Activated Tyrosine Kinase Ack1 Promotes Prostate Tumorigenesis: Role of Ack1 in Polyubiquitination of Tumor Suppressor Wwox

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Abstract
Aberrant activation of tyrosine kinases is linked causally to human cancers. Activated Cdc42-associated kinase (Ack1), an intracellular tyrosine kinase, has primarily been studied for its signaling properties but has not been linked to specific pathologic conditions. Herein, we report that expression of activated Ack1 in LNCaP cells, while minimally increasing growth in culture, enhanced anchorage-independent growth in vitro and dramatically accelerated tumorigenesis in nude mice. Molecular chaperone heat shock protein 90 (Hsp90)–bound Ack1 and treatment of cells with geldanamycin, a Hsp90 inhibitor, inhibited Ack1 kinase activity and suppressed tumorigenesis. Further, we identify the tumor suppressor WW domain containing oxidoreductase (Wwox) as an Ack1-interacting protein. Activated Ack1 tyrosine phosphorylated Wwox, leading to rapid dissociation of the Ack1-Wwox complex and concomitant Wwox polyubiquitination followed by degradation. Tyrosine phosphorylation of Wwox was critical for its degradation, as splice variant Wwox2,5-8 that was not phosphorylated by Ack1 failed to undergo polyubiquitination and degradation. It has been reported that phosphorylation of Wwox at Tyr287 stimulated its proapoptotic activity. We observed that Y33F Wwox mutant was still tyrosine phosphorylated and polyubiquitinated by Ack1 action. Site-directed mutagenesis revealed that activated Ack1 primarily phosphorylated Wwox at Tyr287, suggesting that phosphorylation of distinct tyrosine residues activate or degrade Wwox. Primary androgen-independent prostate tumors but not benign prostate showed increased expression of Ack1 phospho-Wwox and polyubiquitinated Ack1. Taken together, these data indicate that Ack1 activated prostate tumorigenesis in part by negatively regulating the proapoptotic tumor suppressor, Wwox. Further, these findings suggest that Ack1 could be a novel therapeutic target for prostate cancer. (Cancer Res 2005; 65(22): 10514-23)

Introduction
Tyrosine kinases are critical regulators of intracellular signal transduction pathways and their activity is tightly controlled. Dysregulation of tyrosine kinase signaling by chromosomal translocation, overexpression, or activating mutation may result in perturbation of the kinase activity and malignant transformation (1–3). Constitutive tyrosine kinase activation can lead to growth factor–independent cell growth, suppression of apoptosis, angiogenesis, invasion, and metastasis. Thus, tyrosine kinases have proven to be specific molecular targets for the cancer therapeutics development, and inhibitors of the tumor-relevant tyrosine kinases that drive oncogenic behavior have emerged as a novel paradigm for cancer therapy (4, 5).

Prostate cancer is the second leading cause of cancer deaths in American men (6). Androgen deprivation through surgical or medical castration is the current treatment for advanced metastatic disease (7). However, the disease invariably relapses with a median of 24 months, after which it is considered androgen independent (AIcaP) or hormone refractory, with no curative treatment. Cancer progression through the various stages presumably requires altered expression of oncogenes and tumor suppressor genes in the initiated cell (8). Growing evidence suggests that tyrosine kinases play a significant role in prostate cancer progression (9–12), and thus identification of the disease-associated tyrosine kinases, and the mechanism by which they enhance prostate tumorigenesis should produce additional therapeutic targets.

Our previous work identified a transmembrane receptor tyrosine kinase Mer (also known as NYK; ref. 13), belonging to the Mer/Axl/Tyro3 receptor family (14). We observed expression of Mer in macrophages, epithelial cells, and cells of reproductive origin (e.g., testis, ovary, and prostate; refs. 14, 15). Further, we showed that Mer signaling was involved in monocytic ingestion of apoptotic cells (16) in part by regulating Vav1-Rac/Cdc42–mediated cytoskeletal remodeling (15). Gas6 was identified as a ligand for Mer/Axl/Tyro3 family (17, 18) and we have shown that Gas6 binding activates Mer leading to autophosphorylation, tyrosine phosphorylation of guanine nucleotide exchange factors, and accumulation of GTP-bound Rac and Cdc42 (15). Three human prostatic adenocarcinoma cell lines, LNCaP, CWR-R1, and DU-145, express the cell surface 175-kDa Mer protein (13).4 To identify Mer-activated tyrosine phosphoprotein, we used proteomics approach and identified activated Cdc42-associated kinase (Ack1) as a Mer-responsive intracellular tyrosine kinase. To determine what role Ack1 might play in prostate malignancy, we generated constitutively active Ack1 (caAck) and kinase-inactive Ack1 constructs and expressed them in LNCaP cells. Although caAck only modestly increased LNCaP proliferation in culture, we observed dramatic acceleration of xenograft growth of activated Ack1-expressing LNCaP cells. This was suppressed by a heat shock protein 90 (Hsp90) inhibitor that abolished Ack1 kinase activity. Further, we identified one novel mechanism of Ack1-mediated tumorigenesis, tyrosine phosphorylation of the tumor suppressor WW domain containing oxidoreductase (Wwox) leading

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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to its polyubiquitination and degradation. Although others have identified stimulation of Wwox proapoptotic activity by Tyr phosphorylation, the primary Ack1 site of Wwox phosphorylation was at Tyr287. Our analysis of human androgen-independent prostate tumors corroborated this inverse relationship, showing increased tyrosine-phosphorylated Ack1 and decreased Wwox protein. This and the accelerated experimental tumorigenesis define Ack1 as a novel therapeutic target in prostate cancer.

Materials and Methods

Plasmids and site-directed mutagenesis. The full-length Mer cDNA was subcloned into pCDNA3.1 vector (Invitrogen, Carlsbad, CA). The myc-tagged constructs caAck, kinase-dead Ack1 (kdAck), and wAck and Flag-tagged Wwox construct were generated using PCR amplification and subcloned into pcdNA4/A-Myc-His vector (Invitrogen). Mutagenesis was done using GeneEditor system (Promega, Madison, WI). All plasmid constructs were sequenced.

Antibodies, cell lines, virus production, and infection. A synthetic peptide containing the 20 amino acids of Ack1 was used to generate rabbit polyclonal antibody. Anti-phospho-tyrosine (RC20; Transduction Laboratories, San Jose, CA), anti-Hsp90 (Stressgen, San Diego, CA), anti-Wwox polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-myc (Invitrogen) antibodies were purchased. Anti-Wwox monoclonal was kindly provided by Dr. C. Croce (Cancer Center, Ohio State University, Columbus, OH). To generate LNCaP stable lines, respective retrovirus constructs, subcloned into pMSCV (Invitrogen) vector, were transfected in A293T cells with a packaging plasmid pCMV-WSV-G and pUMC3-gagpol using FuGene (Roche, Indianapolis, IN). LNCaP cells were infected for 5 to 6 hours with viral supernatants containing 8 μg/mL polybrene. The transfected cells were selected in the presence of puromycin (2 μg/mL).

Mass spectrometry. Coomassie, silver, or Pro-Q diamond stained gel bands were excised and digested overnight with trypsin using a Progester digestor (Genomic Solutions, Ann Arbor, MI). The peptide extract was lyophilized, resuspended, and spotted on the matrix-assisted laser desorption/ionization target. The spectra were database searched using ABI’s Data Explorer software package (Foster City, CA). The peptide mass fingerprinting and sequence tag data from the time of flight (TOF)/TOF were ranked according to ABI’s GPS Explorer scores, and confident hits were reported.

Immunoprecipitation and immunoblot analysis. HEK293T cells were transfected using FuGene as per manufacturer’s protocol. Thirty-two hours after transfection, cells were lysed in receptor lysis buffer containing 25 mmol/L Tris (pH 7.5), 225 mmol/L NaCl, 1% Triton X-100, 1 mmol/L DTT, 10% glycerol, phosphatase inhibitors (10 mmol/L NaF, 1 mmol/L Na2VO4), and protease inhibitor mix (Roche). Equivalent amounts of protein were incubated with respective primary antibodies and protein A/G-Sepharose (Santa Cruz Biotechnology) for 2 hours or overnight at 4°C. Immunoprecipitates were washed and prepared for immunoblot analysis. The blots were stripped and reprobed with a second set of antibodies to confirm the presence of respective proteins.

Purification of glutathione S-transferase fusion proteins and immunoaffinity assay. Glutathione S-transferase (GST)-caAck and GST-kdAck constructs were transformed into BL21(DE3) cells and plated onto ampicillin plates. Colonies were picked and grown overnight in 10 mL LB containing ampicillin. Culture grown overnight was added to 100 mL fresh LB containing 25 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 0.4% Triton X-100, 1 mmol/L DTT, 15% glycerol, phosphatase inhibitors (10 mmol/L NaF, 1 mmol/L Na2VO4), and protease inhibitor mix. Lysates were incubated with glutathione beads for 2 hours followed by washing the beads with lysis buffer. Affinity purification method was employed to identify Ack1-interacting proteins. LNCaP cell lysate (made using receptor lysis buffer) was incubated with glutathione beads for 2 hours. Glutathione beads were washed and bound proteins were eluted in 10 mmol/L glutathione, subjected to SDS-PAGE, and stained with Coomassie blue. Ack1-interacting protein was identified using mass spectrometry.

Anchorage-independent and cell growth assay. LNCaP cells (1 × 104) that stably expressed caAck, kdAck, wAck, and vector were seeded in 12-well plates in quadruplets in 1 mL top agar. Colonies were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) after 3 to 4 weeks. Cell survival growth assay was performed using LNCaP cells (1 × 103) that stably expressing Ack1 variants were seeded in 96-well plates. Solution of MTT was added to cells and incubated for 2 hours, and cell growth as a function of mitochondrial activity in living cells was measured spectrophotometrically at a wavelength of 570 nm.

Kinase assay. Ack1 kinase activity was determined using phospho-cellulose filter binding assay. Reaction mixtures contained 20 mmol/L Tris-HCl (pH 7.4), 10 mmol/L MgCl2, 0.1 mmol/L Na3VO4, 0.5 mmol/L DTT, 0.25 mmol/L ATP, varying concentrations of peptide substrate (EAIYAFAKKKK), and [γ-32P]ATP (200–400 counts/min/μmol). Reactions were terminated and the samples were spotted on p81 phosphocellulose filter. Incorporation of 32P into peptide was determined using liquid scintillation counting.

Tumorigenicity assay. LNCaP cells (2 × 105) that expressed Ack1 variants were suspended in 100 μL PBS and 100 μL Matrigel (Discovery Labware, Bedford, MA) and injected s.c. into flanks of male nude mice. Tumor volume was measured twice weekly using calipers.

In vivo ubiquitination assays. HEK293T cells were transfected and pretreated with 25 μmol/L MG-132 for 3 to 4 hours. Cells were lysed by RLB buffer containing 1% SDS. Protein samples were diluted 1:5 and immunoprecipitated with anti-Flag beads. Immunoprecipitates were washed and prepared for immunoblot analysis.

Patient tissue samples. Androgen-independent prostate cancer samples were obtained from transurethral resection specimens from men who developed urinary retention from local recurrence during androgen deprivation therapy. Androgen-stimulated benign prostate samples were obtained from radical prostatectomy specimens from men with clinically localized prostate cancer. Histologic diagnoses were confirmed by examination of frozen and corresponding formalin-fixed, paraffin-embedded tissue specimens. Tissue procurement and subsequent studies were done with institutional review board and HIPPA approval.

Results

Ack1 is a major prostate cell phosphoprotein induced by the Mer receptor tyrosine kinase. To identify tyrosine-phosphorylated proteins responding to Mer activation, we treated large-scale cultures of the prostate cancer cell line LNCaP with Gas6 ligand. Protein lysates were affinity purified using phosphotyrosine antibody covalently immobilized on beads. After elution, samples were subjected to SDS-PAGE. Western analysis using anti-phosphotyrosine antibody revealed increased tyrosine phosphorylation of ~120-kDa protein on Gas6 treatment (Fig. 1A). The ~120-kDa band was excised and subjected to in-gel trypsin digestion and liquid chromatography/tandem mass spectrometry sequencing. Database search for the peptide sequences revealed that the 120-kDa protein was Ack1 (Fig. 1B, sequenced peptides shown in bold). Ack1 is a 120-kDa soluble protein (Fig. 1C) with a NH2-terminal kinase domain, a Src homology 3 (SH3) domain, a Cdc42/Rac interactive binding domain (CRIB) domain, and a proline-rich domain at COOH terminus (19). Ack1 specifically interacts with GTP-bound Cdc42 (activated Cdc42) and inhibits both intrinsic and GTPase-activating protein–stimulated GTPase activities of Cdc42 (19). Ack1 activity has been implicated in cell spreading (20), vesicle trafficking (21), axonal guidance (22), and dorsal closure in Drosophila melanogaster (21), vesicle trafficking (22), and dorsal closure in Drosophila melanogaster (23). However, the physiologic role of Ack1 and its molecular mechanism of action are not fully understood, nor has Ack1 been linked to any specific pathologic condition (24).
To ascertain whether endogenous Mer and Ack1 interact in prostate cells, coimmunoprecipitation was done on LNCaP lysates using Mer antibodies followed by an immunoblotting with Ack1 antibodies. Gas6 treatment resulted in tyrosine phosphorylation of endogenous Mer (Fig. 1D, 4) and Ack1 (Fig. 1D, 2). Endogenous Mer and Ack1 specifically associated in phosphotyrosine-dependent manner following Gas6 treatment (Fig. 1D, 1).

**Ack1 autophosphorylation by an intramolecular mechanism.**

To elucidate the molecular mechanism by which Mer regulates Ack1 tyrosine phosphorylation, three myc-tagged Ack1 constructs, wAck, kdAck, and caAck, were generated, each consisted of the kinase domain, SH3 domain, CRIB domain, and proline-rich domain. Each lacked the COOH-terminal region (788-1,036 amino acids; Fig. 2A, 4), as we and others have observed that the full-length Ack1 construct is expressed poorly (25); therefore, we used the truncated constructs. The kdAck construct possesses a point mutation, K158R, in the ATP acceptor site and does not autophosphorylate (Fig. 2A, 4). Nuclear magnetic resonance spectroscopy has shown details of molecular interaction between Ack1 and GTP-bound Cdc42, wherein Leu487 of Ack1 interacts with Leu67 of Cdc42 (26, 27). Interestingly, Leu487 also interacts with residues in its own kinase domain, maintaining an inactive Ack1 (26, 27). Based on this intramolecular interaction...
(27, 28), we made the L487F mutation, which essentially disrupts autoinhibition and results in a caAck (Fig. 2A).

To determine if tyrosine phosphorylation of Ack1 is mediated by an intramolecular or intramolecular mechanism, HEK293T cells were transfected with myc-tagged Ack1 variants with either the full-length Mer or empty vector. If tyrosine phosphorylation occurs in trans (i.e., intermolecular), then kdAck would be phosphorylated when coexpressed with Mer. In contrast, if phosphorylation occurs via an intramolecular (or cis) mechanism, then K158R mutation in the ATP acceptor site would disrupt tyrosine phosphorylation of Ack1. Earlier we have noticed (15) that full-length Mer overexpression resulted in Gas6-independent Mer activation (Fig. 2B, 3). caAck was tyrosine phosphorylated in the absence or presence of active Mer (Fig. 2B, I, lanes 5 and 6). Active Mer induced tyrosine phosphorylation of wAck but not kdAck (Fig. 2B, I, lanes 2 and 4), suggesting that activated Mer does not directly phosphorylate Ack1 but rather facilitates Ack1 autophosphorylation. These results suggest that Ack1 phosphorylation occurs by an intramolecular mechanism.

To assess the potential relevance of Ack1 activation in prostate cancer, LNCaP cells were infected with myc-tagged Ack1-expressing retroviral constructs and stable lines were generated that showed tyrosine-phosphorylated Ack1 in caAck-expressing cells but not kdAck- or vector-expressing cells (Fig. 2C). Longer exposure shows some autophosphorylation of wAck. To determine whether Ack1 autophosphorylation correlated with kinase activation, kinase assays were done. It has been shown that purified Ack1 kinase prefers a peptide substrate also preferred by the Abl kinase (25). This 13-aa peptide (EAIYAAPFAKKKG) was synthesized and used for kinase assays (Fig. 2D). Ack1 immunoprecipitates from LNCaP cells expressing wAck, kdAck, and vector exhibited low kinase activity. In contrast, caAck immunoprecipitated had 7-fold higher kinase activity than kdAck (Fig. 2D), indicating that Ack1 autophosphorylation correlated with kinase activation. 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Activated Ack1 promotes anchorage-independent growth and tumor growth in vivo. To determine biological effects of Ack1 constructs, growth characteristics of the stably transfectected LNCaP cells were monitored. A modest increase in growth rate of the caAck- and wAck-expressing cells was observed compared with the kdAck- and vector-expressing cells (Fig. 3A). We next assessed ability of LNCaP cells expressing Ack1 constructs to form colonies in soft agar. LNCaP cells expressing caAck exhibited a substantial increase in anchorage-independent growth in soft agar. Fewer colonies were observed in kdAck-, wAck-, and vector-expressing cells (Fig. 3B). To further examine the effect of Ack1 activation, we injected wAck-, kdAck-, and vector-expressing LNCaP cells s.c. into flank of nude mice and monitored the increase in tumor volume over a 3-month period. LNCaP cells are poorly tumorigenic into flank of nude mice (Fig. 3C). Tumors were undetectable at this stage in wAck-, kdAck-, and vector-expressing LNCaP cells (compare tumor size of kdAck and caAck; Fig. 3D), although small tumors were observed in some cases when the animals were followed for 90 days. These data indicate that kinase activation of Ack1 results in enhancement of prostate cancer cell tumorigenic properties.
Heat shock protein 90\textsuperscript{3} maintains Ack1 activity. We next attempted to identify Ack1-interacting proteins by biochemical affinity purification. GST-tagged caAck, kdAck, and GST (Fig. 4A) expressed in *Escherichia coli* were purified using glutathione beads. These were then incubated with LNCaP cell lysates. After extensive washing, bound proteins were eluted using glutathione and separated using SDS-PAGE. A band of \( \sim 90 \) kDa isolated from GST-caAck1 complex, not seen in GST alone, was microsequenced (Supplementary Fig. S1) and identified as Hsp90\textsuperscript{\textbullet}\textsuperscript{\textbullet} (Fig. 4B). Hsp90 is a molecular chaperone that plays a critical role in conformational maturation of protein kinases, steroid hormone receptors, growth factor receptors, and transcription factors involved in signal transduction (29). Ack1-Hsp90\textsuperscript{\textbullet}\textsuperscript{\textbullet} association was further confirmed by coimmunoprecipitation experiment. Hsp90\textsuperscript{\textbullet}\textsuperscript{\textbullet} was coprecipitated by myc affinity beads from LNCaP cells that stably express all the three myc-tagged Ack1 constructs but not in vector-expressing cells (Fig. 4C). Immunoprecipitation of untransfected LNCaP cell lysates with Ack1 antibodies also revealed the presence of Hsp90\textsuperscript{\textbullet}\textsuperscript{\textbullet}, indicating endogenous Ack1-Hsp90\textsuperscript{\textbullet}\textsuperscript{\textbullet} interaction (data not shown). Thus, in vivo interaction between Hsp90\textsuperscript{\textbullet}\textsuperscript{\textbullet} and Ack1 seems to be constitutive in LNCaP cells, but Hsp90\textsuperscript{\textbullet}\textsuperscript{\textbullet} may prefer tyrosine-phosphorylated Ack1 (Fig. 4C, lane 3).

We investigated whether Hsp90-specific inhibitors could suppress caAck kinase activity and caAck accelerated tumorigenesis. Geldanamycin binds to a conserved binding pocket inhibiting ATP-dependent chaperone activity of Hsp90; this destabilizes many Hsp90 client proteins, including Raf-1 and HER2 (30, 31). LNCaP cells expressing Ack1 variants were treated with geldanamycin for 8 hours and kinase activity was monitored (Fig. 4D). The caAck kinase activity was reduced to basal level on geldanamycin treatment (Fig. 4D), with no significant decrease in total Ack1 protein, in 8 hours (Fig. 4E, 2), indicating that Hsp90\textsuperscript{\textbullet}\textsuperscript{\textbullet} was involved in maintaining the active Ack1 kinase conformation. This might differ from the proposed mechanism of Hsp90 action for HER2, whereby geldanamycin treatment lead to significant HER2 degradation at 8 hours (Fig. 4E, 3 ref. 32). The apparent increase in HER2 levels between vector and all three Ack1-transfected cell lines was not reproducible (i.e., Ack1 expression does not change HER2 expression levels).

We further tested the role of Ack1 kinase activity in promoting tumorigenesis in vivo by treating stably transfected LNCaP cell lines with geldanamycin. caAck1 LNCaP cells were treated with geldanamycin or DMSO for 8 hours and injected into nude mice. DMSO-treated caAck cells formed large tumors in all mice within 24 days, but the preimplantation geldanamycin treatment substantially delayed caAck LNCaP cell tumorigenesis (Fig. 4F). These results suggest that the mechanism underlying Ack1-mediated tumorigenesis in vivo includes Hsp90\textsuperscript{\textbullet}\textsuperscript{\textbullet}-stabilized Ack1 kinase activity, although we cannot rule out the involvement of additional Hsp90 clients.

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**Figure 4.** Inhibition of Ack1 kinase activity by Hsp90 inhibitor suppress tumorigenesis. **A**, schematic of GST-Ack1 fusion proteins expressing constructs. **B**, lysates of LNCaP cells were incubated with glutathione beads immobilized with GST-caAck, GST-kdAck, and GST alone. Following electrophoresis, tryptic digestion of 90-kDa excised band, and mass spectrometry, Hsp90\textsuperscript{\textbullet}\textsuperscript{\textbullet} was identified (see Supplementary Fig. S1). The sequenced peptides are shown in bold. **C**, lysates of LNCaP cells stably expressing myc-tagged Ack1 variants were treated with geldanamycin (5 \( \mu \text{mol/L} \)) for 8 hours and immunoprecipitation/kinase assays were done. Geldanamycin treatment resulted in abrogation of Ack1 kinase activity. **E**, LNCaP cells were treated with geldanamycin (5 \( \mu \text{mol/L} \)) for 8 hours and subjected to immunoprecipitation followed by immunoblotting as indicated. **F**, Hsp90 inhibition results in loss of tumorigenicity. LNCaP cells stably expressing Ack1 variants were treated with geldanamycin (5 \( \mu \text{mol/L} \)) for 8 hours and injected s.c. into male nude mice \((n = 10)\). Data represent growth of tumors. Points, mean tumor volume; bars, SE. Geldanamycin treatment delayed caAck-mediated xenograft growth of LNCaP cells. Representative of two independent experiments.
Wwox was detected in cells expressing myc-tagged caAck but not in vector (Supplementary Fig. S2). Microsequencing revealed it to be WwoxΔ5-8 (Fig. 5A, peptides are shown in bold). This alternately spliced Wwox lacks exons 5 to 8 and has a COOH terminus whose sequence diverges from full-length Wwox due to translation of a spliced Wwox lacks exons 5 to 8 and has a COOH terminus whose sequence diverges from full-length Wwox due to translation of a spliced Wwox lacks exons 5 to 8 and has a COOH terminus whose sequence diverges from full-length Wwox due to translation of a spliced Wwox lacks exons 5 to 8 and has a COOH terminus whose sequence diverges from full-length Wwox due to translation of a spliced Wwox lacks exons 5 to 8 and has a COOH terminus whose sequence diverges from full-length Wwox due to translation of a spliced Wwox lacks exons 5 to 8 and has a COOH terminus whose sequence diverges from full-length Wwox due to translation of a spliced Wwox lacks exons 5 to 8 and has a COOH terminus whose sequence diverges from full-length Wwox due to translation of a spliced Wwox lacks exons 5 to 8 and has a COOH terminus whose sequence diverges from full-length Wwox due to translation of a spliced Wwox lacks exons 5 to 8 and has a COOH terminus whose sequence diverges from full-length Wwox due to translation of a spliced...
tyrosine phosphorylation promotes Wwox polyubiquitination (Fig. 5D, 6). In contrast, WwoxΔ5-8 was neither tyrosine phosphorylated nor polyubiquitinated when coexpressed with caAck (Fig. 5D, 6, lane 7), suggesting that tyrosine phosphorylation is a prerequisite for Wwox polyubiquitination.

To investigate the potential of activated Ack1 to modulate Wwox steady-state levels, HEK293T cells were cotransfected with myc-tagged caAck1 and Flag-tagged Wwox or WwoxΔ5-8. After 36 hours, cells were treated with cyclohexamide for 3 hours and levels of Wwox were determined by immunoprecipitation followed by Western analysis (Fig. 5E). Coexpression of caAck1 resulted in reduction in Wwox protein (Fig. 5E, 1, lane 2). In contrast, WwoxΔ5-8 was not degraded (Fig. 5E, 1, lane 4), indicating that activated Ack1 mediated Wwox phosphorylation and polyubiquitination resulting in degradation.

To ascertain whether endogenous Wwox is targeted for polyubiquitination, HEK293T and LNCaP cells were transfected with caAck and treated with proteasome inhibitor MG-132 for 3 hours. The cells were harvested and lysates were subjected to immunoprecipitation using Wwox antibodies followed by an immunoblotting with ubiquitin antibodies. MG-132 treatment resulted in detection of polyubiquitinated endogenous Wwox (Fig. 5F, lanes 2 and 4). These results show that Ack1 activation results in polyubiquitination of endogenous Wwox protein in prostate-derived cells.

**Ack1 tyrosine phosphorylates Wwox predominantly at Tyr**

**287** **and not Tyr**

**33.** It has been reported that, during "cell stress," Wwox undergoes tyrosine phosphorylation at Tyr287, located in first WW domain, stimulating its proapoptotic activity (39, 43). To investigate whether the Ack1-dependent Wwox tyrosine phosphorylation occurs at Tyr287, we created a Y287F Wwox mutant (Fig. 6A) and assessed caAck-dependent tyrosine phosphorylation on cotransfection. The Y287F mutant was tyrosine phosphorylated and polyubiquitinated by caAck1 (Fig. 6B, lane 2, 4), indicating that the ubiquitination targeting tyrosine phosphorylation occurs at a different site from the apoptosis activating Y33F tyrosine phosphorylation.

![Figure 6](image.jpg)

**Figure 6.** Activated Ack1-mediated phosphorylation and degradation of Y33F mutant of Wwox. **A**, domain structure of Wwox and its Y33F mutant. Position of point mutation is indicated. **B**, following cotransfection of Flag-tagged-Wwox or Y33F mutant with myc-tagged-Ack1 variants, HEK293T lysates were subjected to immunoprecipitation followed by immunoblotting as indicated. Y33F mutant of Wwox was also phosphorylated and polyubiquitinated by caAck.

The fact that WwoxΔ5-8 is neither phosphorylated nor polyubiquitinated by caAck1 and has a different COOH-terminal sequence suggests that the "ubiquitination targeting" tyrosine phosphorylation site resides in the COOH terminus of Wwox. Site-directed mutagenesis was done to identify tyrosine residue in Wwox that is phosphorylated by activated Ack1. The COOH-terminal region of Wwox possess seven tyrosine residues; thus, seven distinct point mutants were generated, each having single Tyr-to-Phe substitution (Fig. 7A). One Wwox mutant, Y287F, exhibited the most significant loss of tyrosine phosphorylation when coexpressed with activated Ack1 (Fig. 7B), although tyrosine phosphorylation of Y287F mutant was not completely lost (Fig. 7B, lane 2). This indicates that Tyr287 is the predominant but perhaps not the only phosphorylation site.

**Androgen-independent prostate tumor specimens exhibit increased tyrosine-phosphorylated Ack1 and decreased Wwox.**

To investigate the correlation between activated, tyrosine-phosphorylated Ack1 and Wwox levels in human prostate cancer, immunoprecipitation and immunoblotting were used to examine and compare 16 samples of primary androgen-independent prostate cancers (AlCaP) and 16 samples of benign prostate. Whereas some benign prostate specimens exhibited tyrosine-phosphorylated Ack1 (Fig. 8A), AlCaP had significantly higher tyrosine-phosphorylated Ack1 content per milligram of tissue lysate (Fig. 8A, 1). Densitometric quantitation from all samples showed that AlCaP and benign prostate samples had similar total Ack1, but AlCaP exhibited 4- to 5-fold higher levels of tyrosine-phosphorylated Ack1 (Fig. 8D). In contrast, compared with benign prostate samples, Wwox levels were reduced significantly in AlCaP (Fig. 8A, 3), suggesting that the autophosphorylation (i.e., activated) Ack1 regulates Wwox by tyrosine phosphorylation, polyubiquitination, and degradation.

![Figure 7](image.jpg)

**Figure 7.** Activated Ack1 phosphorylates Wwox at Tyr287. **A**, domain structure of Wwox and Tyr-to-Phe point mutants. Position of point mutations is indicated. **B**, following cotransfection of Flag-tagged-Wwox or point mutants with myc-tagged caAck, HEK293T lysates were subjected to immunoprecipitation followed by immunoblotting as indicated.
Activated Ack1 accelerated prostate cancer cell tumorigenesis in vivo in part through a novel mechanism, stimulation of polyubiquitination and degradation of a tumor suppressor Wwox. Moreover, primary tumor specimen provided correlative evidence that these two processes occur clinically. Questions remain, for example, how is Ack1 stimulated in AICaP? One possibility is that Ack1 could acquire somatic mutations within its kinase domain or the autoinhibitory activation loop leading to autoactivation as has recently been detected for a portion of lung cancers (epidermal growth factor receptor and HER4) and hematopoietic tumor types (Janus-activated kinase 2). Tumor-specific point mutations in Ack1 kinase domain or the autoinhibitory activation loop could play a role in Ack1 phosphorylation and degradation of androgen receptor, HER2, Raf-1, and AKT; both indicate that Ack1 tyrosine kinase activity is important of this experimental system’s observations.

In our experiments, geldanamycin/17-AAG–mediated suppression of hormone-naïve and castration-resistant tumors (47). Ack1 promotes prostate tumorigenesis. Wwox has been found to be “underexpressed” in human cancers, which could result from distinct events (e.g., allelic loss, point mutation, or promoter hypermethylation or combination of two or more events resulting in a loss of tumor suppressor activity), as proposed by Knudson (48). Point mutations are rare in Wwox gene (49), but LOH of Wwox allele was observed in variety of cancers, including prostate (49, 50). Our data lead us to speculate that other “second hit” diminishing functional Wwox protein is COOH-terminal Wwox tyrosine phosphorylation at Tyr287, targeting Wwox for polyubiquitination and degradation. It is interesting that Wwox tyrosine phosphorylation at different sites (Tyr25 and Tyr287) seems to have radically different outcomes, activation of apoptosis or targeting for destruction.

WW domains are protein-protein interaction modules that recognize short proline-rich motifs and have been identified in various signaling proteins (51). Src tyrosine kinase was initially recognized short proline-rich motifs and have been identified in various signaling proteins (51). Src tyrosine kinase was initially predicted to be involved in tyrosine phosphorylation of WW domain containing proteins (52). Later, it was shown that Src kinase activation and stress stimuli, including UV irradiation, could lead to Wwox phosphorylation at Tyr25, promoting cell death (39, 43). We observed ubiquitination of Wwox but not WwoxΔ5-8 when coexpressed with caAck. This suggests that Ack1-mediated COOH-terminal tyrosine phosphorylation at Tyr287 would override Wwox proapoptotic action due to degradation, thus promoting tumorigenesis. The inverse correlation between tyrosine phosphorylation of WW domains and Ack1 activity in AICaP implies that Ack1 could accelerate tumorigenesis.

We have observed Mer-dependent Vav2 and Vav3 tyrosine phosphorylation.5 Because Mer does not directly phosphorylate Ack1 (Fig. 2B), it may regulate autophosphorylation and activation indirectly by activating Cdc42. Additionally Ack1 could be activated by other receptor tyrosine kinases (e.g., platelet-derived growth factor receptor), we have shown that Mer interacts with and phosphorylates a guanine nucleotide exchange factor, Vav1, resulting in activation of Cdc42 (15). Thus, other signals, including those from G-protein-coupled or cytokine receptors that activate Cdc42 GDP-to-GTP exchange, could also regulate Ack1 activity.

kdAck does not accelerate xenograft growth, and caAck1-induced tumorigenesis is reversed with geldanamycin-mediated kinase inhibition; both indicate that Ack1 tyrosine kinase activity is necessary. The involvement of Hsp90β chaperone in activity of large number of potentially oncogenic protein kinases has engendered substantial interest in Hsp90β as a cancer chemotherapy target (44). Geldanamycin, a naturally occurring ansamycin antibiotic, and its clinical analogue 17-allylamino-17-demethoxy-geldanamycin (17-AAG) have significant anticancer activities. Recently, it has been shown that Hsp90β derived from tumor cells binds to 17-AAG up to 100 times more tightly that those isolated from normal cells (45). In examining Ack1-Hsp90β complexes, we noted increased amount of Hsp90β in caAck immunoprecipitates compared with wAck or kdAck. This may suggests that Ack1 tumors with their elevated levels of activated Ack1 might be more sensitive to 17-AAG to the extent that Ack1 is a major target of 17-AAG. Hsp90β chaperone activity is required for maturation and stability of number of proteins involved in mediating prostate cancer progression (e.g., androgen receptor, HER2, Raf-1, and AKT; ref. 46). In murine models of prostate cancer, 17-AAG causes inhibition of hormone-naïve and castration-resistant tumors (47). In our experiments, geldanamycin/17-AAG–mediated suppression of tumor progression may be primarily due to kinase inactivation of Ack1 but may also involve a combinatorial effect, including degradation of androgen receptor, HER2, Raf-1, and AKT.

Ack1 binds to a tumor suppressor Wwox and to at least one of its splice isoforms. On Ack1 activation, Wwox is tyrosine-phosphorylated and targeted for ubiquitination-mediated degradation. This observation provides a novel mechanism by which Ack1 could accelerate tumorigenesis. Wwox has been found to be “underexpressed” in human cancers, which could result from distinct events (e.g., allelic loss, point mutation, or promoter hypermethylation or combination of two or more events resulting in a loss of tumor suppressor activity), as proposed by Knudson (48). Point mutations are rare in Wwox gene (49), but LOH of Wwox allele was observed in variety of cancers, including prostate (49, 50). Our data lead us to speculate that other “second hit” diminishing functional Wwox protein is COOH-terminal Wwox tyrosine phosphorylation at Tyr287, targeting Wwox for polyubiquitination and degradation. It is interesting that Wwox tyrosine phosphorylation at two different sites (Tyr25 and Tyr287) seems to have radically different outcomes, activation of apoptosis or targeting for destruction.

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Watanabe et al. (53) showed accumulation of a truncated Wwox protein of approximate molecular weight of 26 kDa by inhibiting proteasomal degradation with MG-132 treatment. The expression

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5 N.P. Mahajan, unpublished data.
Apart from targeting tumor suppressor Wwox for polyubiquitination, Ack1 may have additional mechanisms by which it accelerates prostate tumor growth. The fact that caAck1 modestly stimulates LNCAp growth in cell culture while dramatically enhancing in vivo tumorigenesis indicates that the caAck1 mechanism of action is not simply pro-proliferative. For example, Ack1’s role as a potential downstream effector of GTP-bound Cdc42 on cytokskeletal or integrum dynamics is a possibility, as are alterations in gene expression. Recently, it has been shown that Ras transforms, but not normal cells, are highly reliant on the Ack1 kinase for both survival and growth, suggesting that Ack1 plays a role in transducing Ras signals for transformation of mammalian cells (54). The fact that Ack1 can significantly enhance prostate tumorigenesis in vivo indicates that both upstream and downstream Ack1 signaling pathways may provide potential targets for drug discovery in prostate cancer and paves the way for new therapeutic strategies in advanced prostate cancer.

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