CANCER-ASSOCIATED MUTATIONS ACTIVATE THE NONRECEPTOR TYROSINE KINASE ACK1

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Ack1 is a non-receptor tyrosine kinase that participates in tumorigenesis, cell survival and migration. Relatively little is known about mechanisms that regulate Ack1 activity. Recently, four somatic missense mutations of Ack1 were identified in cancer tissue samples, but the effects on Ack1 activity and function have not been described. These mutations occur in the N-terminal region, the C-lobe of the kinase domain, and the SH3 domain. Here, we show that the cancer-associated mutations increase Ack1 autophosphorylation in mammalian cells without affecting localization, and increase Ack1 activity in immune complex kinase assays. The cancer-associated mutations potentiate the ability of Ack1 to promote proliferation and migration, suggesting that point mutation is a mechanism for Ack1 deregulation. We propose that the C-terminal Mig6 homology region (MHR) (residues 802 to 990) participates in inhibitory intramolecular interactions. The isolated kinase domain of Ack1 interacts directly with the MHR, and the cancer-associated E346K mutation prevents binding. Likewise, mutation of a key hydrophobic residue in the MHR (F820) prevents the MHR-kinase interaction, activates Ack1, and increases cell migration. Thus, the cancer-associated mutation E346K appears to destabilize an autoinhibited conformation of Ack1, leading to constitutively high Ack1 activity.

Ack1 is a non-receptor tyrosine kinase (NRTK) that was first isolated from a human hippocampal expression library due to its specific binding to GTP-bound Cdc42 (1). Ack1 belongs to a family of non-receptor tyrosine kinases that includes Ack1, Tnk and homologous proteins in Drosophila and C. elegans (2-4). Ack1 is a 120 KDa protein with an N-terminal sterile alpha motif (SAM) domain (5), a kinase domain, an SH3 domain, and a Cdc42-binding domain (CRIB) (Fig1A). The large C-terminal portion of Ack1 contains several proline-rich sequences as well as a clathrin binding motif (6), a ubiquitin binding domain (7) and a region homologous to Mig6 (8). The domain architecture of Ack1 is unique among NRTKs; it is the only NRTK with a CRIB domain, and the position of the SH3 domain (C-terminal to the kinase domain) differs from all other families of NRTKs.

Recent data implicate Ack1 in different stages of cancer. Amplification of the Ack1 gene correlates with metastasis and poor prognosis in lung and prostate cancer, and overexpression increases invasiveness (9). In v-Ras-transformed mammalian cells, Ack1 is required for the maintenance of the transformed phenotype (10). Ack1 contributes to prostate tumorigenesis by phosphorylating the tumor suppressor protein Wwox, leading to its degradation (11), and by phosphorylating androgen receptor (12).

Ack1 is ubiquitously expressed in mammals, with highest expression in spleen, thymus and brain (5). Ack1 is phosphorylated and activated in response to a number of stimuli, including EGF, PDGF, bradykinin, agonists of the M3 muscarinic receptor, and integrin-mediated cell adhesion (5,9,13-15). Upon EGF stimulation, Ack1 phosphorylates and activates the guanine exchange factor Dbl (16). In response to Cdc42 activation, Ack1 mediates phosphorylation of p130Cas to promote cell migration (17,18). Other identified substrates of Ack1 include WASP (19) and the sorting nexin SH3PX1 (20). A number of SH3 containing proteins have been identified as ligands for the proline rich region of Ack1, including the SH3 domains of Hck (21), Grb2 (22), SNX9 (20,22), and the WW domain of the E3 ubiquitin ligase Nedd4-2 (23). Although the physiological functions of Ack1 are not completely understood, several studies have focused on the role of Ack1 in EGF receptor trafficking and dynamics. Ack1 is recruited to EGFR...
following EGF stimulation, and overexpression of Ack1 interferes with the proper trafficking of EGFR (24). EGFR stability is regulated by interactions between Ack1 and multiple partner proteins including ubiquitin (7), clathrin heavy chain (6,25), and SH3PX1 (26).

The activities of other NRTKs are controlled through intramolecular interactions, and by conformational changes in the kinase activation loops (27). In Src family kinases, the inactive conformation is stabilized by an interaction between the SH3 domain and a polypeptide type II helix, and an interaction between the SH2 domain and the C-terminal phosphotyrosine (28,29). Activating signals (such as SH3 or SH2 ligands) disrupt these interactions and promote the phosphorylation of Y416 in the activation loop of Src (30-33). In c-Abl, the SH2 and SH3 domains also participate in autoinhibitory interactions. These interactions are stabilized by an N-terminal cap region, and by an interaction between the N-terminal myristoyl group and the base of the kinase catalytic domain (34). Based on the distinctive domain organization of Ack1, the regulatory mechanisms are likely to be divergent from the Src- and Abl- family paradigm.

Using purified Ack1, we previously showed that the major autophosphorylation site is Y284 in the activation loop (21). The crystal structure of the isolated kinase domain of Ack1 has been solved in both the phosphorylated (p-Y284) and unphosphorylated forms (35). In both of these structures, the conformation of the activation loop resembles the conformation seen in other active kinases. Phosphorylation of Y284 in Ack1 stimulates kinase activity (21), but not as strongly as in Src or Abl NRTKs. Thus, the regulatory importance of Ack1 autophosphorylation appears to be intermediate between Src/Abl (strongly phosphorylation-dependent) (35), and kinases such as EGFR or Cdk5 (in which formation of the activated state is phosphorylation-independent) (36-38). Ligands for the SH3 or the CRIB domains of Ack1 do not activate the purified enzyme (21); thus, the regulatory mechanism for Ack1 is poorly understood at present.

Recently, the genes encoding 518 protein kinases were sequenced in a large collection of human cancers to identify somatic mutations (39). Four missense mutations were identified in Ack1: two mutations in the N-terminus (R34L and R99Q) were identified in lung adenocarcinoma and ovarian carcinoma, respectively, a mutation in the kinase catalytic domain (E346K) was identified in ovarian endometroid carcinoma, and a mutation in the SH3 domain (M409I) was found in gastric adenocarcinoma. The probability of each mutation being a driver (i.e., a mutation that confers an advantage to the cancer cell, and that is therefore subject to positive selection pressure) was estimated by comparing the rate of nonsynonymous and synonymous somatic mutations in each gene. The genes were ranked according to their probability of carrying at least one driver mutation, and Ack1 ranked in the top 5%. The effect of these mutations on Ack1 activity and downstream signaling is unknown.

The aims of this study were: (1) to test the effects of the cancer-associated mutations on Ack1 function and activity; and (2) to use the mutations to gain insight into the regulation of Ack1. We report that cancer-associated mutations stimulate Ack1 activity without affecting its subcellular localization, suggesting that point mutations represent a new mechanism for the oncogenic activation of Ack1. Moreover, we propose that an autoinhibitory interaction exists between the kinase domain and the C-terminal Mig6 homology region, and that mutations that disrupt this interaction (such as the cancer-associated mutation E346K) activate Ack1.

**EXPERIMENTAL PROCEDURES**

**Reagents and antibodies**

Bovine serum albumin, leupeptin, aprotinin, PMSF, sodium vanadate, NaF, polybrene, and chloroquine were obtained from Sigma. EGF was from Sigma or Peprotech Inc. Primary antibodies were obtained from the following companies: rabbit polyclonal anti-Ack1 and rabbit polyclonal anti-pY284 Ack1 were from Millipore, mouse monoclonal anti-His6 was from Covance, mouse monoclonal antitubulin clone GTU-88 was from Sigma, rat monoclonal anti-HA high affinity clone 3F10 was from Roche. Horseradish peroxidase linked secondary antibodies (donkey anti-rabbit IgG and sheep antimouse IgG horseradish) were purchased from GE Healthcare. Trypsin-EDTA solution was from Mediatech Inc. Rabbit anti-HA tag antibodies were from Sigma-Aldrich. Mouse anti-EEA1 antibodies were from BD Biosciences (San Jose, CA). Alexa Fluor (AF)-594-transferrin and AF-488- and AF-594-goat anti-mouse and goat anti-rabbit IgG secondary antibodies were from Molecular Probes, Invitrogen (Carlsbad, CA).
Cell culture

Mammalian cells were maintained in DMEM (Cellgro, Mediatech, Inc) supplemented with 10% fetal bovine serum (Sigma) and 1000 IU/ml penicillin, 1000 IU/ml streptomycin, 25ng/ml amphotericin B (Cellgro, Mediatech, Inc). The SF9 insect cells were maintained in SF-900 medium (Gibco) supplemented with 5% fetal bovine serum and antibiotic/antimycotic.

Cloning and site-directed mutagenesis

Plasmid pXJ-HA, encoding full length Ack1 was a kind gift from Dr. Edward Manser (Institute of Molecular and Cell Biology, Singapore). A plasmid encoding EGFP-clathrin light chain A in pEGFP-C3 (40) was the gift of Lois Greene (National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD).

To produce GFP-tagged Ack1, we subcloned the Ack1 gene into pBMN-I-GFP (Addgene plasmid 1736, developed by Garry Nolan). The Mig6 homology region (Ack1 803-880) was amplified by PCR and cloned into pGEX4T-1 (GE Healthcare) using the restriction sites BamHI and NotI. For baculovirus expression of Ack1 kinase domain, a fragment encoding residues 110 to 385 was cloned into the vector pFastBac HTb (Invitrogen) using the restriction sites BamHI and NotI. Site-directed mutagenesis was performed using the Stratagene Quickchange kit, following the manufacturer’s directions.

Cell transfection and Western blotting

Cos7 cells (1x10^6) were plated in 10 cm diameter dishes and transfected for 24 hours using 10-15 µg DNA with TransIT reagent (Mirus) at a ratio of 2 µl TransIT per µg of DNA. After 24 hours, the reagent was removed, and the cells were cultured in DMEM supplemented with 1% fetal bovine serum for an additional 24 hours. The cells were harvested, washed twice in PBS, and lysed using RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% sodium deoxycholate, 1% Nonidet P-40) supplemented with the protease inhibitors leupeptin (10 µg/ml), aprotinin (10 µg/ml), PMSF (200 µM) and the phosphatase inhibitors Na_2VO_4 (0.2 mM) and NaF (10 mM). Lysates (30 µg) were separated by 10% SDS-PAGE, transferred to PVDF membrane, and probed with appropriate antibodies.

For immunofluorescence, the transfection was carried out as follows: Cos7 cells (from American Type Culture Collection (ATCC), Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium with 10% iron-supplemented calf serum (JRH, Lenexa, KS) and penicillin/streptomycin. Transient transfection of cells seeded on acid-washed glass coverslips was performed using fully-deacetylated polyethylenimine (PEI) reagent, prepared from 200 kDa poly(2-ethyl-2-oxazoline) (Sigma-Aldrich, St. Louis, MO) as described (41). Transfection mixtures contained 2 µg DNA and 12 µl PEI reagent per 35 mm dish. Cells were examined one day after transfection.

Fluorescence microscopy

Cells were fixed in phosphate-buffered saline (PBS; 150 mM NaCl, 20 mM phosphate buffer, pH 7.4) containing 3% paraformaldehyde for 30 minutes, permeabilized with PBS containing 0.5% Triton X-100 and blocked with PBS containing 3% bovine serum albumin (BSA) and 10 mM glycine for 1 hour. Cells were incubated with primary antibodies for 1 hour and secondary antibodies for 30 minutes, both in PBS with 3% BSA and 10 mM glycine. Where indicated, cells were incubated with AF-594-Tf (35 µg/ml) for 20 minutes at 37°C before fixation. Cells were photographed and images were captured using a Zeiss Inverted Axiovert 200 M Microscope with a two-photon laser scanning confocal system and a 100x oil immersion objective. Images were processed with Zeiss LSM software.

IP kinase assay

Cell lysates (2 mg) were incubated overnight at 4°C with 2 µg rat high affinity anti-HA antibody (Roche) and 90 µl of protein G-Agarose beads (Roche). The immunocomplexes were washed with PBS supplemented with 1 mM Na_2VO_4. After the last wash, the beads were resuspended in 300 µl of PBS supplemented with 1 mM Na_2VO_4. Aliquots were withdrawn for gel analysis or for kinase assays, which were performed in duplicate. The kinase activity of immunoprecipitated Ack1 was determined using the phosphocellulose paper assay with a synthetic peptide substrate derived from the WASP phosphorylation site (sequence: KVIYDFIEKKKKG) (42). Reaction mixtures contained 20 mM Tris-HCl (pH 7.4), 10 mM MgCl_2, 0.4 mM ATP, 1 mM peptide, and [γ-32P]-ATP (30-50 cpm/pmol). After 30 min at 30°C, the reaction was stopped by the addition of 10% TCA and the samples were spotted on p81 phosphocellulose paper. Incorporation of 32P into peptide was determined by scintillation counting. For gel analysis, proteins were separated by SDS-PAGE, transferred to PVDF...
membranes, and immunoblotted using anti-Ack1 antibodies. The activity was normalized to the total protein immunoprecipitated.

Retrovirus production and infection

The ecotropic virus stock was produced by transfecting subconfluent Phoenix-Eco packaging cells with Ack1 constructs cloned in pBMN-I-GFP. To transfect a 10 cm diameter plate, 10 µg of plasmid DNA was mixed with 438 µl of H2O, 62 µl 2M CaCl2 and 500 µl of 2X HBS (50 mM HEPES pH 7.5, 10 mM KCl, 12 mM dextrose, 280 mM NaCl, Na2HPO4 pH 7.05). The DNA mixture was transferred to a plate containing 4 ml of cell culture medium and 25 µM chloroquine. The plates were incubated at 32°C for 48 hours, and the supernatant containing the retrovirus stock was collected, filtered, aliquoted and stored at -80°C or used to infect cells. NIH-3T3 cells were infected by incubating confluent cells with an inoculum of virus stock in the presence of 4 ug/ml polybrene at 37°C. After 48 hours, cells were visualized using a fluorescence microscope. The GFP positive cells were selected by preparative cell sorting and the infected cells were then used in the migration, anchored growth and nonanchored growth assays.

Transwell migration assay

Migrations assays were performed using 24 well plates with 8.0 µm inserts (Corning). For each cell line (WT or mutant), 200,000 cells were seeded in triplicate or quadruplicate in 200 µl of complete culture medium in the top compartment. The bottom compartment contained 500 µl of complete culture media. The cells were allowed to migrate for 6 hours at 37°C. Afterwards, the inserts and media were removed, and the cells that migrated to the bottom compartment were collected in 100 µl of trypsin-EDTA solution (0.25% Trypsin and 2.21 mM EDTA in HBSS). The cell suspension was combined with 100 µl media containing 10% fetal bovine serum and the cells were counted using a hemocytometer.

Cell growth curves

For the anchored growth curves, 20,000 cells were seeded in duplicate in 12 well plates. Each day, the cell culture medium was aspirated and cells were detached with trypsin for 5-10 min. The cell suspensions were combined with complete culture medium to a final volume of 500 µl or 1 ml. The cells were counted in a hemocytometer.

For the non-anchored growth, 20,000 cells were seeded in duplicate in 24 well ultra low attachment plates (Corning). The plates were incubated at 37°C for 6 days. Cells growing in solution were transferred to a fresh tube, centrifuged, treated with 150 µl of trypsin-EDTA solution for 15-30 min, and counted in a hemocytometer.

Protein expression and purification

The pFastbac HTb vector encoding the Ack1 kinase domain (wild type or E346K) was used to transfect Sf9 cells using the Bac-to-Bac system (Invitrogen). Baculovirus stocks were produced by transfecting Sf9 cells. After 2 rounds of amplification, virus titers on the order of 10^9 pfu/ml were obtained and used to infect 400 ml of Sf9 cells at 2x10^6 cell/ml with MOI~5. After 48 hours, infected cells were collected and lysed in a French pressure cell in 20 mM Tris-HCl buffer (pH 8.0), containing 5 mM 2-mercaptoethanol, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM PMSF, and 1 mM Na3VO4. Cell lysates were centrifuged at 40,000 g for 30 min, filtered using a 0.8 µm filter, and applied to a 4 ml Ni-NTA column (Qiagen). The column was washed with 120 ml of 20 mM Tris-HCl buffer (pH 8.0) containing 2 mM imidazole, 0.5 M NaCl, 10% glycerol, 5 mM 2-mercaptoethanol, 2 mM Na3VO4 and further washed with 40 ml of 20 mM Tris-HCl buffer (pH 8.0) containing 1 M NaCl followed by a last wash with 40 ml of 20 mM Tris-HCl buffer (pH 8.0). The His6-tagged proteins were eluted with 20 mM Tris-HCl buffer (pH 8.0) containing 100 mM imidazole, 5 mM 2-mercaptoethanol and 10% glycerol. The 1 ml fractions containing Ack1 kinase domain were pooled, supplemented with 20% glycerol, aliquoted and stored at -80°C.

The Mig6 homology region (WT or F820A) was expressed as a GST fusion in E.coli BL21 cells. Expression was induced by adding 0.25 mM IPTG for 4 hours at 30°C. The cells were collected and lysed in a French press cell in lysis buffer containing 50 mM Hepes buffer (pH 7.4), 100 mM NaCl, 100 mM EDTA, 1% Triton X-100, 10 mM DTT, 10% glycerol, 5 µg/ml aprotinin, 5 µg/ml leupeptin, and 1 mM PMSF. The lysates were centrifuged at 11,000 g for 10 minutes and the supernatant was incubated for 2 hours at 4°C with 7 ml of glutathione-agarose beads preequilibrated in 50 mM Hepes buffer (pH 7.4) containing 100 mM EDTA. The beads containing the immobilized Mig6 homology region were washed once with 50 ml lysis buffer, three times with 50 ml.
50 mM Hepes buffer (pH 7.4) containing 100 mM EDTA, and twice with 50 mM Tris-HCl buffer (pH 8.0).

**Binding reactions**

Purified WT or mutant Ack1 kinase domain (100 pmol) was incubated for 2 hours at 4°C with immobilized GST-Mig6 homology region (WT or E346K, 160 ng) in a final volume of 500 µl of buffer A (50 mM Hepes pH 7.4, 0.1 M NaCl, 100 mM EDTA, 1% Triton X-100, 10 mM DTT and 10% glycerol). After binding, the beads were washed once with 1 ml of buffer A, once with 1 ml of buffer containing 50 mM Hepes pH 7.4, 1 M NaCl, 100 mM EDTA, 1% Triton X-100, 10 mM DTT and 10% glycerol, and once more with 1 ml of buffer A. Bound proteins were eluted with SDS-PAGE loading buffer and separated by 12% SDS-PAGE. The proteins were then transferred to a PVDF membrane and analyzed by Western blotting with anti-His6 antibodies.

**RESULTS**

**Cancer–associated mutations activate Ack1**

To evaluate the effect of the cancer-associated mutations on Ack1 activity in cells, we introduced the point mutations into full-length Ack1 by site-directed mutagenesis. We expressed wild-type and mutant forms of Ack1 in Cos7 cells, and analyzed their activities in whole cell lysates by probing for autophosphorylation of Y284. As a negative control, we also analyzed kinase-dead Ack1 (K158R). The level of Ack1 autophosphorylation was increased by all the cancer-associated mutations. The N-terminal mutations R34L and R99Q increased Ack1 autophosphorylation by 3- and 4-fold, respectively. The mutation in the C-lobe of the kinase domain (E346K) produced a 6-fold increase, and the mutation located in the SH3 domain (M409I) produced a 3-fold increase in the level of Ack1 autophosphorylation (Fig. 1B). To measure kinase activity towards an exogenous substrate, we immunoprecipitated the Ack1 proteins and incubated them with a synthetic peptide substrate in an *in vitro* assay (Fig. 1C). The peptide used was derived from the Ack1 phosphorylation site of WASP; this is the best *in vitro* peptide substrate reported to date (42). EGF treatment increased Ack1 activity towards the Tyr-containing peptide, confirming earlier observations on EGF activation of Ack1 (5). The activity of Ack1 was also increased by the cancer-associated mutations. The degree of activation ranged from 4.2-fold for E346K to 9.6-fold for M409I. The kinase dead Ack1 (K158R) was not autophosphorylated in cells and was inactive in the IP-Kinase experiment, which underscores the role of autophosphorylation in Ack1 activation.

**Cell localization studies**

One potential explanation for the increased activity of the cancer-associated mutant forms of Ack1 would be a change in subcellular localization. To test this possibility, we examined the localization of wild-type and mutant Ack1 proteins in COS7 cells by fluorescence microscopy. The Ack1 proteins all had similar distribution patterns: all were localized to small discrete puncta and larger amorphous perinuclear structures (Fig. 2). As reported earlier for wild-type HA-Ack1 (6,7,24), all the Ack1 proteins showed partial colocalization with EEA1 on early endosomes (Fig. 2). Also as reported earlier for the wild-type protein (ref. 24), Ack1 and all the mutants were also present on larger amorphous perinuclear structures. These did not stain for EEA1, but often appeared to surround and embed EEA1-positive endosomes (Fig. 2). These structures are probably the same as large reticular complexes and endosome-associated tubules seen in Ack1-overexpressing cells by electron microscopy (24), and may be endosome-derived tubules. Furthermore, all the Ack1 proteins caused a dramatic accumulation of clathrin (visualized using co-expressed GFP-clathrin light chain A) on the amorphous structures (Fig. S1) and inhibited transferrin internalization (Fig. S2), as reported earlier for wild type Ack1 (6). These results suggested that Ack1 over-expression induced the accumulation of endosome-associated tubular structures that sequestered clathrin, inhibiting transferrin internalization by reducing the amount of clathrin available for coated pit formation at the plasma membrane. All the cancer-associated mutants showed the same behavior as wild-type Ack1, suggesting that activation of the mutants did not stem from differences in cellular localization.

**Effects of the cancer-associated mutations on cell growth and migration**

Next, we analyzed the growth properties of NIH3T3 cells overexpressing wild type Ack1 or the cancer-associated mutants. In order to obtain homogeneous populations of Ack1-expressing cells, we generated retroviruses encoding GFP along with the various forms of Ack1. GFP fluorescence was used
to monitor the infection, and GFP-positive cells were selected by preparative FACS. The adherent cells were grown on a normal cell culture surface to measure anchored growth, as well as on ultra low attachment plates to measure nonanchored growth (Fig. 3A). We confirmed the expression and autophosphorylation of Ack1 in these cell lines (Fig. 3B). As in the transiently-transfected cells, autophosphorylation of the cancer-associated mutant forms of Ack1 was higher than wild-type.

Figure 4A shows the anchored growth curves of the cells. Compared to cells infected with control virus (EV), cells expressing wild-type or mutant forms of Ack1 were able to grow to higher saturation densities before significant cell death was observed (~300,000 cells/well for EV compared to ~700,000 cells/well for Ack1), suggesting a partial loss of contact inhibition. Cells expressing the kinase dead version of Ack1 (K158R) exhibited similar growth properties as cells expressing WT Ack1, indicating that the effect of Ack1 on anchored cell growth is independent of kinase activity.

The ability of cells to grow in suspension is a hallmark of cell transformation. We studied the growth of the infected cells in conditions that prevent their attachment to any surface (Fig. 4B). The initial number of cells seeded in each well was 20,000. After 6 days in culture, the cells infected with control virus exhibited significant cell death; their number was reduced by 50%. Cells expressing wild type Ack1 survived, although without multiplying. The cells expressing the cancer-associated mutations R34L, R99Q or M409I did not differ significantly from the cells expressing WT Ack1 (Fig 4B). However, the cells expressing E346K Ack1 were able to multiply, and the population nearly doubled in the period of time they were in culture (Fig. 4B). Thus, the mutation E346K increased the ability of Ack1 to override anchorage dependence.

We examined the cells for signs of apoptotic cell death at different stages of growth, corresponding to early exponential phase (2 days) and late stationary/death phase (8 days). After 2 days in culture, there was no apparent apoptosis, as judged by cleavage of caspase 3 and poly(ADP-ribose) polymerase (Fig. S3). On the other hand, after 8 days in culture, cells expressing vector alone showed significant apoptosis, while cells expressing Ack1 (wild-type or mutants) had reduced levels (Fig. S3). These results are consistent with the growth curves shown above in Fig. 4.

Overexpression of Ack1 in cancer cell lines increases the invasiveness of the cells in vitro and in vivo (9). We studied the migration properties of the Ack1-expressing cells in a transwell migration assay (Fig. 5). Consistent with previous findings, the expression of WT Ack1 increased cell migration as compared with the cells infected with control virus. The cancer-associated mutations produced a range of effects. R34L and M409I produced the largest increase over WT, followed by E346K. The migration of cells infected with the kinase dead Ack1 (K158R) was similar to control cells, indicating that most of the observed effect is due to the kinase activity. Thus, the dependence on kinase activity differed in the three different assays. The effect of Ack1 on anchored cell growth did not depend on kinase activity (Fig. 4A), and as a consequence, the cancer-associated mutants behaved similarly to WT. In the nonanchored growth experiments (Fig. 4B), the kinase-dead form of Ack1 promoted growth as efficiently as wild-type, but the E346K mutation gave additional stimulation. Finally, cell motility did depend on Ack1 kinase activity (Fig. 5), and the cancer-associated mutants with higher in vitro activity also enhanced the migration rate in intact cells. These functional data on the cancer-associated mutations show that increased Ack1 kinase activity has the potential to be manifested in anchorage independence and increased motility in cancer cells.

Although relatively few downstream partners of Ack1 have been identified, we carried out experiments to identify potential alterations in signaling in cells expressing the cancer-associated mutants. Wiskott-Aldrich syndrome protein (WASP) is a Cdc42 effector that plays an important role in the formation of new actin filaments. Phosphorylation of WASP at Y256 and S242 by Ack1 stimulates actin polymerization mediated by WASP (19). We examined WASP Y256 phosphorylation in NIH3T3 cells using a phosphospecific antibody (Fig. 6A). Phosphorylation of WASP was increased in cells expressing the R99Q, E346K, and M409I mutants relative to wild-type Ack1, consistent with the observed enhanced migration (although R34L did not show enhanced WASP phosphorylation). The Crk-associated substrate (Cas) has also been shown to be a substrate of Ack1 (9). In NIH3T3 cells we observed elevated Cas phosphorylation by the E346K mutant and (to a lesser extent) the M409I mutant of Ack1 (Fig. 6B). There was a small increase in Erk phosphorylation by several of the mutants relative to wild-type Ack1 in NIH3T3 cells (Fig. S4). A fuller
mechanistic understanding of the growth- and migration-promoting effects of the Ack1 mutants will be possible when additional downstream signaling pathways of Ack1 have been identified.

A mechanism for intramolecular regulation of Ack1

Next, we focused on the mechanism of Ack1 activation by the cancer-associated mutation E346K. This mutation lies in the large C-terminal lobe of the kinase catalytic domain. Sequence comparisons indicate that the kinase domain of Ack1 is very similar to the kinase domain of the epidermal growth factor receptor (EGFR) (43). Activation of EGFR is achieved by formation of an asymmetric dimer in which one of the kinases acts as an activator of the other (44). Mig6 is a feedback inhibitor of EGFR, and it has been shown to bind to the kinase domain of EGFR and inhibit its activity (45). Structural studies showed that Mig6 inhibits EGFR by blocking the formation of the activating dimer (8). The C-terminal region of Ack1 shows high sequence homology with Mig6 (Fig. 7A), and this region is able to bind to EGFR (7). Furthermore, the Mig6-binding region is conserved in sequence and structure in Ack1. Thus, we hypothesize that an intramolecular interaction exists in Ack1 between the kinase domain and Mig6 homology region, analogous to the Mig6/EGFR interaction. Using the structure of EGFR bound to Mig6, Zhang et al. superimposed the solved structure of Ack1 kinase domain on the EGFR kinase domain, and created a model of the Ack1-Mig6 interaction. (Fig. 7B) (8). In the model, the side chain of E346 is oriented towards the interface with the Mig6 peptide. Thus, mutations at this position could potentially destabilize the inhibited conformation.

Mutations designed to disrupt the intramolecular interaction activate Ack1

In order to test this hypothesis, we introduced point mutations into Ack1 at positions analogous to sites that are important in the Mig6/EGFR interaction (8): L120 and L197 are located in the N-lobe of the Ack1 kinase domain, V365 is in the C-lobe of the kinase domain, and F820 and Y826 are located in the Mig6 homology region (Fig. 8A). For comparison, we included a mutation in Y284, the major autophosphorylation site. The V365R and F820A mutations produced large increases in Ack1 autophosphorylation when they were expressed in cells (Fig. 8B). The Y826A produced a smaller but significant increase, while the L120 and L197 mutations had no effect. When the V365R and F820A mutants were tested in a peptide phosphorylation assay they also showed increased kinase activity (Fig. 8C). This activation appeared to be stronger than the activation caused by the N-terminal mutations (R34L and R99Q), as the increases for V365R and F820A surpassed the activation produced by EGF stimulation (compare Fig1C and Fig. 8C).

As shown above, the cancer-associated mutations potentiated the effects of Ack1 to promote migration, proliferation, and anchorage independent growth. We expected that if the Mig6 homology region-kinase domain interaction were functionally significant, then the introduction of an activating artificial mutation would be able to recapitulate the effects of the naturally occurring mutations. We selected F820A, because the analogous Phe makes a key contact in the Mig6-EGFR structure (8), and because of the strong activation of Ack1 by this mutation (Fig. 8). Indeed, the F820A mutation produced an increase in both cell migration (Fig. 9A) and non anchored growth (Fig. 9B) as compared with the effect caused by wild type Ack1.

Direct binding between Ack1 kinase domain and Mig6 homology region

To test for a direct interaction between the kinase domain and Mig6 homology region, we introduced the Ack1 kinase domain into a recombinant baculovirus, expressed the protein in insect cells, and purified it to homogeneity. We expressed the Mig6 homology region (Ack1 residues 803-880) in bacteria as a GST fusion protein and purified it. The immobilized Mig6 homology region was able to bind to the purified kinase domain (Fig. 10A). The introduction of the F820A mutation into the GST-Mig6 homology region prevented the binding to the Ack1 kinase domain (Fig. 10A). We also produced the E346K mutant form of the kinase domain in insect cells, containing the cancer-associated mutation in the C-lobe. Binding of E346K to immobilized Mig6 homology region was reduced as compared to the binding to the wild type kinase domain (Fig.10B).

To test whether the E346K mutation increases the intrinsic catalytic activity of Ack1, we carried out kinetic experiments on the isolated catalytic domains, using varying concentrations of the WASP peptide substrate. The in vitro activity of purified E346K Ack1 kinase domain (kcat/kM~20 min⁻¹μM⁻¹) was somewhat lower than the activity measured for WT Ack1 (kcat/kM~70 min⁻¹μM⁻¹), demonstrating that the
mutation itself does not increase the kinase activity. These in vitro data indicate that there is a direct association between the two regions of Ack1, and that mutations in the Mig6 homology region (F820A) or the kinase domain (E346K) interfere with binding. Collectively, our data suggest a model in which this interaction is autoinhibitory (Fig 10C); the E346K mutation would activate Ack1 by destabilizing this conformation.

**DISCUSSION**

Ack1 has previously been implicated in tumorigenesis (11,12), cell survival (10), and metastasis (9). The Ack1 gene is amplified and overexpressed in a number of tumors, and the copy number change correlates with later-stage, more aggressive tumors (9). Prior to this study, it was not clear whether mutations represent an additional mechanism for the oncogenic activation of Ack1. The four non-synonymous mutations in Ack1 studied in this paper were identified in a study in which 210 cancer tissue samples were screened for somatic mutations in protein kinase genes (39). Using this approach, Ack1 emerged as a likely candidate to carry driver mutations. We show here that point mutations activate Ack1, and that cells expressing the cancer-associated mutants exhibit aspects of the transformed phenotype. Mutations located in the N-terminus (R34L and R99Q), in the C-lobe of the kinase domain (E346K), and in the SH3 domain (M409I) activated Ack1 in functional and biochemical assays. Our data reinforce the previous characterization of Ack1 as an oncogene, and suggest that the somatic mutations may contribute to cancer development.

Consistent with published data (9), we found that the overexpression of wild type Ack1 promotes the transformed phenotype. Cells overexpressing WT Ack1 showed higher levels of migration than control cells (Fig. 5). The expression of WT Ack1 also caused loss of contact inhibition in cells growing attached to a surface, and allowed the cells to survive in non-anchored conditions (Figs. 4A,B). Our studies were carried out in NIH3T3 cells, and they are similar to results obtained in human mammary epithelial cells and in 4T1 mouse mammary tumor cells, suggesting that Ack1 promotes cell growth and migration independently of cell context.

The two cancer-associated mutations located in the N-terminus of Ack1 increased the level of Ack1 autophosphorylation in cells (Fig. 1B). This was due to enhanced Ack1 kinase activity, since the mutants also phosphorylated a synthetic peptide at a higher rate (Fig.1C). Neither of the N-terminal mutations displayed any significant enhancement of anchored or non-anchored growth over the level observed with WT Ack1. On the other hand, the R34L mutation did increase the effect of Ack1 on cell migration. The N-terminus of Ack1 contains a region that was identified as a SAM domain (Sterile Alpha Motif) and as a membrane localization motif (5). The observed activation of Ack1 by these mutations could potentially have been explained by mislocalization of Ack1 due to the disruption of the membrane localization domain. However, the intracellular localization of Ack1 was not affected by the cancer-associated mutations located in the N-terminus (Fig. 2). Thus, our data indicate that the R34L and R99Q mutations increase Ack1 autophosphorylation and activity. While the mechanism is not clear, one possibility is that the N-terminal mutations disrupt an inhibitory interaction. This putative interaction could be either intramolecular or intermolecular (in the latter case, involving other molecules or the formation of an Ack1 homodimer). SAM domains share an overall secondary structure rich in alpha helices and have been found in more than 1000 proteins, including the Eph family of tyrosine kinase receptors (46,47), diacylglycerol kinase delta (DGKδ) (48) and the transcriptional repressor TEL (49,50). The three-dimensional structures of several SAM domains have been solved, and they form dimeric and polymeric associations. Although the biological roles of these interactions are not yet clear for all the SAM-containing proteins, DGKδ was reported to be regulated by SAM mediated polymerization (48). The deletion of the N-terminus portion of Ack1 reduces the ability of full length Ack1 to undergo autophosphorylation (5). We speculate that the N-terminal SAM domain may be involved in protein-protein interactions that play a role in Ack1 regulation, and that cancer-associated mutations in this region disrupt the normal regulation mechanism, rendering the enzyme more active. Future studies will be focused on the role of the N-terminal region in Ack1 regulation.

The cancer-associated mutation M409I, located in the SH3 domain of Ack1, activated cellular autophosphorylation of Ack1 and promoted increased cell migration as compared to WT Ack1. On the other hand, the effect of this mutation on cell proliferation did not significantly differ from the effect of WT
Ack1. The reason for this discrepancy is not clear; however, the functional assays that we used measure different biological processes. Ack1 may activate several downstream effectors and pathways through its different domains. Therefore, it is possible that the SH3 domain is involved in the regulation of motility-related, rather than cell proliferation pathways. The methionine residue mutated in Ack1 is not conserved in other closely related SH3 domains, and it is not predicted to lie in the binding site for polyproline-containing ligands. Thus, the molecular basis for Ack1 activation by M409I is not understood presently.

The cancer-associated mutation E346K is located in the C-lobe of the kinase domain. We found that the expression of E346K Ack1 potentiated the effects of the expression of WT Ack1 on migration and non-anchored growth. Thus, our functional data suggested that the C-terminal portion of Ack1 plays a role in its regulation. Next, we focused on the study of this region to gain insight into a regulatory mechanism that involves the kinase domain and the C-terminal Mig6 homology region.

Relatively little information was previously available concerning Ack1 regulation. Ack1 autophosphorylation produced modest activation in a construct containing the N-terminus, the catalytic domain, the SH3 domain and the CRIB domain of Ack1 (21). Also, the addition of Cdc42 (ligand for CRIB domain) or proline peptides (ligands for SH3 domain) did not activate this purified construct in vitro. However, in cells expressing full length Ack1, the co-expression of Cdc42 did activate Ack1 (21). The introduction of point mutations into the CRIB domain and SH3 domain in full length Ack1 expressed in cells also produced increased autophosphorylation, suggesting that the different domains of Ack1 participate in regulatory interactions (5).

The structure of the kinase domain of EGFR in a complex with the inhibitor protein Mig6 (8), suggested that the C-lobe of the kinase domain could be important for an intramolecular interaction with the Mig6 homology region located in the C-terminus of Ack1 (Fig. 7B). Based on previous studies on EGFR, we introduced point mutations designed to disrupt this putative interaction. We found that point mutations located either in the C-lobe of the kinase domain (V365R) or in the Mig6 homology region (F820A) resulted in the activation of full length Ack1 (Figs. 8B and 8C). Although the mutation F820A is not a naturally occurring mutation, it was able to recapitulate the functional effects that we observed for the cancer-associated mutations. Likewise, it produced a 50% increase in cell migration and non anchored growth (Figs. 9A and 9B). The ability of F820A mutation to produce a large (~80-fold) increase in kinase activity suggests that previous work on Ack1, which was carried out with a construct lacking the MHR, underestimated the dynamic range of kinase activation.

In order to further test the interaction predicted by the model and suggested by the cell transfection experiments, we conducted binding experiments using purified GST-tagged Mig6 Homology region (MHR) and purified His6-tagged Ack1 kinase domain. The purified kinase domain binds to the purified WT MHR (Figs. 10A, 10B). This interaction is prevented by the F820A mutation in the MHR (Fig. 10A) and by the E346K mutation in the kinase domain (Fig. 10B). The interaction between these two minimal segments provides additional evidence for a direct interaction between the kinase domain and the MHR in the context of full-length Ack1. Based on the data presented in this paper, we propose a model for Ack1 regulation in which the MHR interacts with C-lobe of the kinase domain to stabilize a down-regulated structure (Fig. 10C). The Mig6 region might interact directly with the Ack1 active site as pictured in Fig. 10C, or alternatively act indirectly to position an inhibitory segment in the active site. In preliminary in vitro experiments, the GST-MHR did not inhibit the purified Ack1 kinase domain (V.P.E. and W.T.M., unpublished observations), suggesting that additional regions of Ack1 are needed for direct or indirect autoinhibition.

The C-terminal region of Ack1 is also involved in interactions with EGFR and other upstream and downstream signaling molecules. Thus, as observed for other NRTKs such as Src (51), Abl (52) and FAK (53), enzymatic activation of Ack can be coupled to interactions between the noncatalytic domains and allosteric regulators, effectors, and potential substrates.

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REFERENCES


FIGURE LEGENDS

Figure 1. Cancer-associated mutations activate Ack1 in cells and in vitro. A, Domain architecture of Ack1. The positions of the cancer-associated mutations are indicated. B, Western blot analysis. Lysates from Cos 7 cells expressing wild type or mutant forms of Ack1 were probed with anti-Ack1(pY284), anti-Ack1, and anti-tubulin antibodies, as indicated. Densitometry readings were used to calculate the ratios of phosphorylated to total Ack1. C, Immunoprecipitation-kinase assay. Cos 7 cells expressing Ack1 were stimulated with EGF (50 ng/ml for 5 minutes) or left untreated. Ack1 was immunoprecipitated from these cells, or from cells expressing Ack1 mutants, with anti-HA antibodies. The immunoprecipitated proteins were used in duplicate in vitro kinase reactions with $^{32}$P-ATP and the WASP synthetic peptide. Activities were measured in duplicate with the phosphocellulose paper assay, and were normalized to 1.0 for WT Ack1. The error bars show standard deviations.

Figure 2. Subcellular localization of Ack1. Cos7 cells expressing wild-type HA-tagged Ack1 (A) or the indicated mutant (B-E) were prepared for immunostaining and confocal microscopy as described in Materials and Methods, detecting Ack1 proteins with anti-HA antibodies (left panels; green) and endogenous EEA1 (red) as indicated. Merged images are shown to the right of the EEA1 panels. Enlarged views of the boxed regions in the merged images are shown to the right of the merged images. All Ack1 proteins partially co-localized with EEA1, but were also seen in larger, amorphous structures that often surrounded punctate endosomes. Scale bar: 10 µm.

Figure 3. NIH3T3 cells expressing Ack1. A, NIH 3T3 cells were infected with retroviruses encoding GFP and Ack1 (wild type or mutants) and were cultured in anchored or non anchored conditions. GFP fluorescence was used to select for infected cells by FACS. B, Lysates from NIH3T3 cells expressing wild type or mutant forms of Ack1 were probed with anti-Ack1(pY284), anti-Ack1, and anti-tubulin antibodies, as indicated. Densitometry readings were used to calculate the ratios of phosphorylated to total Ack1.

Figure 4. Effects on cell growth. A, Anchored growth: 20,000 NIH 3T3 cells infected with retroviruses carrying Ack1 (wild-type or mutant) or a control virus (EV) were seeded in 12-well plates and counted at the indicated times. The figure is representative of 2 experiments performed in duplicate. B, Non anchored growth: 20,000 NIH 3T3 cells infected with the indicated Ack1 retrovirus or a control virus (EV) were seeded in 24-well ultra-low attachment plates and counted after 6 days. The figure is representative of 2 experiments performed in duplicate. Error bars: standard deviation.

Figure 5. Cancer-associated mutations increase cell migration. 200,000 NIH 3T3 cells (prepared as for Figure 3) were seeded in the top chambers of 24-well plates that contained transwell inserts. After 6 hours, the cells that migrated through the membranes were trypsinized and counted. The percentage of cells that migrated was calculated and normalized to the percent migration of control cells (EV). The figure represents the combined results of 3 experiments performed in triplicate or quadruplicate. Error bars: SEM.

Figure 6. WASP and Cas phosphorylation in Ack1-expressing cells. (A) Lysates from NIH3T3 cells expressing wild type or mutant forms of Ack1 were probed by Western blotting with anti-WASP (pY284), anti-WASP, and anti-tubulin antibodies, as indicated. Densitometry readings were used to calculate the ratios of phosphorylated to total WASP. (B) Cas was isolated by immunoprecipitation
from NIH3T3 cell lysates. The immunoprecipitated proteins were separated by SDS-PAGE and analyzed by Western blotting with anti-Cas and anti-pY249 Cas antibodies. Densitometry readings were used to calculate the ratios of phosphorylated to total Cas. Tubulin immunoblotting was performed on cell lysates. The figure is representative of 3 experiments.

**Figure 7. A model for Ack1 intramolecular regulation.** A, Amino acid sequence conservation between residues 700 to 910 of Ack1 (accession number: Q07912) and residues 264 to 435 of Mig6 (accession number: Q9UJM3). Green shading represents amino acid identity or conservative substitution, and the boxed region represents the fragment that was purified and used in this study. B, Three dimensional model of Ack1 kinase domain (blue) bound to Mig6 peptide (green) (8). The cancer-associated mutation E346K, and additional residues targeted for mutagenesis, are indicated.

**Figure 8. Activation of Ack1 by mutations designed to disrupt the intramolecular interaction.** A, Schematic diagram of Ack1 with sites of mutation indicated. B, Lysates from Cos 7 cells expressing wild type or mutant forms of Ack1 were analyzed by Western blotting with anti-Ack1 (pY284), anti-Ack1 and anti-tubulin antibodies. C, Immunoprecipitation-kinase assay: The following forms of Ack1 were immunoprecipitated from Cos 7 cells: WT (unstimulated or EGF-stimulated), V365R, F820A. Immunoprecipitated proteins were analyzed *in vitro* as described in the legend to Figure 1.

**Figure 9. A mutation in the Mig6-homology region recapitulates effects of cancer-associated mutations.** A, NIH 3T3 cells infected with retrovirus carrying F820A Ack1 were analyzed in a cell migration assay, as described in the legend to Figure 5. B, Non anchored growth: the same cells were grown in ultra low attachment plates and counted after 6 days, as described in the legend to figure 4B.

**Figure 10. Direct binding of Mig6-homology region to the Ack1 kinase domain.** A, GST fusion proteins containing the Ack1 Mig6-homology region (MHR) (wild type or F820A) were immobilized on glutathione-agarose beads and incubated with purified His6-tagged Ack1 kinase domain. The bound proteins were eluted from the beads with Laemmli buffer, separated by SDS-PAGE, transferred to a PVDF membrane, and analyzed by Western blotting using anti-His6 antibodies. The PVDF membrane was stained with Ponceau S to show the GST proteins. Immobilized GST was used as a control. B, GST-MHR was incubated with the His6-tagged kinase domain (WT or E346K). Bound proteins were eluted with Laemmli buffer, separated by SDS-PAGE, transferred to a PVDF membrane, and analyzed by Western blotting using anti-His6 antibodies. The PVDF membrane was stained with Ponceau S to show GST proteins. Input proteins were detected by anti-His6 Western blotting. C, Cartoon depicting the proposed interaction between MHR and kinase domain.
Figure 1
Figure 3
Figure 4
Figure 5
**Figure 6**

A

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ratio pWASP/WASP

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B

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ratio pCas/Cas

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Figure 7
**Figure 9**

**A**

Cell migration

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**B**

Non anchored growth

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Figure 10
Cancer-associated mutations activate the nonreceptor tyrosine kinase Ack1
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