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Identification of otubain 1 as a novel substrate for the *Yersinia* protein kinase using chemical genetics and mass spectrometry

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Abstract ***Yersinia*** **encodes a protein kinase, YpkA, which disrupts the actin cytoskeleton. Using an approach termed chemical genetics, we identified a 36-kDa substrate for YpkA in both J774 lysates and bovine brain cytosol. Mass spectrometry analysis identified this substrate as FLJ20113, an open reading frame that corresponds to otubain 1, a deubiquitinating enzyme implicated in immune cell clonal anergy. We demonstrate that otubain 1 is phosphorylated by YpkA in vitro and interacts with YpkA and actin in vivo. Identification of otubain 1 as a YpkA substrate suggests that regulation of immune cell anergy may be a survival mechanism for** ***Yersinia*****.**

Keywords: *Yersinia* pathogenesis; Chemical genetics; Phosphorylation; Protein kinase

1. Introduction

Pathogenic *Yersinia* cause a variety of diseases including the bubonic plague. *Yersinia* utilize a Type III secretion system to translocate effector proteins into host cells [1]. These effectors, referred to as Yops (*Yersinia o*uter membrane *p*roteins), include YopH, YopJ, YopE, YopT, YopM, and YpkA (YopO). The Yops function to disrupt mammalian signaling cascades, resulting in disturbance of the host innate immune system allowing for bacterial survival [1].

YpkA (*Yersinia* protein kinase A) has similarity to the mammalian serine/threonine kinases [2]. YpkA is essential for virulence, as disruption of YpkA results in an avirulent strain of *Yersinia* [2]. We demonstrated that YpkA is produced as an inactive kinase in bacteria, and is activated by actin in the host cell where it disrupts the actin cytoskeleton [3]. The only substrate for YpkA identified to date is actin [3], and we hypothesize that YpkA phosphorylates additional substrates which are involved in rearranging the actin cytoskeleton or inhibiting phagocytosis, thus ensuring survival of *Yersinia*.

Here, we report the use of chemical genetics to identify substrates for YpkA. This approach allows for the identification of novel protein kinase substrates by altering the kinase active site to accept a chemically modified form of ATP [4]. This method permits one to screen a crude extract for substrates with little background phosphorylation, as the mutated kinase is the only kinase capable of efficiently using the chemically modified ATP [5]. Chemical genetics has been employed successfully for various kinases including JNK and v-Src [5,6]. Using a mutant form of YpkA, which shows a pronounced preference for γ-^{32}P N^6-phenethyl ATP, we show selective phosphorylation of actin and another protein with an apparent size of 36-kDa by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE). Using mass spectrometry we identified this protein as otubain 1, a member of the family of ovarian tumor (OTU)-like cysteine proteases involved in the ubiquitin pathway [7]. We demonstrate phosphorylation of otubain 1 by YpkA in vitro and further demonstrate a connection between otubain 1 and the actin cytoskeleton. These data suggest a role for otubain 1 in the pathogenic effects initiated by YpkA.

2. Materials and methods

2.1. Constructs

YpkA constructs have been previously described [3]. Otubain 1 was PCR amplified from human expressed sequence tag (EST) clone 5300681 (Research Genetics) and subcloned into both pGEX-6*p*-2 (Amersham Pharmacia) for bacterial expression and pCDNA3 (Invitrogen) with an N-terminal HA tag for mammalian expression.

2.2. Expression of recombinant proteins

YpkA was expressed and purified as previously described [3]. The vector pGEX-6*p*-2-otubain 1 was transformed into Novablue DE3 *E. coli*-competent cells (Stratagene) for protein expression. Otubain 1 was expressed and purified in the same manner as YpkA [3]. His-tagged nucleoside diphosphate kinase (NDK, a gift from Justin Blethrow and David Morgan, University of California, San Francisco) was purified as previously described [5].

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Abbreviations: Yop, *Yersinia* outer membrane protein; YpkA, *Yersinia* protein kinase A; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; OTU, ovarian tumor; NDK, nucleoside diphosphate kinase; MALDI-MS, matrix-assisted laser desorption ionization-mass spectrometry; TOF, time of flight; PSD, post source decay; MBP, myelin basic protein; EST, expressed sequence tag

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2.3. Radiolabeling of N^6-ATP Analogs

N^6-benzyl ADP, N^6-cyclopentyl ADP, and N^6-phenethyl ADP were radiolabeled with His-NDK resin as previously described [5].

2.4. Kinase assays

Recombinantly expressed YpkA (1.5 μg) and either J774 cell extract (3 μg), bovine brain fractionated proteins, or purified actin were incubated in an in vitro kinase assay as previously described [3].

2.5. Preparation of mammalian extracts

J774 macrophages were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2 mM glutamine, and 100 μg/ml penicillin/streptomycin (Invitrogen). Approximately 10^7 cells were washed once with phosphate buffered saline and resuspended in homogenization buffer (10 mM Tris, pH 7.5/10% sucrose/2 μg/ml aprotinin/2 μg/ml leupeptin/0.2 mM phenylmethylsulfonyl fluoride). Cells were homogenized with 30 passes in a Dounce homogenizer. The cell lysate was centrifuged at $5000 \times g$ for 20 min to collect unbroken cells and nuclei. Bovine brain cytosolic proteins were isolated as previously described [3].

2.6. Protein identification by matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS)

The p36 band was excised from a stained SDS/PAGE gel and digested with trypsin (Promega). Following in gel trypsin digestion, extracted tryptic peptides were analyzed by matrix-assisted laser desorption ionization/time of flight (MALDI-TOF) mass spectrometry on a Voyager-DE Pro time of flight instrument (Applied Biosystems) in linear positive mode to generate a peptide mass map. The peptide mass values were used to search a non-redundant database (NCBInr) using the software tool MS-Fit. MALDI-post source decay (PSD) was performed on selected parent ions in reflectron mode for sequence determination. PSD spectra were acquired in 8–10 segments with mirror ratios ranging from 1.0 to 0.25, and stitched together using the Data Explorer software.

2.7. Cell culture and western blot analysis

HEK293 cells were transfected using the FuGENE 6 transfection kit (Roche Molecular Biochemicals) and lysed as described previously [3]. N-terminally FLAG epitope-tagged K269A-YpkA was immunoprecipitated using anti-FLAG M2 Sepharose resin (Sigma). Actin was affinity purified using DNase I-Sepharose affinity resin (Worthington). Affinity purified samples were separated by SDS/PAGE, transferred to nitrocellulose membranes and probed with the appropriate primary and secondary antibodies followed by chemiluminescence detection.

3. Results and discussion

3.1. Modification of YpkA for chemical genetics

Serine/threonine and tyrosine kinases contain a conserved hydrophobic amino acid in subdomain V termed the "gatekeeper residue" that is positioned adjacent to the N^6 position of the adenine ring in the ATP binding pocket [4]. Substitution of this residue with small amino acids enables the mutated kinase to accept chemically modified ATP analogs that contain a bulky moiety at the N^6 position [6]. These chemically modified ATP analogs cannot be utilized by wild type kinases due to the conservation of bulky side chain of the gatekeeper residue that sterically clash with the substituent added to the N^6 position in the ATP analogs. This methodology, termed chemical genetics, allows for the identification of substrates for a kinase of interest as one can use a mutant kinase with radiolabeled N^6-modified ATP in a crude extract with little background phosphorylation compared to similar experiments when a wild type kinase and radiolabeled ATP are used [4].

Alignment of subdomain V of YpkA with those of other kinases revealed that methionine 211 is likely to be the conserved gatekeeper residue in YpkA (Fig. 1A). In order to determine whether methionine 211 is the gatekeeper residue of YpkA, we mutated methionine 211 to either glycine (M211G) or alanine (M211A) and tested mutant YpkA kinase activity in the presence of actin and the artificial substrate myelin basic protein (MBP) using either radiolabeled ATP or radiolabeled N^6-modified ATPs. Mutation of methionine 211 to glycine in YpkA expands the ATP specificity of YpkA from ATP to N^6-modified ATP (Fig. 1B). Wild type YpkA utilizes the N^6-modified ATPs much less efficiently than it utilizes ATP as determined by poor MBP phosphorylation and a lack of autophosphorylation in the presence of N^6-modified ATPs (Fig. 1B). Mutation of methionine 211 to alanine in YpkA alters the ATP specificity to a lesser degree, presumably due to the presence of the side chain methyl group on alanine within the ATP binding pocket (Fig. 1B). These data demonstrate that methionine 211 is the conserved gatekeeper hydrophobic residue within subdomain V of YpkA.

3.2. Identification of a novel 36-kDa substrate for YpkA

YpkA M211G was added to a J774 macrophage extract with radiolabeled (γ-^{32}P) N^6-phenethyl ATP, as YpkA M211G showed slightly increased phosphorylation of MBP with this analog. Control experiments show that wild type YpkA phos-

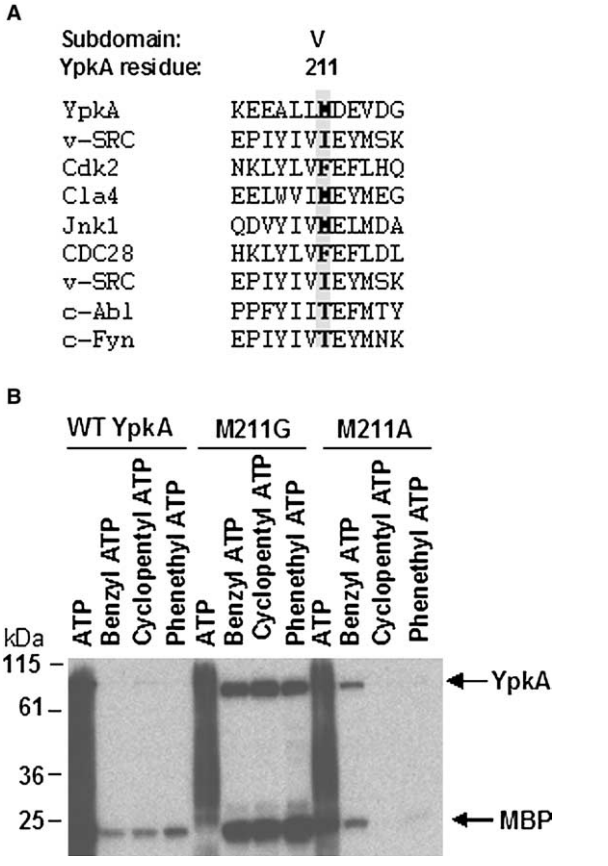

Fig. 1. Mutation of methionine 211 in YpkA alters its ATP specificity. (A) Alignment of subdomain V of YpkA with other serine/threonine and tyrosine kinases. (B) Recombinant wild-type, M211G, or M211A YpkA (1.5 μg) was incubated with actin (1 μg), MBP (1 μg), and either radiolabeled (γ-^{32}P) ATP or radiolabeled (γ-^{32}P) N^6 ATP analogs shown.

phorylates actin as expected (Fig. 2A, lane 1). Remarkably, little background phosphorylation was seen in extracts incubated with radiolabeled (γ-^{32}P) N^6-phenethyl ATP in the absence or presence of YpkA M211G (Fig. 2A), demonstrating the specificity of the chemical genetics approach. YpkA M211G specifically phosphorylated itself and actin as expected, as well as a 36-kDa protein (p36) present in the J774 extract (Fig. 2A). Interestingly, during the purification of actin as an activator of YpkA [3], we routinely observed a 36-kDa protein within bovine brain extracts that co-eluted with actin from a gel filtration column that was phosphorylated in the presence of wild type YpkA (data not shown). To further analyze the nature of p36 we fractionated bovine brain extract and followed its phosphorylation by YpkA using the chemical genetics approach. Fractions containing actin and p36 were incubated with M211G YpkA and radiolabeled (γ-^{32}P) N^6-phenethyl ATP. As shown in Fig. 2B, YpkA M211G phosphorylated a 36-kDa protein within fractionated bovine brain extracts. Thus, the chemical genetics approach confirmed a novel 36-kDa protein substrate for YpkA present in both J774 and bovine brain extracts.

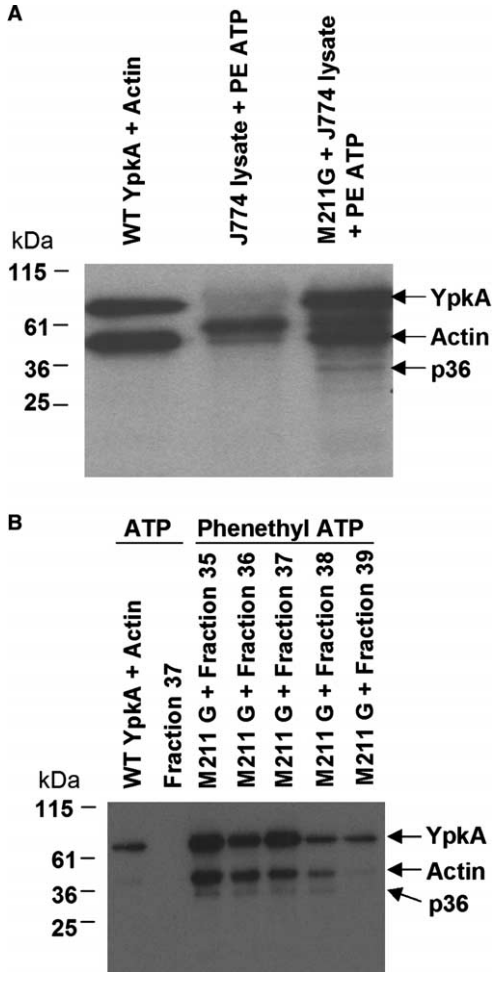

Fig. 2. YpkA phosphorylates a 36-kDa protein in macrophages and brain extract. (A) J774A macrophage lysates (5 μg protein) were incubated with radiolabeled N^6 phenethyl (PE) ATP and M211G YpkA. (B) Bovine brain cytosol fractions from a gel filtration column were incubated with radiolabeled N^6 phenethyl ATP and M211G YpkA. Fractions 35–39 were previously found to contain both actin and p36 (data not shown).

3.3. Identification of p36 as human open reading frame FLJ20113/Otubain 1

Bovine brain extract fractions containing p36 were subjected to SDS/PAGE and the band corresponding to p36 was excised and subjected to in-gel trypsin digestion. The pool of tryptic peptides was analyzed by MALDI-TOF mass spectrometry (Fig. 3). The mass fingerprint of p36 best matched a human open reading frame FLJ20113 (Fig. 3A). MALDI-PSD (PSD), which analyzes product ion fragments originating through metastable decomposition of the precursor ion, was performed on two p36 peptides, and confirmed the FLJ20113 identification (Fig. 3B and C).

As the analyzed sample was from bovine brain and the identified protein was a human ORF, the human FLJ20113 sequence was used to search the EST database for related bovine sequences. Sequence alignment searches revealed that FLJ20113 bears 99% identity to other predicted amino acid sequences from higher eukaryotes, including bovine. Importantly, the peptides identified by MALDI-TOF and PSD sequencing are identical between FLJ20113 and the bovine orthologue. FLJ20113 shares significant similarity to the OTU cysteine proteases [8]. Borodovsky et al. isolated FLJ20113 (termed HSPC263 in the study) as a novel deubiquitinating enzyme [9]. Recently, the protein encoded by FLJ20113 was named otubain 1 (OTU-domain ubiquitin aldehyde-binding protein) as a member of a family of cysteine proteases involved in ubiquitin signaling capable of cleaving tetra-ubiquitin at the iso-peptide bond [7].

3.4. Otubain 1 is a substrate for YpkA and interacts with the actin cytoskeleton

To verify that YpkA can phosphorylate otubain 1, different amounts of otubain 1 were incubated with YpkA and actin. YpkA is capable of phosphorylating otubain 1 in vitro only when actin is present to activate YpkA (Fig. 4A). To test the interaction between otubain 1 and YpkA, HEK293 cells were co-transfected with HA-tagged variants of wild type or mutant (D88A, C91S) otubain 1 and FLAG-epitope tagged YpkA K269A, a kinase dead mutant [3]. YpkA was immunoprecipitated using anti-FLAG M2 affinity resin and anti-HA immunoblot analysis confirmed that otubain 1 associates with YpkA (Fig. 4B).

To examine whether otubain 1 interacts with the actin cytoskeleton, HEK293 cells were transfected with HA-tagged variants of otubain 1. Actin was affinity purified using a DNase-I affinity resin, and co-precipitated otubain 1 was detected by immunoblot analysis using an HA antiserum. Otubain 1 was detected in the actin affinity purification, indicating that it interacts with either actin or an actin-associated protein (Fig. 4B). The strongest otubain/actin interaction observed was between the actin cytoskeleton and the C91S mutant of otubain 1. The C91S mutant may act as a substrate-trapping mutant as has been suggested for other mutated cysteine proteases including caspase-1 [10], suggesting that a substrate for otubain is either actin or an actin-associated protein.

We have identified otubain 1 as a novel substrate for the *Yersinia* kinase YpkA using chemical genetics coupled to mass spectrometry. YpkA phosphorylation of otubain 1

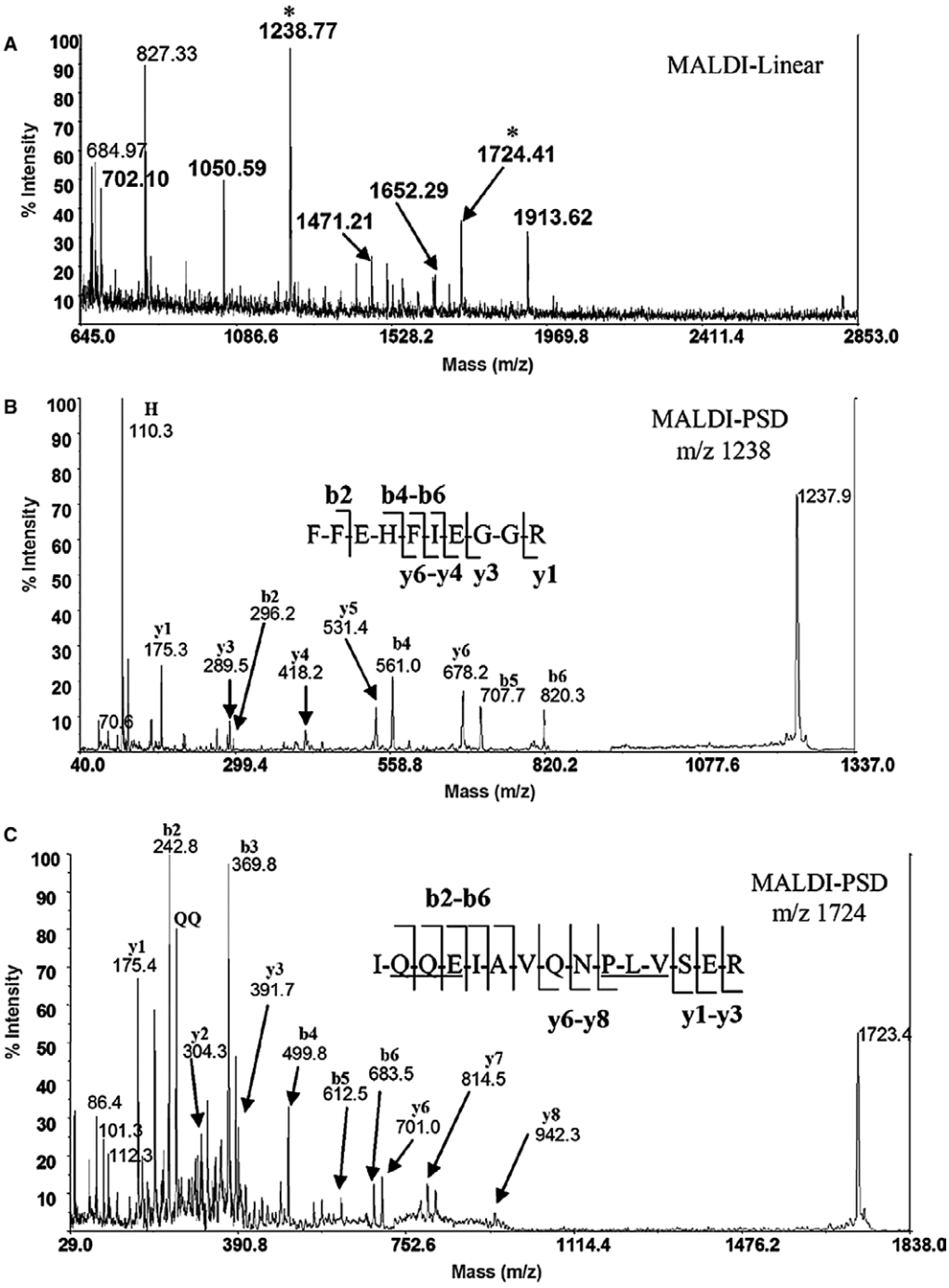

Fig. 3. Identification of p36 as FLJ20113 using MALDI-TOF and MALDI-PSD mass spectrometry. (A) MALDI-TOF mass fingerprint of the tryptic pool of p36 peptides. The two peptides analyzed by MALDI-PSD are marked with an asterisk. (B) MALDI-PSD spectrum for *m/z* 1238. (C) MALDI-PSD spectrum for *m/z* 1724. The observed C-terminal (y) and N-terminal (b) fragment ions are indicated on the spectrum and peptide sequence.

may regulate otubain in numerous ways including proteolytic activity, localization, or protein stability. Interestingly, otubain 1 is phosphorylated in HeLa cells by an unknown serine/threonine kinase [11], suggesting otubain 1 is regulated by phosphorylation in vivo. As otubain 1 has been shown to interact with the E3 ligase GRAIL, leading to GRAIL degradation and repression of T-cell anergy [12], it is tempting to speculate that otubain 1 phosphorylation by YpkA may disrupt the GRAIL-otubain 1 complex in macrophages, stabilize GRAIL, and induce macrophage anergy. This disruption of GRAIL signaling would suppress phagocytosis and increase *Yersinia* survival. Current efforts directed towards examining all possible consequences of otubain 1 phosphorylation by YpkA should aid in understanding the molecular details of how this mechanism is advantageous for *Yersinia* pathogenesis.

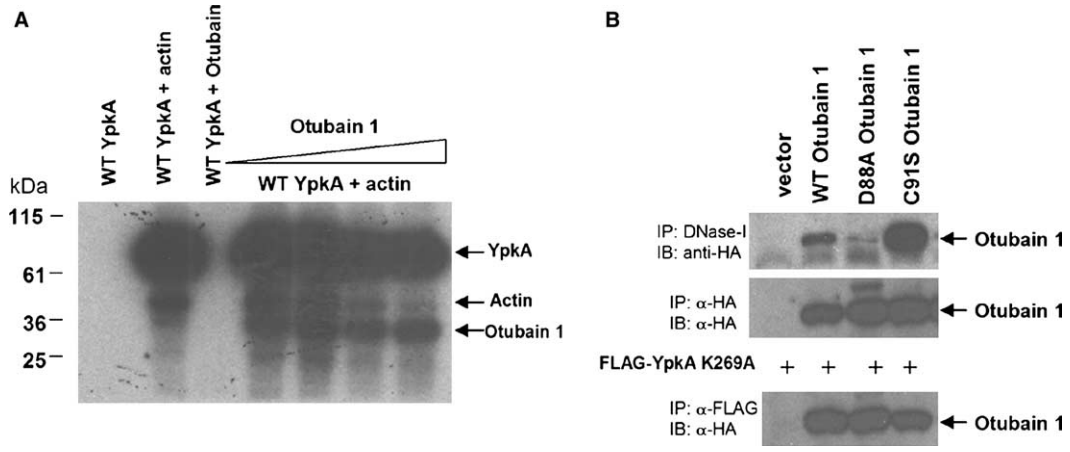

Fig. 4. Otubain 1 is a substrate for YpkA and interacts with YpkA and the actin cytoskeleton. (A) Recombinant otubain 1 was added in increasing concentrations (250 ng, 625 ng, 1.25 μg and 2.5 μg) with recombinant YpkA (1.5 μg) and actin (1 μg) in in vitro kinase assays as described [3]. (B) HEK293 cells were transfected with the otubain 1 constructs indicated alone (*top and middle panels*, 5 μg) or co-transfected with FLAG-epitope-tagged YpkA K269A (*bottom panel*, 2.5 μg each). YpkA was immunoprecipitated using anti-FLAG M2 affinity resin (*bottom panel*). Actin was affinity purified using a DNase-I sepharose affinity resin (*top panel*). Otubain 1 was immunoprecipitated using an anti-HA antibody followed by incubation with Protein A-agarose (*middle panel*). Immunoprecipitated otubain 1 was detected using an anti-HA antibody.

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