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Lysine^{11}-Linked Polyubiquitination of the AnkB F-Box Effector of *Legionella pneumophila*

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The fate of the polyubiquitinated protein is determined by the lysine linkages involved in the polymerization of the ubiquitin monomers, which has seven lysine residues (K^6, K^{11}, K^{27}, K^{29}, K^{33}, K^{48}, and K^{63}). The translocated AnkB effector of the intravacuolar pathogen *Legionella pneumophila* is a bona fide F-box protein, which is localized to the cytosolic side of the *Legionella*-containing vacuole (LCV) and is essential for intravacuolar proliferation within macrophages and amoebae. The F-box domain of AnkB interacts with the host SCF1 E3 ubiquitin ligase that triggers the decoration of the LCV with K^{48}-linked polyubiquitinated proteins that are targeted for proteasomal degradation. Here we report that AnkB becomes rapidly polyubiquitinated within the host cell, and this modification is independent of the F-box domain of AnkB, indicating host-mediated polyubiquitination. We show that the AnkB effector interacts specifically with the host E3 ubiquitin ligase Trim21. Mass spectrometry analyses have shown that AnkB is modified by K^{11}-linked polyubiquitination, which has no effect on its stability. This work shows the first example of K^{11}-linked polyubiquitination of a bacterial effector and its interaction with the host Trim21 ubiquitin ligase.

Legionella pneumophila, the causative agent of Legionnaires' disease, infects a wide array of protozoan hosts in the aquatic environment (1–4). Following inhalation of aerosols containing *L. pneumophila*, the bacteria replicate within alveolar macrophages, after remodeling their phagosome into a rough endoplasmic reticulum-derived vacuole termed the *Legionella*-containing vacuole (LCV) (5, 6). Mechanisms of LCV biogenesis are unknown, but they are dependent on the type IV Dot/Icm secretion system (7, 8). The ~300 translocated effector proteins injected by the Dot/Icm system into the host cell manipulate a myriad of host cellular processes, which enable intracellular proliferation in evolutionarily distant hosts (5, 6, 9). Bioinformatic analyses have revealed that many of the *L. pneumophila* translocated effector proteins share a resemblance to eukaryotic proteins, which is thought to have occurred through horizontal gene transfer and adaptation to the host (10–12).

Unlike most Dot/Icm-translocated effectors, the eukaryote-like F-box effector protein AnkB is required for intracellular proliferation in human macrophages and amoebae in the AA100/130b strain and the Paris strain but not in the strain Philadelphia-derived Lp02 strain (13–15). Following its translocation, AnkB is anchored to the cytosolic face of the LCV membrane through host-mediated farnesylation (16), where it triggers polyubiquitination of the LCV (14, 17). The F-box domain of AnkB interacts directly with the SCF1 (SKP1/CUL1/F-box) E3 ubiquitin ligase complex, which is acquired by the LCV (18). The interaction with the SCF1 complex results in AnkB-mediated decoration of the LCV with lysine^{48}-linked polyubiquitinated proteins, which are subjected to host-mediated proteasomal degradation (19). This results in a robust rise in the levels of free cellular amino acids above the threshold needed as the main sources of carbon and energy for intravacuolar replication of *L. pneumophila* (19–21).

Ubiquitin is a highly conserved, eukaryotic, 76-amino-acid protein that can be covalently linked to a lysine residue in a substrate protein, resulting in ubiquitination of the protein. The process of ubiquitination begins with an E1 ubiquitin activation enzyme (22), followed by an E2 ubiquitin-conjugating enzyme (23). This process is completed by an E3 ubiquitin ligase that transfers ubiquitin from E2 to a lysine residue of the substrate protein (24). The substrate protein can be monoubiquitinated, or ubiquitin can be polymerized through any of the 7 lysine residues (K^6, K^{11}, K^{27}, K^{29}, K^{33}, K^{48}, and K^{63}) of ubiquitin to form a polyubiquitin chain on the substrate protein (25, 26). The fate of the polyubiquitinated protein depends on the lysine residue within ubiquitin in the polyubiquitin chain (25, 26). The most studied polyubiquitin chains are the K^{48}-linked chains, which result in proteasomal degradation of the substrate protein, while K^{63}-linked polyubiquitin chains are involved in nondegradation processes such as alteration of protein function or subcellular location (27, 28). The fate of polyubiquitination through K^6, K^{11}, K^{27}, K^{29}, and K^{33} linkages is not well defined. However, it has been shown that some of these modes of polyubiquitination may lead to proteasomal degradation (29), cell cycle regulation (30), DNA repair, cellular signaling, and regulation (31, 32). The tumor suppressor Beclin 1 undergoes proteasomal degradation through K^{11}-linked polyubiquitination (33). The majority of the knowledge on K^{11}-linked polyubiquitination has come from the anaphase-promoting complex (APC/C) E3 ubiquitin ligase (34, 35). APC/C promotes proteasomal degradation of substrates through K^{11}-linked polyubiquitin chains, which play a major role in cell cycle regulation (36, 37). Interestingly, major histocompatibility complex class I (MHC-I) is polyubiquitinated through a mixture of K^{11}- and K^{63}-linked polyubiquitin chains to undergo receptor internalization (38).

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It is becoming increasingly apparent that bacterial pathogens manipulate eukaryotic cellular ubiquitination machinery for various purposes, and many translocated bacterial effectors become polyubiquitinated by the host cell machinery (19, 39). The *Salmonella enterica* SopA effector is ubiquitinated by HsRMA1, which results in its proteasomal degradation (40, 41), while the SopB effector is ubiquitinated by TRAF6 through K^{63}-linked polyubiquitination, which alters subcellular localization (42, 43). The SopE and SptP effectors of *Salmonella* are ubiquitinated following translocation and are degraded by the proteasome (44). Ubiquitination of the *L. pneumophila* effector SidH leads to its degradation (45, 46). Interestingly, the *Pseudomonas aeruginosa* A2 phospholipase ExoU has been shown to be modified by a diubiquitin chain, which could result in altered trafficking (47). In addition to diubiquitination, the enzymatic functions of ExoU have been shown to be activated by interactions with ubiquitin following translocation, indicating another role for ubiquitin modification of bacterial proteins (48). These few examples highlight various established polyubiquitinations of bacterial effectors. To date, there has been no example of any bacterial effector protein polyubiquitination through K^6, K^{11}, K^{27}, K^{29}, or K^{33} linkages.

The Trim21 E3 ubiquitin ligase plays a major role in the regulation of the innate immune response by ubiquitinating interferon regulatory factor 3 (IRF3), IRF5, IRF7, and IRF8 (49–53). Trim21 interacts with the F-box protein Skp2 as well as the Cul1 component of the SCF1 E3 ubiquitin ligase complex to promote ubiquitination of p27 (54). While most Trim21 substrates are degraded by the proteasome, certain substrates have been shown to be monoubiquitinated (55). Polyubiquitination of IRF8 with unknown lysine linkages by Trim21 does not lead to its degradation (56). Trim21 also promotes signaling of immune pathways through K^{63}-linked polyubiquitinated chains (57). In addition, Trim21 has been shown to catalyze K^{48}-linked as well as K^{63}-linked polyubiquitination. However, Trim21 has not been shown to synthesize K^{11}-linked polyubiquitinated chains.

Here we report polyubiquitination of the *L. pneumophila* translocated F-box effector AnkB through K^{11}-linked polyubiquitination, which does not affect its half-life. We show that the E3 ubiquitin ligase Trim21 is a specific binding partner for AnkB.

MATERIALS AND METHODS

Cell cultures and bacterial strains. *Escherichia coli* strain DH5α was used for cloning and plasmid preparation purposes. HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (BioWest) and 200 mM L-glutamine (Corning) at 37°C in a 5% CO_2 atmosphere.

Transfection of HEK293T cells. The *ankB* gene was cloned into the mammalian expression vector p3XFlag-CMV-10. Generation of 3×Flag-tagged AnkB substitution mutant $AnkB^9L^{10}P$, 3×Flag-tagged AnkBΔF-box, and 3×Flag-tagged AnkH was described previously (17, 58). Hemagglutinin (HA)-tagged Trim21 was a kind gift from Yong-Jun Liu at the University of Baylor. HEK293T cells were grown to ~70% confluence and plated onto poly-L-lysine-treated 6-well plates. Following 24 h of incubation, HEK293T cell monolayers were transfected with ~2 μg plasmid DNA/well by using polyethylenimine (Polysciences) for 24 h.

***In vivo* coimmunoprecipitation.** HEK293T cells were transfected with 3×Flag-tagged AnkB, $AnkB^9L^{10}P$, AnkBΔF-box, AnkH, and HA-tagged Trim21 for 24 h and collected in lysis buffer containing 50 mM Tris (pH 7.4), 0.25 M NaCl, 0.5% Triton X-100, 1 mM EDTA, 50 mM NaF, 0.1 mM Na_3VO_4, and EDTA-free protease inhibitor cocktail (Roche). Flag-tagged and HA-tagged proteins were immunoprecipitated by using anti-Flag M2 agarose (Sigma) or anti-HA affinity gel (Sigma) according to the manufacturer's instructions.

Antibodies and Western blot analysis. Immunoprecipitated proteins were heated at 99°C for 5 min in sample buffer, separated by 10.4 to 15% SDS-PAGE, and transferred onto a polyvinylidene difluoride (PVDF) membrane. Anti-Flag (Sigma) used at a 1:1,000 dilution, antiactin (Proteintech) used at 1:15,000, antiubiquitin (Cell Signaling) used at 1:1,000, anti-lysine 48 ubiquitin (Cell Signaling) used at 1:1,000, and anti-lysine 63 ubiquitin (Cell Signaling) used at 1:1,000 were incubated overnight in 5% milk at 4°C. Anti-HA (Cell Signaling) was used at 1:1,000 and incubated in 5% bovine serum albumin (BSA) overnight at 4°C.

Cycloheximide inhibition. HEK293T cells plated in 6-well plates were transfected with 3×Flag-AnkB for 24 h. The proteasomal inhibitor MG132 (Selleckchem) used at 20 μM was added to the indicated cells 2 h prior to cycloheximide treatment at 100 μg/ml. At the indicated time points, cells were lysed and subjected to SDS-PAGE and immunoblotting with anti-Flag and antiactin antibodies.

Nano-LC-MS2 data collection. In collaboration with Steve Gygi, Ryan Kunz, and Ross Tomaino at the Harvard University Taplin Mass Spectrometry Facility, mass spectrometry (MS) data were collected on an Orbitrap Fusion mass spectrometer equipped with an Easy Nano-LC 1000 instrument for sample handling and liquid chromatography (LC). Peptides were separated on a 75-μm by 30-cm hand-pulled fused silica microcapillary column with a needle tip diameter of <10 μm and packed with 1.8-μm 120-Å GP-C_{18} beads (Sepax Technologies Inc.). The column was equilibrated with buffer A (3% ACN plus 0.125% FA). Peptides were loaded onto the column with 100% buffer A. Separation and elution from the column were achieved by using a 90-min 3 to 25% gradient of buffer B (100% ACN plus 0.125% FA). Survey scans of peptide precursors from *m/z* 400 to 1,400 were performed at 120K resolution (at *m/z* 200), with AGC of 50k, a maximum injection time of 100 ms, monoisotopic precursor selection turned on, charge state of 2 to 6, and dynamic exclusion for 45 s with a 10-ppm tolerance. Tandem MS (MS/MS) was performed in top-speed mode (2-s cycles) starting with the most intense precursor having an intensity of >5k. Parent ions were isolated in the quadrupole (*m/z* 0.7 isolation window). Collision-induced dissociation was performed in the ion trap with a rapid scan rate, 35% collision energy, AGC of 10k, maximum injection time of 35 ms, and the parallelizable time turned on.

Mass spectrometry data analysis. A suite of in-house software tools was used for .RAW file processing, controlling peptide and protein false discovery rates, and assembling peptide-level data into protein-level data (59). MS/MS spectra were searched by using the SEQUEST algorithm (60) against a composite protein database consisting of all protein sequences from the UniProt human database along with common contaminating proteins in both the forward and reverse directions. SEQUEST parameters used to search the MS data were a precursor tolerance of 50 ppm, a fragment ion tolerance of 1 Da, fully tryptic cleavage, 2 missed cleavages, variable modifications of oxidized methionine (15.9949 Da), alkylation of cysteine (57.0214 Da), and a diglycine motif on lysine (114.0429 Da). A target-decoy strategy was used to determine false discovery rates (61). The peptide-level false discovery rate was restricted to <1% by using linear discriminate analysis based on several different SEQUEST parameters, including an Xcorr of ≥1.0, deltaXcorr, charge state, and a minimum peptide length of 7 amino acids (59). An algorithm similar to Ascore was used for diglycine localization and site quantification (62).

RESULTS

Ubiquitination of the *L. pneumophila* translocated effector AnkB. To determine whether the AnkB effector of *L. pneumophila* strain AA100/130b undergoes any posttranslational modifications within eukaryotic cells, we immunoprecipitated ectopically expressed Flag-tagged AnkB and analyzed it by Western blotting with anti-Flag antibodies. In addition to the native AnkB band at ~23 kDa, there were 2 distinct bands at ~37 kDa and ~75 kDa as well as a smear of high-molecular-mass proteins recognized by

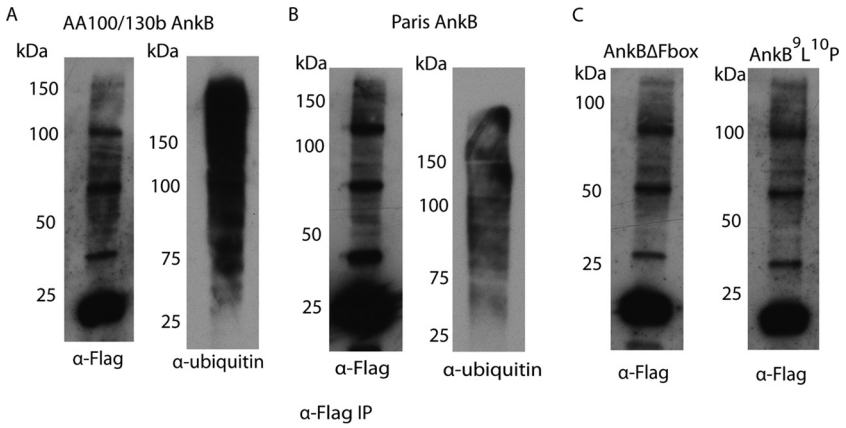


FIG 1 Ubiquitination of ectopically expressed AnkB. (A) HEK293T cells were transfected with Flag-tagged AnkB from *L. pneumophila* strain AA100/130b. Cell lysates were immunopurified with anti-Flag agarose and analyzed by immunoblotting with anti-Flag and antiubiquitin antibodies. (B) HEK293T cells were transfected with Flag-tagged AnkB from *L. pneumophila* strain Paris. Cell lysates were purified with anti-Flag agarose and analyzed by immunoblotting with anti-Flag and antiubiquitin antibodies. (C) HEK293T cells were transfected with AnkB variants, Flag-tagged AnkB^{9}L^{10}P or Flag-tagged AnkBΔF-box. Cell lysates were purified with anti-Flag agarose and analyzed by immunoblotting with anti-Flag antibodies.

anti-Flag antibodies (Fig. 1A), which suggested that the distinct bands at ~37 kDa and ~75 kDa may correspond to the potential addition of 2 and 6 ubiquitin monomers to AnkB (Fig. 1A). Reprobing this blot with antiubiquitin antibodies showed that the higher-molecular-mass species were AnkB modified by ubiquitin (Fig. 1A).

To ensure that ubiquitination of AnkB was not limited to *L. pneumophila* strain AA100/130b only, we utilized AnkB of the *L. pneumophila* Paris strain (14), which lacks the C-terminal CaaX motif (16). We performed immunoprecipitation (IP) of Flag-tagged AnkB of strain Paris and immunoblotted the immunoprecipitate with anti-Flag antibodies. Similar to AA100/130b-derived AnkB, the Paris strain AnkB protein showed distinct bands and a high-molecular-mass smear that were determined to be ubiquitinated AnkB (Fig. 1B). We tried to determine whether AnkB is also ubiquitinated during infection. The two major limitations of this approach are that AnkB is poorly expressed during infection and is present only on the LCV membrane, and only ~20% of the cells become infected. We utilized up to 5×10^8 infected cells, but the amount of AnkB detected from the whole sample was very minimal after IP.

AnkB is a bona fide F-box protein (14, 17), and its F-box domain interacts directly with the SKP1 component of the host SCF1 E3 ubiquitin ligase complex on the LCV (14, 17). To determine if the F-box domain of AnkB was required for its ubiquitination, we transfected HEK293T cells with an AnkB variant lacking the F-box domain (AnkBΔF-box) and immunoprecipitated the protein. Western blot analysis showed that deletion of the F-box domain of AnkB did not have an impact on its ubiquitination, as evident from the banding pattern of higher-molecular-mass species of AnkB (Fig. 1C). To ensure that deletion of the F-box domain did not have an impact on the structure of AnkB, we utilized an AnkB substitution mutant (AnkB^{9}L^{10}P/AA) within the F-box domain that does not bind to SKP1. Following immunoprecipitation of AnkB^{9}L^{10}P/AA, the Western blot showed that AnkB^{9}L^{10}P/AA was ubiquitinated similarly to native AnkB, as evident from the banding pattern of higher-molecular-mass species of AnkB (Fig. 1C). Taken together, these results indicate that AnkB is ubiquitinated and that its ubiquitination is independent of the interaction of its F-box domain with the host SCF1 E3 ubiquitin ligase complex.

AnkB interacts with the E3 ubiquitin ligase Trim21. The ubiquitination of the AnkB variant lacking the F-box domain as well as the AnkB^{9}L^{10}P/AA substitution variant allowed us to exclude a role for the SCF1 E3 ubiquitin ligase in AnkB ubiquitination. Therefore, we sought to determine the E3 ubiquitin ligase that interacts with AnkB. We immunoprecipitated Flag-tagged AnkB from HEK293T cells and performed MS analysis on the proteins that coimmunoprecipitated with AnkB. Our mass spectrometry data showed that the RING finger protein Ro52 (Trim21) copurified with AnkB at a 1:2 ratio. To confirm that AnkB directly interacts with Trim21, we cotransfected HEK293T cells with Flag-tagged AnkB and HA-tagged Trim21. Following cell lysis, Flag-tagged AnkB was coimmunoprecipitated with anti-Flag antibody, and the immunoprecipitate was immunoblotted with anti-HA tag antibody. The data showed a band corresponding to the molecular mass of Trim21 that coimmunoprecipitated with AnkB (Fig. 2A). When the coimmunoprecipitation was reversed through immunoprecipitation with anti-HA antibody and the immunoprecipitate was immunoblotted with anti-Flag antibodies, a band with a molecular mass corresponding to AnkB was detected (Fig. 2B). To determine if the Trim21-AnkB interaction was specific for AnkB, we utilized the *L. pneumophila* translocated effector protein AnkH (58) as a control in the coimmunoprecipi-

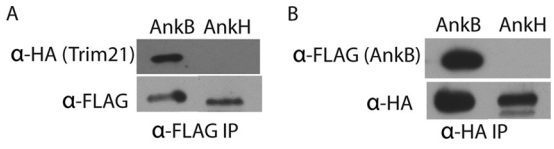


FIG 2 AnkB directly interacts with the E3 ubiquitin ligase Trim21. (A) Flag-tagged AnkB or Flag-tagged AnkH was cotransfected with HA-tagged Trim21 in HEK293T cells. Flag-tagged AnkB or Flag-tagged AnkH was coimmunoprecipitated with anti-Flag agarose and subsequently immunoblotted with anti-HA antibodies and anti-Flag antibodies. (B) HA-tagged Trim21 was coimmunoprecipitated by using anti-HA agarose and subsequently immunoblotted with anti-Flag antibodies and anti-HA antibodies.

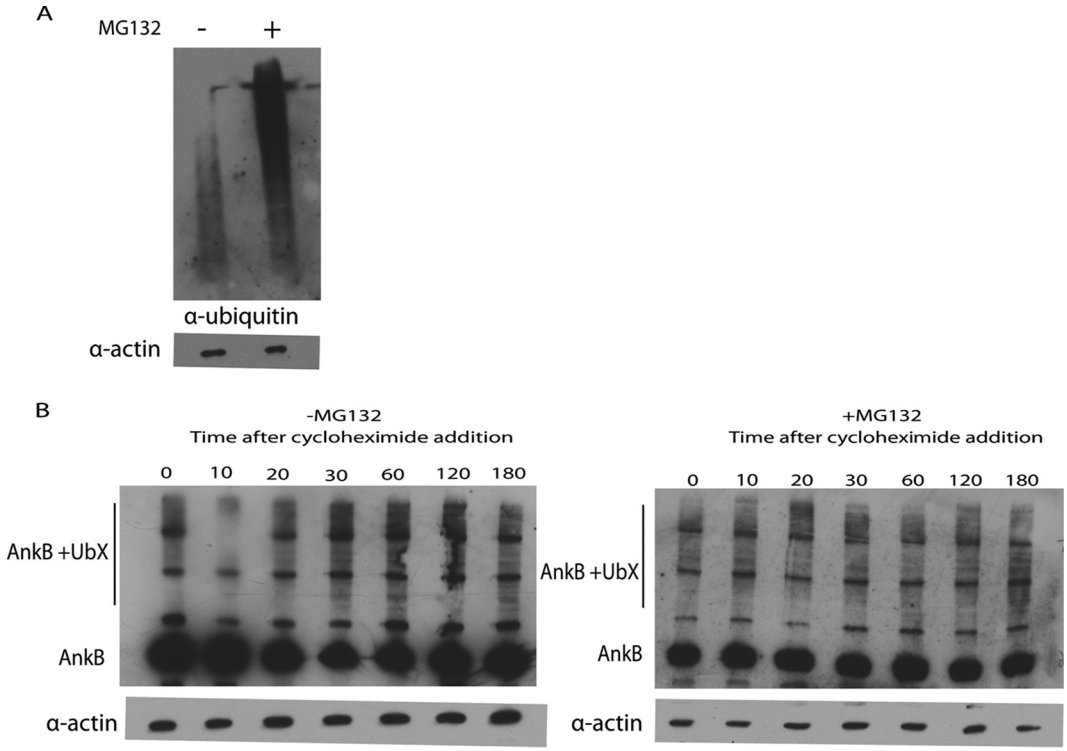


FIG 3 Ubiquitinated AnkB is not degraded by the proteasome. (A) HEK293T cells were untreated or treated with MG132 for 3 h. Cells were lysed, and the resulting immunoblot was probed with antiubiquitin and antiactin antibodies. (B) HEK293T cells transfected with Flag-tagged AnkB were treated with the protein synthesis inhibitor cycloheximide. To inhibit proteasomal degradation, a subset of cells was pretreated for 2 h with MG132 prior to cycloheximide treatment. At the indicated time points, cells were lysed, and equivalent amounts of protein were subjected to immunoblotting with anti-Flag and antiactin antibodies.

tation. Flag-tagged AnkH was coimmunoprecipitated with anti-Flag antibodies, and the resulting immunoprecipitate was immunoblotted with anti-HA antibodies. Unlike AnkB, Trim21 did not coimmunoprecipitate with AnkH (Fig. 2A). This was confirmed by reversing the coimmunoprecipitation and Western blotting, which also showed a lack of an interaction of Trim21 with the AnkH control (Fig. 2B). Taken together, these results indicate that AnkB specifically interacts with the E3 ubiquitin ligase Trim21. This is the first example of an interaction of a bacterial effector with the host Trim21 ubiquitin ligase.

Ubiquitinated AnkB is not degraded by the proteasome. When proteins are polyubiquitinated through K^{48}-linked polymerization of ubiquitin, they are degraded by the proteasome (25). In general, ectopically overexpressed proteins in eukaryotic cells are modified by K^{48}-linked polyubiquitination to degrade the overexpressed protein. To determine whether ubiquitination of AnkB led to its proteasomal degradation, we utilized the proteasomal inhibitor MG132 and determined the stability of AnkB in the presence or absence of the protein synthesis inhibitor cycloheximide. To ensure that the MG132 proteasome inhibitor blocked proteasomal degradation, treated or untreated cells were lysed, and a Western blot of the total cell lysate was probed with antiubiquitin and reprobed with antiactin antibodies (Fig. 3A). As expected, cells treated with MG132 had a large increase in the amount of ubiquitinated proteins compared to untreated cells (Fig. 3A). In contrast, our data showed that upon proteasomal inhibition, ubiquitinated AnkB was stable over the 3-h experiment (Fig. 3B). Upon proteasomal inhibition by MG132, the amounts of unmodified AnkB decreased slightly over time, as some of it was modified and shifted to a higher molecular mass. At 20 to 30 min, the ubiquitination of AnkB became more prominent, as the higher-molecular-mass modified species of AnkB bands exhibited higher intensity than that at 10 min. Therefore, ubiquitination of AnkB does not result in its proteasomal degradation (Fig. 3B).

Polyubiquitin linkages of polyubiquitinated AnkB. Ubiquitin contains 7 lysine residues, all of which can be utilized to polymerize ubiquitin on the substrate protein (25). K^{48}-linked polyubiquitination and K^{63}-linked polyubiquitination are the most common and most studied modes of polyubiquitination. We sought to determine the chain linkage of the ubiquitin polymers of ubiquitinated AnkB by mass spectrometry. Depending on the lysine residue within ubiquitin that contains the ubiquitin-ubiquitin linkage, a characteristic mass shift increase of 114 Da (diglycine) is observed by mass spectrometry (63). Flag-tagged AnkB was ectopically expressed in HEK293T cells and immunoprecipitated by using anti-Flag antibody. Immunoprecipitated proteins were resolved by SDS-PAGE and visualized by Coomassie blue staining (Fig. 4A). Of the several bands corresponding to AnkB with a different number of ubiquitin moieties, the most prominent band at ~75 kDa (AnkB plus 6 ubiquitin moieties) was analyzed by mass spectrometry to determine the modified lysine residues within AnkB and ubiquitin. The resulting spectra showed ubiquitination of AnkB on K^{67} in the peptide (residues 63 to 75) (Fig. 4B). More than 90% of the b and y ions with +1, +2, and +3 charges were detected, which provided clear evidence for the ubiq-

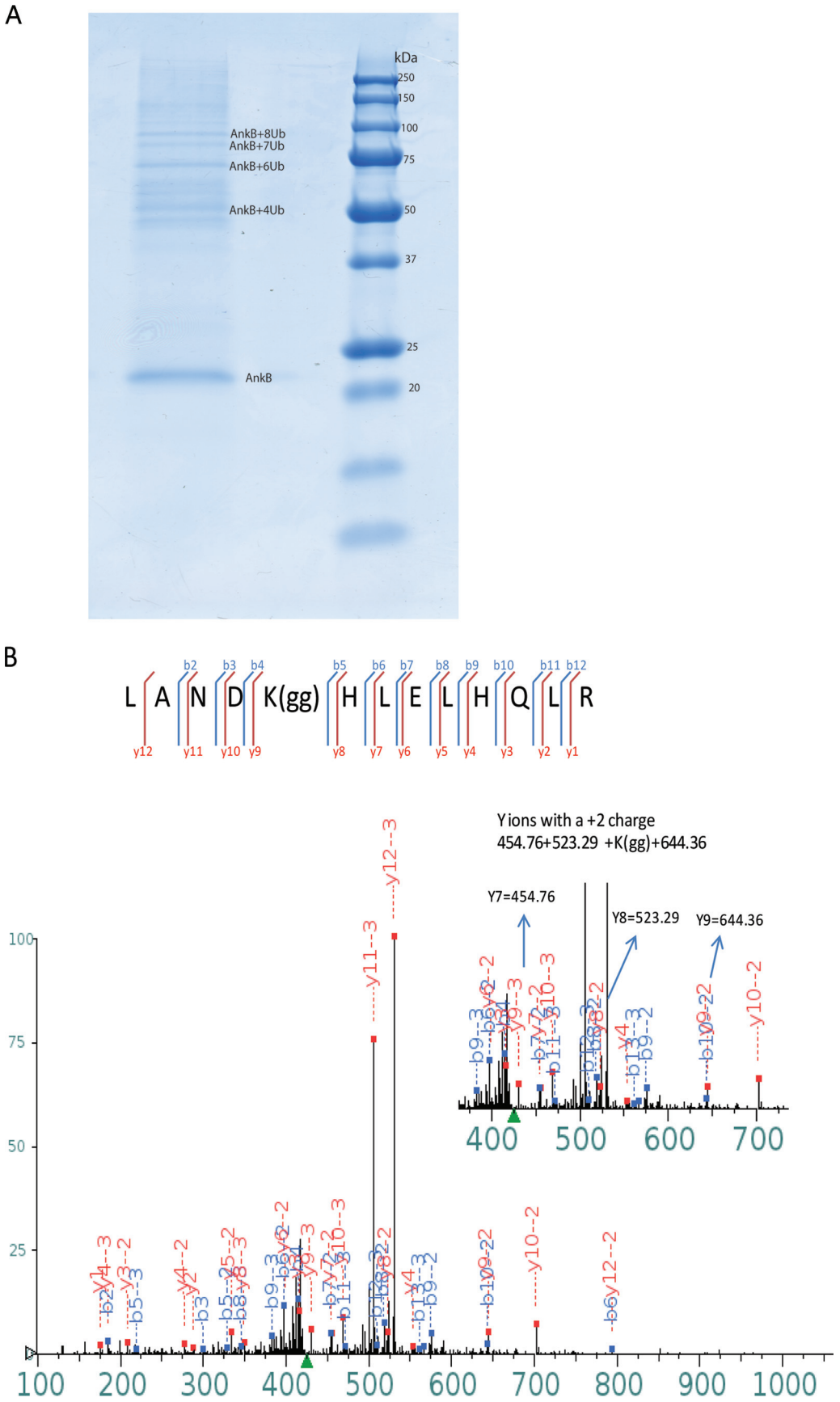
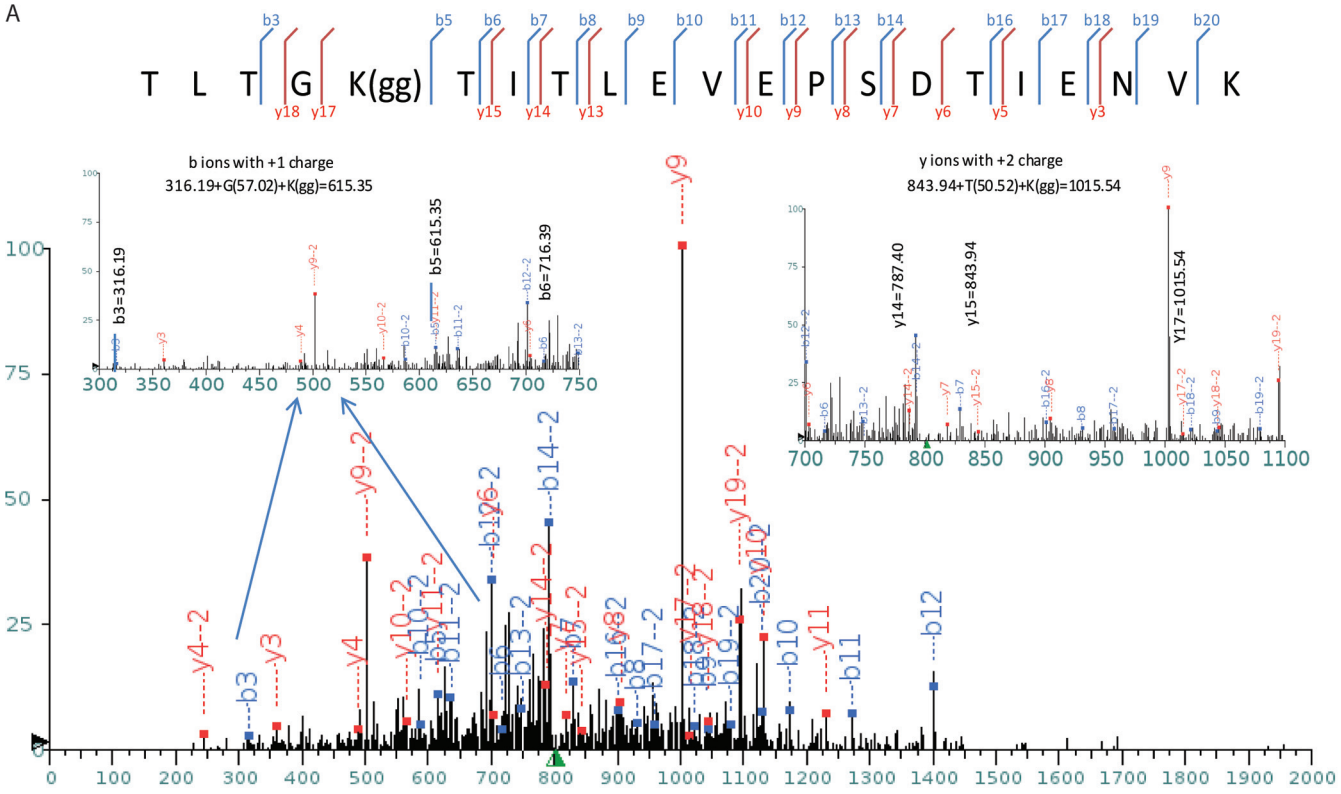


FIG 4 AnkB is ubiquitinated on lysine 67. (A) Flag-tagged AnkB was immunoprecipitated from transfected HEK293T cells and analyzed by SDS-PAGE and Coomassie blue staining. (B) The band at ~74 kDa (AnkB plus 6 ubiquitin [Ub] moieties) was analyzed by mass spectrometry, and the resulting spectra within the ubiquitinated AnkB peptide (residues 63 to 75) are shown with the b ions (blue lines) and y ions (red lines). The y ions with a +2 charge are shown with the mass/charge ratio calculated to show ubiquitination on lysine 67 of AnkB.

A



B

Sequence

TLTGK*TITLEVEPSDTIENVK

Predicted Fragmentation Pattern

+1

Seq	#	b: Δ Error	b	y	y: Δ Error	+1
T	1	---	102.055	---	---	21
L	2	---	215.139	2301.218	---	20
T	3	5.821	**316.187**	2188.134	---	19
G	4	---	373.208	2087.087	---	18
K*	5	85.122	**615.346**	2030.065	---	17
T	6	256.643	**716.394**	1787.927	---	16
I	7	-196.005	**829.478**	1686.880	---	15
T	8	806.436	**930.525**	1573.796	---	14
L	9	-432.663	**1043.610**	1472.748	---	13
E	10	162.235	**1172.652**	1359.664	---	12
V	11	211.158	**1271.721**	**1230.621**	10.310	11
E	12	115.312	**1400.763**	**1131.553**	66.598	10
P	13	---	1497.816	**1002.510**	98.048	9
S	14	---	1584.848	**905.457**	-1026.256	8
D	15	---	1699.875	**818.425**	206.895	7
T	16	---	1800.923	**703.398**	167.206	6
I	17	---	1914.007	602.351	---	5
E	18	---	2043.049	**489.267**	-1862.492	4
N	19	---	2157.092	**360.224**	916.759	3
V	20	---	2256.161	246.181	---	2
K	21	---	---	147.113	---	1

Sequence

TLTGK*TITLEVEPSDTIENVK

Predicted Fragmentation Pattern

+2

Seq	#	b: Δ Error	b	y	y: Δ Error	+1
T	1	---	51.531	---	---	21
L	2	---	108.073	1151.113	---	20
T	3	---	158.597	**1094.571**	153.093	19
G	4	---	187.108	**1044.047**	752.896	18
K*	5	---	308.177	**1015.536**	-681.273	17
T	6	---	358.701	894.467	---	16
I	7	---	415.243	**843.943**	707.057	15
T	8	---	465.766	**787.401**	-634.924	14
L	9	---	522.308	736.878	---	13
E	10	330.235	**586.830**	680.336	---	12
V	11	-939.702	**636.364**	**615.814**	-675.680	11
E	12	342.185	**700.885**	**566.280**	182.311	10
P	13	-1205.642	**749.412**	**501.759**	672.194	9
S	14	-463.667	**792.928**	453.232	---	8
D	15	---	850.441	409.716	---	7
T	16	365.404	**900.965**	352.203	---	6
I	17	429.971	**957.507**	301.679	---	5
E	18	-372.176	**1022.028**	**245.137**	-3746.872	4
N	19	-149.147	**1079.050**	180.616	---	3
V	20	825.741	**1128.584**	123.594	---	2
K	21	---	---	74.060	---	1

FIG 5 AnkB is polyubiquitinated through lysine[11] of ubiquitin. (A) The ~74-kDa ubiquitinated AnkB band was analyzed by mass spectrometry for ubiquitination of ubiquitin. The resulting spectrum of the modified ubiquitin peptide (residues 7 to 27) is shown, along with the b ions (blue lines) and y ions (red lines) detected. The b ions with a +1 charge and y ions with a +2 charge are shown with the mass/charge ratio calculated to show ubiquitination on lysine[11] of ubiquitin. (B) Predicted fragmentation pattern and mass-to-charge ratios of the +1 and +2 charges of the ions within the spectra.

uitination of AnkB on K^{67} (see Fig. S1 in the supplemental material). Within the same ~75-kDa band, ubiquitin was ubiquitinated on K^{11} in the peptide (residues 7 to 27) (Fig. 5A). The b ions with a +1 charge and the y ions with a +2 charge surrounding the modified lysine residue of ubiquitin are shown, and the calculation provided direct evidence for ubiquitination of ubiquitin on K^{11} (Fig. 5A and B). Although phosphorylation of proteins has been shown to be a signal for protein ubiquitination (64), we did not detect phosphorylated AnkB in the MS analysis.

To determine the role of host cell ubiquitination of AnkB in its function, we generated a substitution mutant of the ubiquitinated K residue in AnkB (AnkB-K^{67}R) and determined whether this abolished the ubiquitination of AnkB. However, following immunoprecipitation from HEK293T cells, the results showed that AnkB ubiquitination was identical to that of native AnkB (Fig. 1). This is not surprising, since in many eukaryotic proteins and the SopB effector of *Salmonella*, ubiquitination can shift from one K residue to another after substitution of the modified residue within a protein (42, 65). Taken together, AnkB is ubiquitinated on K^{67} through K^{11}-linked polyubiquitination, but the lysine residue involved does not have to be the K^{67} residue.

DISCUSSION

Pathogenic bacteria have evolved elaborate mechanisms to counteract and manipulate eukaryotic ubiquitination machinery to subvert the immune response, acquire nutrients through proteasomal degradation, and alter the subcellular localization of effector proteins (39). In this report, we highlight novel K^{11}-linked polyubiquitination of the *L. pneumophila* F-box effector protein AnkB. Our data obtained by IP as well as by MS analyses of the ubiquitinated species show that they are all AnkB species modified by ubiquitin (Fig. 4A), and no other proteins were detected. This is the first example of a bacterial effector undergoing K^{11}-linked polyubiquitination. In all but one of the reported examples of K^{11}-linked polyubiquitination, the substrate protein is degraded by the proteasome (36, 38, 66). Our data showed that K^{11}-linked polyubiquitinated AnkB is not degraded by the proteasome. This suggests that K^{11}-linked polyubiquitination of AnkB could play a unique role in the fate or function of AnkB in addition to the established role of K^{11}-linked polyubiquitination as a proteasomal degradation signal.

Previous reports have shown that F-box proteins undergo autoubiquitination, acting as regulatory mechanisms in most cases (67, 68). However, ubiquitination of AnkB is independent of its F-box domain that interacts with the SCF1 ubiquitin ligase. This raises the question of whether other F-box proteins are ubiquitinated in a manner that does not lead to proteasomal degradation. The ubiquitination of F-box proteins has probably been overlooked due to the fact that F-box proteins cause ubiquitination of their substrates. Perhaps, the ubiquitination of F-box proteins may impact their interaction with specific substrates. The ubiquitination of F-box proteins could alter their subcellular localization, which could allow their interaction with specific substrates localized to vesicles or the plasma membrane. When the ubiquitinated lysine residue in AnkB was replaced with arginine, ubiquitination of AnkB was not affected. This switching of the modified lysine residue upon its substitution is a common process that has been repeatedly observed in other F-box proteins and has been also shown for SopB of *Salmonella* (42, 65) Since there are a total of 19 lysine residues within AnkB, we chose not to replace all of them, since this would most likely impact the structure and function of the protein independent of the effect of the lack of ubiquitination.

Trim21 has been shown to play a major role in the proteasomal degradation of opsonized intracellular viral particles and is required to prevent certain fatal viral infections (69, 70). Recently, it was shown that Trim21 is recruited to opsonized *Salmonella enterica*, which stimulates NF-κB activation, but whether it interacts with a bacterial effector or ubiquitinate proteins on the *Salmonella*-containing vacuole is not known (57). While we propose the recognition of a bacterial effector protein for ubiquitination by Trim21, this could imply an additional mechanism similar to the ubiquitination of viral particles. Due to effector proteins being exposed to eukaryotic cytosolic enzymes during bacterial infection, Trim21 would have access to bind and ubiquitinate them. The Trim21-AnkB interaction is the first example of a Trim21 interaction with a bacterial effector. However, whether the Trim21-AnkB interaction is exhibited on the LCV membrane and whether Trim21 is the only enzyme capable of modifying AnkB are still to be determined.

While translocated bacterial proteins have been shown to be ubiquitinated, primarily for proteasomal degradation, K^{11}-linked polyubiquitination of AnkB does not affect its stability. To date, K^{11}-linked polyubiquitination has been observed only on eukaryotic proteins and has been primarily implicated in proteasomal degradation associated with cell cycle regulation. However, RIPA is modified through K^{11}-linked chains in a manner thought to be involved in tumor necrosis factor (TNF) signaling (66). With the few proteins experimentally shown to be modified through K^{11}-linked polyubiquitination, the possible outcomes of this emerging mode of polyubiquitination remain to be defined. It is possible that K^{11}-linked polyubiquitination of AnkB alters its subcellular localization or affects its interaction with substrates that bind the region of AnkB where ubiquitination is exhibited. Future studies will decipher the biological significance of this unique mode of polyubiquitination of the F-box protein.

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