinases in vitro. J Biol Chem. 1993;268:25146–51. [PubMed: 8227078]
38. Zhang Y, Zheng QC. Exploring the interactions of EGFR with phosphorylated Mig6 by molecular dynamics simulations and MM-PBSA calculations. J Theor Biol. 2018;447:118–25. [PubMed: 29574142]

39. Boopathy GTK, Lynn JLS, Wee S, Gunaratne J, Hong W. Phosphorylation of Mig6 negatively regulates the ubiquitination and degradation of EGFR mutants in lung adenocarcinoma cell lines. Cell Signal. 2018;43:21–31. [PubMed: 29196224]
40. Li N, Wei M. Conversion of MIG6 peptide from the nonbinder to binder of lung cancer-related EGFR by phosphorylation and cyclization. Artif Cells Nanomed Biotechnol. 2017;45:1023–8. [PubMed: 27346601]
41. Yu XD, Yang R, Leng CJ. Truncation, modification, and optimization of MIG6(segment 2) peptide to target lung cancer-related EGFR. Comput Biol Chem. 2016;61:251–7. [PubMed: 26967626]
42. Hopkins S, Linderoth E, Hantschel O, Suarez-Henriques P, Pilia G, Kendrick H, et al. Mig6 is a sensor of EGF receptor inactivation that directly activates c-Abl to induce apoptosis during epithelial homeostasis. Dev Cell. 2012;23:547–59. [PubMed: 22975324]
43. Salomon AR, Ficarro SB, Brill LM, Brinker A, Phung QT, Ericson C, et al. Profiling of tyrosine phosphorylation pathways in human cells using mass spectrometry. Proc Natl Acad Sci USA. 2003;100:443–8. [PubMed: 12522270]
44. Ibarrola N, Molina H, Iwahori A, Pandey A. A novel proteomic approach for specific identification of tyrosine kinase substrates using [13C]tyrosine. J Biol Chem. 2004;279:15805–13. [PubMed: 14739304]
45. Olsen JV, Blagoev B, Gnad F, Macek B, Kumar C, Mortensen P, et al. Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. Cell. 2006;127:635–48. [PubMed: 17081983]
46. Maity TK, Venugopalan A, Linnoila I, Cultraro CM, Giannakou A, Nemati R, et al. Loss of MIG6 accelerates initiation and progression of mutant epidermal growth factor receptor-driven lung adenocarcinoma. Cancer Discov. 2015;5:534–49. [PubMed: 25735773]
47. Celniker G, Nimrod G, Ashkenazy H, Glaser F, Martz E, Mayrose I, et al. ConSurf: using evolutionary data to raise testable hypotheses about protein function. Isr J Chem. 2013;53:199–206.
48. Berezin C, Glaser F, Rosenberg J, Paz I, Pupko T, Fariselli P, et al. ConSeq: the identification of functionally and structurally important residues in protein sequences. Bioinformatics. 2004;20:1322–4. [PubMed: 14871869]
49. Grovdal LM, Johannessen LE, Rodland MS, Madshus IH, Stang E. Dysregulation of Ack1 inhibits downregulation of the EGF receptor. Exp Cell Res. 2008;314:1292–300. [PubMed: 18262180]
50. Jones S, Cunningham DL, Rappoport JZ, Heath JK. The non-receptor tyrosine kinase Ack1 regulates the fate of activated EGFR by inducing trafficking to the p62/NBR1 pre-autophagosome. J Cell Sci. 2014;127:994–1006. [PubMed: 24413169]
51. Smith TF, Waterman MS. Identification of common molecular subsequences. J Mol Biol. 1981;147:195–7. [PubMed: 7265238]
52. Waterhouse AM, Procter JB, Martin DM, Clamp M, Barton GJ. Jalview Version 2—a multiple sequence alignment editor and analysis workbench. Bioinformatics. 2009;25:1189–91. [PubMed: 19151095]

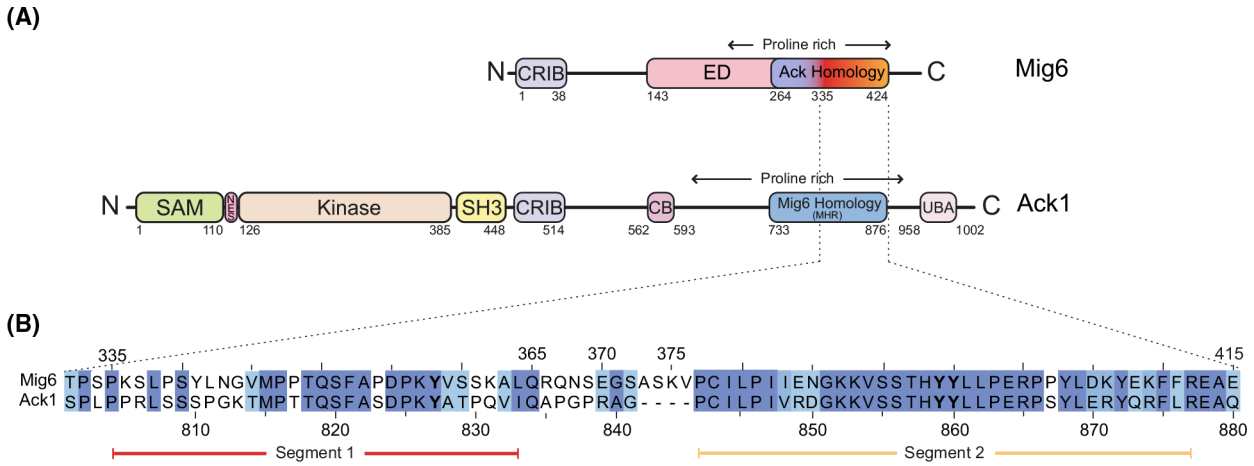

Fig. 1.

Comparison of linear structures of human Mig6 and Ack1. (A) Top, Mig6. CRIB, Cdc42/Rac interactive and binding domain; ED, endocytic domain. The Ack homology domain contains sequences important in binding EGFR, namely segment 1 (335–365) and segment 2 (377–413), depicted in red and orange, respectively. Bottom, Ack1. SAM, Sterile alpha motif; NES, nuclear export signal; SH3, Src homology 3 domain; CB, clathrin-binding motif; MHR, Mig6 homology region; UBA, ubiquitin-associated domain. (B) Sequence homology between Ack1 residues 800–880 and Mig6 residues 332–415. Pairwise sequence alignment was generated by EMBOSS Water [51] using the Smith–Waterman algorithm and visualized with JALVIEW [52] using the BLOSUM62 colouring scheme. Residue numbers are labelled according to UniProt (Q07912 and Q9UJM3). The dark blue columns represent the alignment of identical amino acid residues, whereas the lighter colours represent the alignment of less conserved positions.

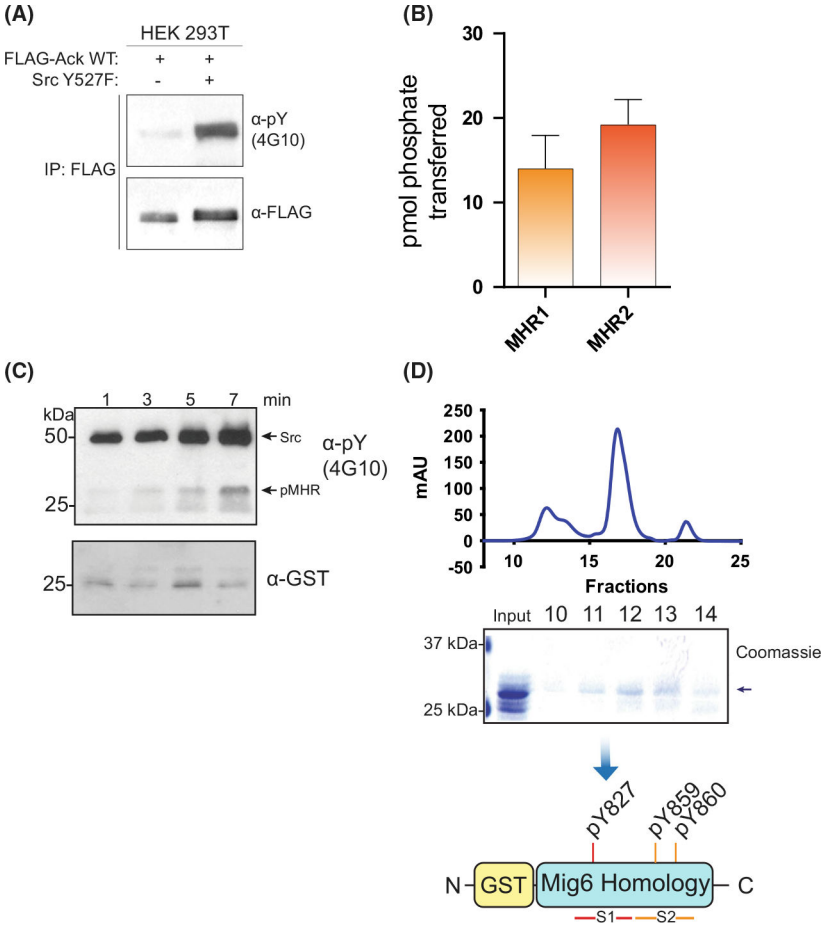

Fig. 2.

Src phosphorylation of MHR. (A) FLAG-tagged Ack1 was transfected into HEK293T cells alone, or together with a constitutively active version of Src (Y527F). Immunoprecipitation (IP) reactions were performed with anti-FLAG antibody followed by Western blotting with anti-phosphotyrosine and anti-FLAG antibody. (B) MHR-derived peptides (0.75 mM) were tested as Src substrates in a radiometric kinase assay. (C) Phosphorylation of MHR by Src *in vitro*. GST-MHR (10 μM) was incubated with Src (1 μM) for the indicated times, and the results were analysed by Western blotting with anti-pTyr antibody. (D) Identification of Src-phosphorylated residues in panel (C). The reaction mixture from panel (C) was fractionated on a Superose 12 10/300 GL column. The fractions containing the phosphorylated MHR were pooled, concentrated, and subjected to LC–MS/MS with chymotrypsin and lysC digestion. S1, segment 1; S2, segment 2.

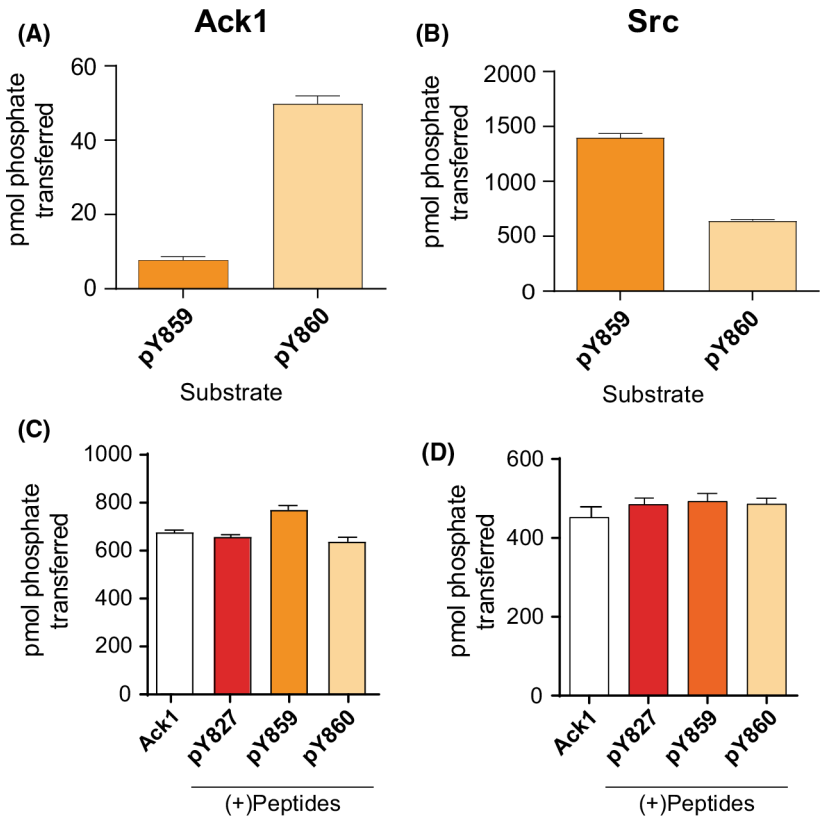

Fig. 3.

Synthetic phosphopeptides tested as substrates or modulators of catalytic activity. (A) Phosphorylation of the synthetic phospho-peptides (Table 1) (0.75 mM) by Ack1 KD-SH3 was measured using the phosphocellulose-binding assay. (B) Similar experiments were performed with Src. (C) The indicated phosphopeptides (0.75 mM) were added to *in vitro* kinase reactions with full-length (FL) Ack1 immunoprecipitated from HEK29T cells and 0.8 mM WASP peptide substrate (open bar, Ack1). The phosphopeptides were added to the reaction at a concentration of 20, 50, and 100 μM as modulators. The collared bars show the reactions with 100 μM designated phosphopeptides. (D) The phosphopeptides were added to kinase reactions with the purified Ack1 KD-SH3 and WASP peptide (open bar, Ack1). The phosphopeptides were tested at a concentration of 20, 50, and 100 μM as modulators. Collared bars show the reactions with 100 μM designated phosphopeptides.

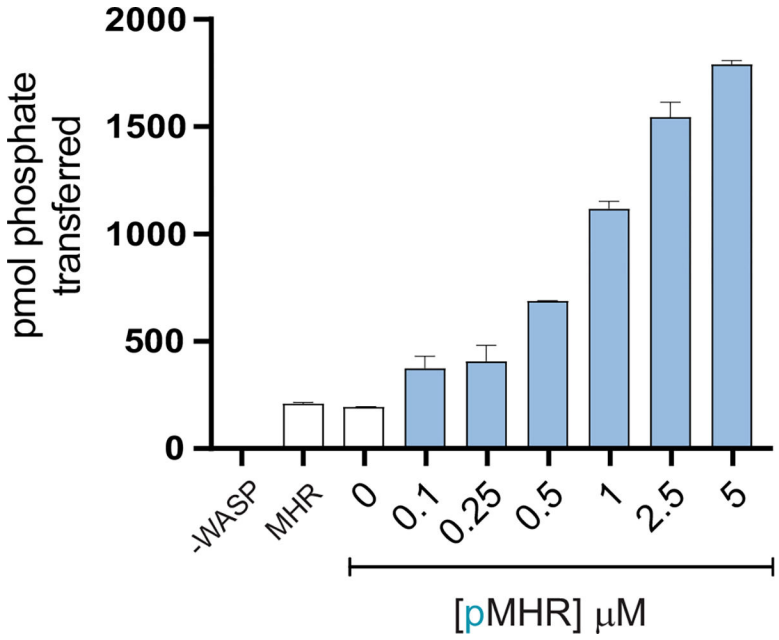

Fig. 4.

Effects of phospho-GST-MHR on Ack1 kinase domain activity. GST-tagged phospho-MHR (filled bars) that was isolated as described in Fig. 2D was added to reactions containing 1 μM Ack1 KD-SH3, [γ^{32}P]-ATP, and 0.8 mM WASP peptide at the indicated concentrations. Ack1 activity was measured with the phosphocellulose paper-binding assay. Controls (open bars) include a reaction without WASP and a reaction with unphosphorylated MHR (5 μM).

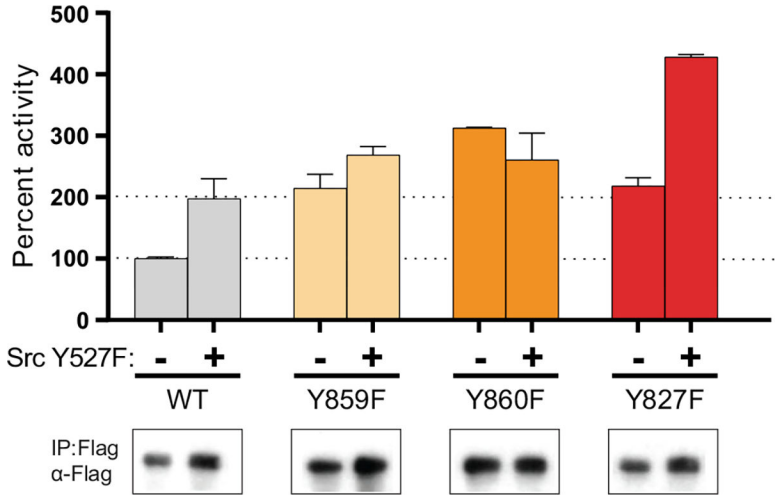

Fig. 5.

Mutation of Y859 and Y860 to a nonphosphorylatable residue prevents the full-length Ack1 from further activation by Src. HEK293T cells were transfected with FLAG-tagged Ack1 (wild-type, Y859F, Y860F, or Y827F) alone or together with a constitutively active form of Src (Y527F). The Ack1 constructs were immunoprecipitated using anti-FLAG and their activities toward WASP peptide were determined using the phosphocellulose paper-binding assay with [γ^{32}P]-ATP. The activities are expressed relative to WT Ack1 in the absence of Src, which is set at 100%.

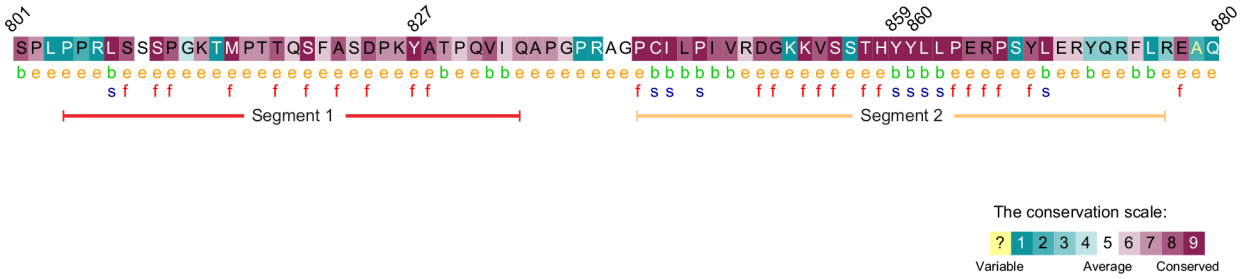

Fig. 6.

ConSurf results for segments 1 and 2 of MHR. Labels based on the neural-network algorithm from ConSurf. e, an exposed residue; b, buried residue; f, predicted functional residue; s, predicted structural residue (highly conserved and buried).

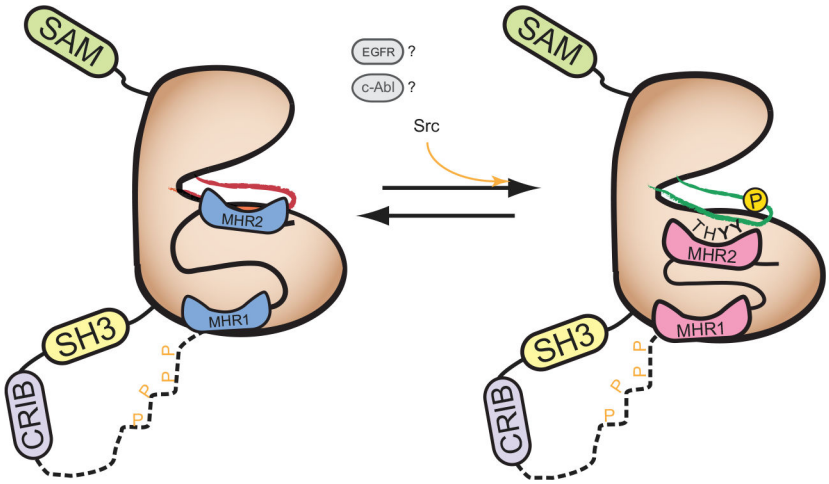

Fig. 7.

Schematic representation of the proposed model for MHR–KD interaction. MHR is depicted as blue and pink, respectively, in the autoinhibited (with red activation loop) and active (with green activation loop) form of Ack1 KD. MHR1, MHR segment 1. MHR2, MHR segment 2. The circled P on the activation loop represents phosphate. The Ps on the dashed line represent the proline-rich sequences. MHR is proposed to interact with the kinase domain to form an autoinhibitory structure. MHR1 interacts with the C-lobe of KD while MHR2 interacts directly with the Ack1 active site. Upon phosphorylation on the activation loop (Y284) by Ack1 or Src, MHR2 is repositioned to release the autoinhibitory interactions. In this model, this release might be enhanced by the Src phosphorylation of MHR (Y859, Y860). EGFR or c-Abl are alternative candidate enzymes for MHR phosphorylation.

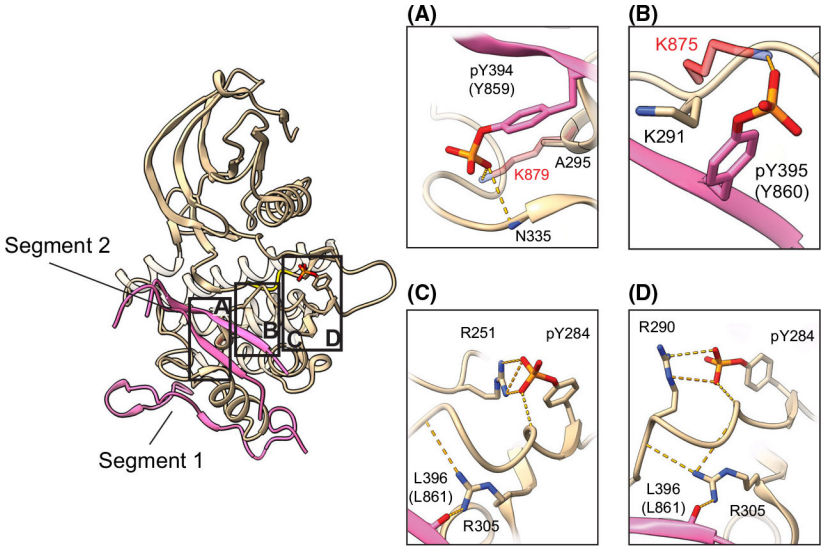

Fig. 8.

Ack1 KD-MHR interactions. Left, 3D model of the phosphorylated (Y284) Ack1 kinase domain (beige, PDB: 4EWH) bound to the Mig6 protein encompassing segments 1 and 2 (pink, PDB: 4ZJV). Residues of the pink structure are labelled according to their position in the Mig6 sequence with the corresponding position in Ack1 MHR in parentheses. (A) When Mig6 is doubly phosphorylated (pY394–pY395), pY394 is coordinated by K879 (red) of EGFR KD. In Ack1 KD, this residue is replaced by A295 (outlined in black). (B) Mig6 pY395 is coordinated by K875 (red) of the EGFR KD active site. This lysine is conserved in Ack1 (K291). However, in both active and inactive structures of Ack1 KD, K291 is oriented differently than EGFR K875, which might explain the stimulatory effect of phospho-MHR on Ack1 kinase activity. (C, D) In the model, the phosphorylated form of segment 2 could make interactions favouring the activated form of Ack1 KD with L396 (L861 in MHR). In Ack1, there are three arginines near the active site (R251, R290, R305) while the equivalent position to R290 in EGFR is glycine (G874).

Table 1.

Peptides used in this study. MHR1, peptide derived from segment 1 of MHR; MHR2, peptide derived from segment 2 of MHR; WASP, Wiskott–Aldrich syndrome protein.

Peptide	Amino acid sequence
WASP	K V I **Y** D F I E K K G
pY859	R D G K K V S S T H pY **Y** L L P E
pY860	R D G K K V S S T H **Y** pY L L P E
MHR1	R L S S S P G K T M P T T Q S F A S D P K **Y** A
MHR2	T H **Y** **Y** L L E R P S **Y** L E R **Y** Q R F L R E A