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Proteasome activity in a naïve mosquito cell line infected with *Wolbachia pipientis* wAlbB

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Abstract

We used *Wolbachia pipientis* strain wAlbB from *Aedes albopictus* Aa23 cells to infect clonal *Ae. albopictus* TK-6 cells, which are resistant to 5-bromodeoxyuridine. Infected TK-6 cells were cultured in medium containing 5-bromodeoxyuridine to select against Aa23 cells that might have persisted in the inoculum. Infected TK-6 lines retained the *Wolbachia* infection for 5 mo, indicating that their metabolic processes support *Wolbachia* growth and multiplication. To investigate early events after *Wolbachia* infection, we labeled infected cells with ^{35}S [methionine/cysteine]. Patterns of labeled proteins on sodium dodecyl sulfate gels were similar in control and infected cells, with the exception of a 29-kDa protein. Tandem mass spectrometry revealed that the 29-kDa band included α and β subunits of the 26S proteasome. Independent confirmation of the up-regulation of the proteasome was established by probing Western blots with a monoclonal antibody to the proteasome-associated co-factor, ubiquitin. *Wolbachia*'s loss of metabolic pathways for the synthesis of most amino acids and retention of pathways for their uptake and metabolism suggest that proteasome activation provides a mechanism whereby controlled degradation of intracellular host proteins would increase availability of amino acids to support establishment and maintenance of the *Wolbachia* infection.

Keywords

Wolbachia; *Aedes albopictus*; Mosquito cell line; Proteasome; Ubiquitin; Mass spectrometry

Introduction

Genome analysis reveals that *Wolbachia* and other members of the Rickettsiales differ from free-living bacteria in their loss of most of the pathways for the biosynthesis of amino acids while retaining pathways for the uptake of amino acids as organic nutrients. Moreover, *Wolbachia*'s ability to metabolize amino acids suggests that this obligate intracellular microbe uses amino acids sequestered from its host as an energy source (Wu et al. 2004; Hotopp et al. 2006). These predictions provide a conceptual basis for the experimental investigation of metabolic interactions between *Wolbachia* and its host in cultured insect cell lines that maintain the *Wolbachia* infection.

The *Aedes albopictus* cell line known as Aa23 was derived from *Wolbachia*-infected *Ae. albopictus* mosquito embryos and is persistently infected with *Wolbachia pipientis* strain wAlbB (O'Neill et al. 1997). Aa23 cells have been a source of infectious inoculum that has

been transferred to other insect cell lines (Dobson et al. 2002; Rasgon et al. 2006). Conversely, Aa23 cells can be made *Wolbachia*-free by treatment with tetracycline. Cells thus cured, designated Aa23T, can be reinfected with diverse strains of *Wolbachia*, including isolates from the fruit fly, *Drosophila simulans*, the mosquito, *Culex pipiens*, and the moth, *Cadra cautella* (Dobson et al. 2002). In aggregate, these in vitro studies indicate that *Wolbachia* can become established and persist over several passages in diverse insect cell lines regardless of their prior exposure to *Wolbachia*. Likewise, an individual cell line can productively support diverse strains of *Wolbachia*.

Most insect cell lines consist of a heterogeneous population of cell types. The Aa23 cell line contains at least two cell types (O'Neill et al. 1997) and has a relatively long population doubling time even in medium containing 20% fetal bovine serum (Fallon 2008; Fallon and Hellestad 2008). We began this study by investigating whether *wAlbB* from Aa23 cells could be stably transferred to a clonal population of TK-6 cells, which have a deficiency in thymidine kinase activity (Mazzacano and Fallon 1992, 1995). TK-6 cells were derived from *Ae. albopictus* C7-10 cells (Fallon 1997) after mutagenesis and selection for resistance to the thymidine analog, 5-bromodeoxyuridine (BrdU).

When previously uninfected (naïve) TK-6 cells were inoculated with *wAlbB* from Aa23 cells and labeled with ^{35}S [methionine/cysteine], the predominant difference in labeled proteins was increased synthesis of an approximately 29-kDa protein. We analyzed this band using mass spectrometry, which revealed peptides corresponding to α and β subunits of the 26S proteasome, a large 2.4-MDa proteolytic complex responsible for the degradation of most intracellular proteins (DeMartino and Gillette 2007).

To verify that *Wolbachia* infection was accompanied by the up-regulation of the 26S proteasome, we took advantage of the 100% amino acid sequence identity between mosquito and bovine ubiquitin to probe Western blots with a commercially available antibody. Using either spot blots, or conventional Western blots, we showed increased protein ubiquitination in infected cells. These results support the hypothesis that *Wolbachia* growth and proliferation are accompanied by increased degradation of host cell proteins by the ubiquitin–proteasome pathway.

Materials and Methods

Cell lines and culture conditions

Cells were maintained in Eagle's medium supplemented with nonessential amino acids, vitamins, glutamine, and penicillin/streptomycin as detailed by Shih et al. (1998). For TK-6 cells and infected derivatives, heat-inactivated fetal bovine serum was added to a final concentration of 5%; for Aa23 cells, the serum concentration was 20%. These formulations are called E-5 and E-20, respectively. Adaptation of Aa23 cells (O'Neill et al. 1997) to Eagle's medium has been described previously (Fallon 2008). Cells were maintained at 28–30°C under a 5% CO₂ atmosphere. For infection (see below), TK-6 cells were plated in advance and grown to confluence in 60-mm plates containing 4 ml of E-5 medium. Cells were typically labeled with ^{35}S [methionine/cysteine], 25 $\mu\text{Ci}/\text{ml}$ (Tran [^{35}S] Label; ~1,100 Ci/mmol; MP Biomedicals, Solon, OH). At appropriate time points, cells were resuspended in the culture medium, harvested by centrifugation, washed in phosphate-buffered saline (Dulbecco and Vogt 1954), and pellets were resuspended by sonication in 0.1% sodium dodecyl sulfate (SDS). Radioactivity was determined by scintillation counting, and samples containing equal amounts of label were reconstituted in SDS sample buffer and boiled prior to electrophoresis. For some experiments, samples were prepared in RIPA buffer (PBS containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing HALT protease inhibitor complex (Thermo Scientific, Waltham, MA), but no differences in labeling patterns were observed.

Preparation of the wAlbB inoculum

Stock cultures of Aa23 cells were diluted tenfold in two 180-cm² flasks, each containing 40 ml of E-20 medium. Initially, a robust patchy monolayer forms; after 2 wk, when cells formed attached aggregates (Fallon 2008), an additional 40 ml of E-20 was added to each flask. Cell growth was monitored microscopically over the course of 2 to 4 wk, during which the patchy monolayer from the initial seeding, plus additional cells that grow after refeeding, converted to aggregates of floating cells. Aggregates and attached cells were pooled, harvested by centrifugation, resuspended in 16 ml (0.1 volume relative to starting material) of E-5 medium, and distributed in 1 ml aliquots to sterile tubes for sonication (5 s in a Kontes GE 70.1 ultrasonic processor, at an amplitude setting of 40) under sterile conditions. Sonicated material was pooled, filtered in 5-ml portions through 2.7- μ m syringe filters, and added to TK-6 recipient cells (0.3 ml of inoculum per 60-mm plate containing 4 ml of E-5 medium). A slide was made for Giemsa staining, and the infected status of the inoculum was verified by light microscopy. For control treatments, the inoculum was boiled for 5 min and cooled on ice prior to addition to the cells.

Infected cells

Three infected lines, designated TW-180, 280, and 380, were obtained from infected TK-6 cells after two rounds of selection in E-5 medium containing BrdU at a final concentration of 80 μ g/ml. The BrdU selection was initiated 7 d after infection and continued for 4 wk, after which the cells were subcultured by tenfold dilution into fresh E-5 medium at 10-d intervals.

Mass spectrometry

A ~29-kDa Coomassie blue-stained band (Fig. 2) that directly correlated to the mass as well as increased expression observed in the methionine-labeled sample was excised and subjected to tryptic digestion (Kinter and Sherman 2000). Peptides were rehydrated in water/acetonitrile (ACN)/formic acid (FA) 95:5:0.1 and loaded using a Paradigm AS1 autosampler system (Michrom Bioresources, Inc., Auburn, CA). Each sample was subjected to Paradigm Platinum Peptide Nanotrap (Michrom Bioresources, Inc.) pre-column (0.15 $\times$ 50 mm, 400- μ l volume) followed by an analytical capillary column (75 μ m $\times$ 12 cm) packed with C₁₈ resin (5 μ m, 200 Å Magic C18AG, Michrom Bioresources, Inc.) at a flow rate of 250 nl/min. Peptides were fractionated on a 60 min (10–40% ACN) gradient on a flow MS4 flow splitter (Michrom Bioresources, Inc.). Mass spectrometry (MS) was pre-formed on a LTQ (Thermo Electron Corp., San Jose, CA). Ionized peptides eluting from the capillary column were subjected to an ionizing voltage (2.0 kV) and selected for MS/MS using a data-dependent procedure alternating between an MS scan followed by four MS/MS scans for the four most abundant precursor ions.

Database searching and protein identification

Sequest (ThermoFinnigan, San Jose, CA, version 27, rev. 13) and X! Tandem (www.thegpm.org; version 2007.01.01.1) were used to search the *Aedes*_NCBI_011508_CTM database, 37,114 entries. The search was repeated with a combined *Aedes*, *Wolbachia* reference sequence database (rs_wolbachia_aedes_v200808_cRAP database, 23,725 entries) to determine if any *Wolbachia* proteins were present in the excised band. Sequest and X! Tandem were searched with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 1.00 Da. Oxidation of methionine was specified as a variable modification. Scaffold (version Scaffold-01_07_00, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications with filters set to a protein identification probability, 99.9%; minimum number of peptides, 3; and minimum identification probability, 95%. By these criteria, 13 proteins were identified, including the trypsin precursor, mosquito actin (42 kDa), and bovine serum albumin (66 kDa), which were excluded from further consideration.

Western blots

Samples were electrophoresed on 12% polyacrylamide SDS gels as described by Laemmli (1970). Gels were stained in Coomassie brilliant blue, photographed, dried, and exposed to X-ray film for autoradiography or transferred directly to Whatman PRO-TRAN nitrocellulose (0.45- μ m pore size; PerkinElmer Life and Analytical Sciences, Boston, MA) for Western blots. Filters were blocked by incubation in 5% nonfat dry milk containing 0.1% Tween-20 at room temperature for 1 h. Primary antibody [mouse monoclonal antibody to full-length bovine ubiquitin (Ub-P4D1) from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, catalog # sc-8017] was added at a 1:1,000 dilution, and incubation was continued overnight at 4°C, with gentle shaking. Filters were washed in 20 mM Tris-HCl, pH 7.5, containing 0.5 M NaCl and 0.1% Tween-20 for 15 min and three times for 5 min. Filters were placed in 5% milk containing 0.1% Tween 20 and secondary antibody (goat anti-mouse IgG, coupled to horse radish peroxidase; sc-2005) at a 1:1,000 dilution for 1 to 3 h at room temperature, washed as described above, and developed using SuperSignal West Pico detection system (Thermo Scientific) and Kodak Biomax X-OMAT AR film, typically with a 5-min exposure.

Results

Ae. albopictus TK-6 cells are a suitable recipient for wAlbB

In contrast to Aa23 cells, which have a doubling time of 45–70 h in medium containing 20% serum (Fallon and Hellestad 2008), TK-6 cells have a doubling time of 30–32 h in medium containing 5% serum (Mazzacano et al. 1992). We reasoned that their more rapid growth and reduced serum requirement might facilitate investigation of metabolic events accompanying *Wolbachia* infection. Moreover, passage of infected cells in medium containing BrdU provided a rigorous means of eliminating any Aa23 cells that persisted through preparation of the inoculum.

We evaluated proliferation of *Wolbachia* from Aa23 cells in three lines (designated TW-180, TW-280, and TW-380) derived from infected TK-6 cells by assaying polymerase chain reaction amplifiable *Wolbachia* DNA in the cell cultures. Two primer sets, designed to amplify *Wolbachia* DNA and host cell DNA targets of similar size, were used in a single reaction as described previously (Fallon 2008). As was the case with Aa23 cells, *Wolbachia* DNA persisted in the culture medium of infected TW-280 cells (Fig. 1, passages 2 through 12), while cellular DNA degraded during prolonged culture and was barely detectable in the early samples (Fig. 1, passages 2 through 7). By passage 12, *Wolbachia* DNA was barely detectable, and levels declined as passage continued, while the band representing cellular DNA remained robust. Lines TW-180 and TW-280, which were established earlier and were therefore at a higher passage number than the TW-380 line, spontaneously lost wAlbB after approximately 5 mo. Because the three TW lines were comparable in their cytological and growth properties, we discontinued the experiment before the TW-380 cells were subcultured to the passage number at which wAlbB was lost from TW-180 and TW-280 cells. The wAlbB infection is considerably more stable in Aa23 cells, which, despite variability in infection levels (O'Neill et al. 1997; Fallon 2008), has remained stable in our hands for over 2 yr. Overall, the Aa23 line has maintained *Wolbachia* for more than a decade, during which time it has been distributed to a number of laboratories and maintained in a variety of culture media.

Long-term persistence of wAlbB in Aa23 cells over years, as opposed to months for the TK-6 cells, may reflect subtle physiological adaptations to *Wolbachia* in the infected embryonic tissues from which the Aa23 line was derived. Likewise, eventual loss of infection in TW-180 (not shown) and TW-280 (Fig. 1) cells suggested that the metabolic accommodations between wAlbB and the naïve host cell are less well-adjusted, resulting in eventual loss of the

Wolbachia infection. We reasoned that this instability might facilitate detection of changes in protein synthesis during establishment of *wAlbB* during the first week after inoculation.

Up-regulation of an ~29-kDa band in infected TK-6 cells

To investigate intracellular changes early after infection, we labeled infected cells with ^{35}S [methionine/cysteine] and harvested cells at various times to examine changes in protein synthesis on SDS polyacrylamide gels. Against a background of largely identical protein labeling, a single ~29-kDa band showed substantially increased radioactivity in infected cells, relative to cells treated with boiled inoculum (Fig. 2, left panel, *note* the open arrow). This band was also detectable by Coomassie blue staining, and a corresponding band was absent in the control lane (Fig. 2, right panel). In independent experiments (not shown), we verified that TK-6 cells treated with boiled inoculum and untreated TK-6 cells expressed identical patterns of labeled proteins. The up-regulated 29-kDa band was detected in more than six independent experiments initiated with independent preparations of live inocula from Aa23 cells, suggesting that the response was invariant.

When the 29-kDa stained band was recovered from SDS gels and processed for mass spectrometry, MS/MS data analyzed at the highest levels of stringency in the Scaffold protein analysis software revealed ten peptides that originated from mosquito proteins with an approximate mass of 29 kDa. The highest ranking non-ribosomal protein candidate was the β subunit of the 26S proteasome (accession no. EAT45312), which had an identity probability of 100% for each of two preparations, with five unique peptides and 20% (56/280 amino acids) protein coverage, indicated by boxed residues in Fig. 3. The β subunit has a calculated molecular mass of 30.4 kDa. The α subunit (accession no. ABF18446) was also present among the top ten candidates, with probabilities of 100% and 94% for the two samples, 15% coverage in one sample (Fig. 3), and 4% coverage in the other. Reduced coverage of the 26.9-kDa α subunit is consistent with its lower mass, which would reduce its representation in the excised band. These bands were of particular interest because they are components of a multi-protein complex, the 26S proteasome, whose activity potentially increases the availability of amino acids to *Wolbachia* via controlled degradation of host cell proteins.

We anticipated that both *Wolbachia* and *Aedes* proteins would be present in the band recovered after SDS gel electrophoresis. However, when search parameters were expanded to include the reference sequence database of *Wolbachia*, no proteins with a high confidence level were identified, which suggests that either the excised band contained no *Wolbachia* protein or that levels of *Wolbachia* protein contained in the excised slice were below the detection range of the mass spectrometer.

Because proteasome activity is responsible for normal turnover of cytoplasmic proteins during cellular homeostasis, we sought independent experimental evidence for increased proteasome activity in infected cells. Proteins destined for degradation are targeted to the 26S proteasome by covalent attachment of the 76 amino acid co-factor, ubiquitin. Amino acid sequence identity between mosquito and bovine ubiquitin allowed use of a monoclonal antibody to bovine ubiquitin to probe Western blots of extracts from infected and uninfected mosquito cells. In Fig. 4, the left panel shows that a large number of proteins in infected cells show increased labeling with ubiquitin (lanes 2, 3, and 4), relative to uninfected controls (lane 1). Note that ^{35}S [methionine/cysteine]-labeled bands in Fig. 4, right panel, exposed from the filter used for the Western blot verifies that lanes 1 through 4 contain comparable levels of transferred protein. Comparison of the Western blot with the labeled bands also reveals that distribution of ubiquitinated proteins differs from that of labeled bands, suggesting that proteins are not uniformly destined for proteolytic degradation.

Discussion

The present study provides a novel insight into a mechanism by which *Wolbachia* interacts with host cellular machinery in a naïve cell line. Continual turnover of proteins by regulated proteolysis is an important component of overall cellular homeostasis, growth, and metabolism. Proteins destined for degradation by the 26S proteasome are tagged by covalent attachment of the 76 amino acid cofactor, ubiquitin. Within the 24-MDa complex, the core 20S proteasome houses a proteolytic chamber in which the α and β subunits mediate catalytic degradation of ubiquitin-tagged proteins, releasing peptides of three to 20 residues. These peptides are further hydrolyzed to amino acids by cytoplasmic enzymes, while ubiquitin itself is recycled (for reviews, *see* Lee and Goldberg 1998; Lecker et al. 2006; DeMartino and Gillette 2007).

While this work was in progress, Brennan et al. (2008) reported use of a proteomics approach based on two-dimensional polyacrylamide gels to identify differential up-regulation of host antioxidant proteins in Aa23 cells compared with Aa23 cells cured after seven passages in the presence of rifampicin. In their analysis, proteins with *pI* values ranging from 5 to 8 were separated on a second dimension gel with discrimination between 47 and 12 kDa. Although the α subunit of the proteasome has a *pI* of 4.72, and would be undetectable on their gels, the β subunit has a calculated *pI* of 6.34, assuming absence of phosphorylation. Brennan et al. (2008) do not describe an up-regulated protein that matches these parameters. Likewise, in studies related to those reported here, we have not detected the 29-kDa band in tetracycline-cured Aa23 cells reinfected with *Wolbachia*, while the band was present when naïve C7-10 cells were infected with *Wolbachia*. We suggest that the absence of the 29-kDa band in reinfected Aa23 cells may reflect unique reciprocal adaptations between *Wolbachia* and its host cells that evolved during establishment of the infected Aa23 cell line.

The overall similarity in protein labeling in *Wolbachia*-infected cells compared with uninfected control cells and the absence of *Wolbachia* proteins in the excised 29-kDa band support the hypothesis that the metabolic needs of *Wolbachia* impose minimal disruption of host cell metabolism once the infection becomes established. It remains to be determined whether activation of intracellular protein degradation via the proteasome remains detectable as the naïve cell becomes adapted to *Wolbachia* and whether this activity imposes a subtle metabolic cost that leads to the eventual loss of *Wolbachia* from TW-280 cells (Fig. 1). We anticipate that the identification of ubiquitin-tagged proteins in infected TK-6 cells, and the evaluation of their roles in cell metabolism, will lead to an improved understanding of this widespread bacterium and its diverse effects on insect reproduction.

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References

- Brennan LJ, Keddie BA, Braig HR, Harris HL. The endosymbiont *Wolbachia pipientis* induces the expression of host antioxidant proteins in an *Aedes albopictus* cell line. *PLoS ONE* 2008;35:e2083.10.1371/journal.pone.0002083 [PubMed: 18461124]
- DeMartino GN, Gillette TG. Proteasomes: machines for all reasons. *Cell* 2007;129:659–662.10.1016/j.cell.2007.05.007 [PubMed: 17512401]
- Dobson SL, Marsland EJ, Veneti Z, Bourtzis K, O'Neill SL. Characterization of *Wolbachia* host cell range via the in vitro establishment of infections. *Appl Environ Microbiol* 2002;68:656–660.10.1128/AEM.68.2.656-660.2002 [PubMed: 11823204]

- Dulbecco R, Vogt M. Plaque formation and isolation of pure lines with poliomyelitis virus. *J Exp Med* 1954;99:167–182.10.1084/jem.99.2.167 [PubMed: 13130792]
- Fallon, AM. Transfection of cultured mosquito cells. In: Crampton, JM.; Beard, CB.; Louis, C., editors. *Molecular biology of insect disease vectors*. Chapman and Hall; New York: 1997. p. 430-443.
- Fallon AM. Cytological properties of an *Aedes albopictus* mosquito cell line infected with *Wolbachia* strain wAlbB. *In Vitro Cell Dev Biol—Animal* 2008;44:154–161.10.1007/s11626-008-9090-4.0
- Fallon AM, Hellestad VJ. Standardization of a colorimetric method to quantify growth and metabolic activity of *Wolbachia*-infected mosquito cells. *In Vitro Cell Dev Biol—Animal* 2008;44:351–356.
- Hotopp JC, Lin M, Madupu R, Crabtree J, Angiuoli SV, Eisen J, Seshadri R, et al. Comparative genomics of emerging human *Ehrlichiosis* agents. *PLoS Genetics* 2006;22:e21.10.1371/journal.pgen.0020021 [PubMed: 16482227]
- Kinter, M.; Sherman, NE. Protein sequencing and identification using tandem mass spectrometry. Wiley-Interscience; New York: 2000. The preparation of protein digests for mass spectrometric sequencing experiments; p. 147-165.
- Laemmli UK. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685.10.1038/227680a0 [PubMed: 5432063]
- Lecker SH, Goldberg AL, Mitch WE. Protein degradation by the ubiquitin–proteasome pathway in normal and disease states. *J Am Soc Nephrol* 2006;17:1807–1819.10.1681/ASN.2006010083 [PubMed: 16738015]
- Lee DH, Goldberg AL. Proteasome inhibitors: valuable new tools for cell biologists. *Trends Cell Biol* 1998;8:397–403.10.1016/S0962-8924(98)01346-4 [PubMed: 9789328]
- Mazzacano CA, Fallon AM. Thymidine kinase-deficient mutants of *Aedes albopictus* mosquito cells. *In Vitro Cell Develop Biol—Animal* 1992;28:455–458.10.1007/BF02634051
- Mazzacano CA, Fallon AM. Evaluation of a viral thymidine kinase gene for suicide selection in transfected mosquito cells. *Insect Mol Biol* 1995;4:125–134.10.1111/j.1365-2583.1995.tb00017.x [PubMed: 7551194]
- O'Neill SL, Pettigrew MM, Sinkins SP, Braig HR, Andreadis TG, Tesh RB. In vitro cultivation of *Wolbachia pipientis* in an *Aedes albopictus* cell line. *Insect Mol Biol* 1997;6:33–39.10.1046/j.1365-2583.1997.00157.x [PubMed: 9013253]
- Rasgon JL, Ren X, Petridis M. Can *Anopheles gambiae* be infected with *Wolbachia pipientis*? Insights from an in vitro system. *Appl Environ Microbiol* 2006;72:7718–7722.10.1128/AEM.01578-06 [PubMed: 17028229]
- Shih KM, Gerenday A, Fallon AM. Culture of mosquito cells in Eagle's medium. *In Vitro Cell Develop Biol—Animal* 1998;34:629–630.10.1007/s11626-996-0010-1
- Wu M, Sun LV, Vamathevan J, Riegler M, Deboy R, Brownlie JC, et al. Phylogenomics of the reproductive parasite *Wolbachia pipientis* wMel: a streamlined genome overrun by mobile genetic elements. *PLoS Biol* 2004;2:E69.10.1371/journal.pbio.0020069 [PubMed: 15024419]

Figure 1.

Gradual loss of *Wolbachia* during continued passage of TW-280 cells. Working stocks of cells were maintained in E-5 medium and subcultured to provide additional working stocks that were further subcultured and replicate flasks, which were maintained in the incubator under normal growth conditions, without further manipulation, for up to 5 mo. After passage 14, total DNA was harvested from the set of flasks representing passages 2 through 14 and assayed for *Wolbachia* DNA (*upper band*) and mosquito DNA (*lower band*) using the polymerase chain reaction (PCR) and primer sets S12F/S7R and S6.97F/S6.560R as described previously (Fallon 2008). The lanes representing passages 12, 13, and 14 were processed in the same PCR reaction, run on the same gel, and photographed. To construct the figure, these three lanes were electronically aligned with samples from the earlier passages. Similar loss of the *Wolbachia* infection was observed in a second independent line of infected cells.

Figure 2.

Increased synthesis of a 29-kDa band in wAlbB-infected TK-6 cells. *Left panel* Cells were infected with live (*lane 1*) or boiled (*lane 2*) inoculum prepared from Aa23 cells (passage 39 in E-20 medium). On day 4, ^{35}S [methionine/cysteine] was added (25 $\mu\text{Ci}/\text{ml}$) and cells were harvested 5 d later. Samples were electrophoresed on 12% SDS polyacrylamide gels; an autoradiogram is shown. *Values* indicate molecular mass markers, and the *open arrow* in the *left panel* indicates the 29-kDa protein of interest. *Right panel* shows Coomassie blue-stained gel from lysates prepared in parallel from the same batch of cells, but without radiolabeled precursors.

SEQUENCE, 95% PROB	SEQUEST Xcorr, deltaCn	X! TANDEM
(R)TLNYDFQPGTTGVLQSK(S)		3.80
(R)TLNYDFQPGTTGVLQSK(S)	5.59, 0.65	5.96
(R)TLNYDFQPGTTGVLQSK(S)	2.54, 0.29	-0.04
(R)TVPVVAANTMLK(Q)		3.38
(R)TVPVVAANTMLK(Q)		3.08
(K)DGVILGADTR(A)	3.22, 0.36	1.14
(K)DGVILGADTR(A)	3.30, 0.36	0.40
(K)DGVILGADTR(A)	2.67, 0.20	0.34
(K)DGVILGADTR(A)	3.30, 0.40	0.55
(R)ATEGPIVADK(N)	2.77, 0.55	1.23
(K)DGAQYLR(T)	2.54, 0.33	-0.11
(K)DGAQYLR(T)	2.22, 0.37	-0.72

ALPHA subunit, 26930 Da

MFLTRSEYDR	GVNTFSPEGR	LFQVEYAIEA	IKLGSTAIGI	CTQEGVVMAY	EKRITSPLME	60
PTKVEKIVEV	DRHIGCATSG	LMADSRTLDD	RARVECQNHV	FVYDERMSVE	SCAQAIVSSLA	120
IQFGSDDDTG	SAMSRPFGVA	ILFAGIENG	PQLWHMDPSG	TYIRYDAKAI	GSGSEGAQQN	180
LQEYYLQTM	IKEAINLALS	TLKQVMEEKL	NSTNVEVMTM	TPGGLFRMFT	KEEVEEYINN	240

MS

BETA subunit, 30421 Da

MMTDIARNPE	APGFSFENCR	RNAQLVKQGF	VAPKMIKTGT	TIVGIIYKDG	VILGADTRAT	60
EGPIVADKNC	EKIHYLAKNM	YCCGAGTAAD	TEMTTQMIAS	NLELHRLNTG	RTVPVVAANT	120
MLKQFLFRYQ	GYVSAALVLG	GVDNTGSYIY	CIYPHGSTD	K	LAAMSVFESR	180
WKPDMEEEEG	KKLVRDAIAA	GVFNDLGSGS	NIDLCVIKKD	GAQYLRTFEE	ANKKGTRTLN	240

YDFQPGTTGV LQSKSFKIDI TEECVRTLIP VGGVESMDTA

Figure 3.

MS/MS analysis of tryptic peptides from the 29-kDa band shown in Fig. 2. MS/MS peptides and associated statistics for the β subunit are shown on top. Boxed peptides identified by MS/MS for the α (gi|157105496) and β (gi|108881087) subunits of the *Ae. aegypti* 26S proteasome and their positions within the complete amino acid sequences are shown below.

Figure 4.

Increased ubiquitination of proteins in infected cells. The *left panel* shows a Western blot incubated with anti-ubiquitin and stained with a secondary antibody coupled with horseradish peroxidase. The *right panel* shows an autoradiogram (4 d at -80°C) of the nitrocellulose filter. *Lanes 1 and 2* TK-6 cells labeled continuously with ^{35}S [methionine/cysteine] for 9 d after inoculation with boiled or live *Wolbachia*, respectively; *lanes 3 and 4*, TK-6 cells 12 and 14 d after inoculation with live *Wolbachia*.