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Essential function of VCP/p97 in infection cycle of the nucleopolyhedrovirus AcMNPV in *Spodoptera frugiperda* Sf9 cells

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ABSTRACT

The protein VCP/p97 (also named CDC48 and TER94) belongs to a type II subfamily of the AAA + ATPases and controls cellular proteostasis by acting upstream of proteasomes in the ubiquitin-proteasome protein degradation pathway. The function of VCP/p97 in the baculovirus infection cycle in insect cells remains unknown. Here, we identified VCP/p97 in the fall armyworm *Spodoptera frugiperda* (Sf9) cells and analyzed the replication of the Autographa californica multiple nucleopolyhedrovirus, AcMNPV, in Sf9 cells in which the VCP/p97 function was inhibited. The specific allosteric inhibitor of the VCP/p97 ATPase activity, NMS-873, did not deplete VCP/p97 in infected cells but caused a dose-dependent inhibition of viral DNA synthesis and efficiently suppressed expression of viral proteins and production of budded virions. NMS-873 caused accumulation of ubiquitinated proteins in a manner similar to the inhibitor of proteasome activity, Bortezomib. This suggests the essential function of VCP/p97 in the baculovirus infection cycle might be associated, at least in part, with the ubiquitin-proteasome system.

1. Introduction

Baculoviruses contain circular, double-stranded DNA genomes of 80 to 180 kb and infect insects of the orders Lepidoptera, Hymenoptera, and Diptera. In infected species they produce two types of mature viruses, budded virions (BV) required for systemic infection of insect tissues and occlusion derived viruses (ODV) for transmission in insect populations (for review see (Rohrmann, 2013)). Baculoviruses cause stress in infected cells as demonstrated by the induction of the apoptotic pathway (Clem, 2007; Clem et al., 1991; Schultz and Friesen, 2009), signal kinases (Chen et al., 2009; Katsuma et al., 2007; Schultz and Friesen, 2009; Xiao et al., 2009), the DNA damage response (Huang et al., 2011; Mitchell and Friesen, 2012), oxidative stress (Micheal and Subramanyam, 2013; Wang et al., 2001), and the heat shock response (Breitenbach and Popham, 2013; Iwanaga et al., 2014; Lyupina et al., 2010, 2011; Tung et al., 2016). Although, baculoviruses encode most of factors required for replication in cell nuclei, they utilize host cell systems to maintain proteostasis. The conserved VCP protein, also named p97, CDC48, or TER (Transitional Endoplasmic Reticulum) ATPase, plays an important role in control of the cellular proteome. VCP/p97 is a member of a type II subfamily of the AAA + ATPase family (extended family of ATPases associated with various cellular activities) and contains two tandem ATPase domains and forms a double ring-shaped homohexamer structure inside cells (for review see (Hanzelmann and Schindelin, 2017; Ripstein et al., 2017; van den Boom and Meyer, 2017)). VCP/p97 acts as an unfoldase/segregase and disassembles protein aggregates in an ubiquitin-dependent manner by substrate threading through the central channel of the p97 hexamer (Bodnar and Rapoport, 2017b). Recent data suggest an involvement of VCP/p97 in the infection cycle of several DNA and RNA viruses (Arita et al., 2012; Lin et al., 2017; Panda et al., 2013; Phongphaew et al., 2017; Wang et al., 2017; Wong et al., 2015; Wu et al., 2016; Yi and Yuan, 2017). VCP/p97 is a key member of the endoplasmic reticulum (ER)-associated protein degradation (ERAD) pathway. It pulls poly-ubiquitinated, misfolded proteins out of the ER and transfers them to the proteasome (for review see (Bodnar and Rapoport, 2017a; Brodsky, 2012; Locke et al., 2014; Stein et al., 2014; van den Boom and Meyer, 2017)). Induction of the GRP78 homolog by baculoviruses infection suggests that it activates ERAD in infected cells (Lyupina et al., 2011). The functional association of VCP/p97 with proteasomes is conserved

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in evolution. In Archaea, the VCP/p97 homolog forms a stable complex with the 20S proteasome (Barthelme et al., 2014; Barthelme and Sauer, 2012). The essential role of the host cell proteasomes and ubiquitin-proteasome system (UPS) in the baculovirus replication cycle was shown in several laboratories (Katsuma et al., 2011; Lyupina et al., 2013; Xue et al., 2012). In order to control UPS, baculoviruses encode ubiquitin (Guarino et al., 1995; Ma et al., 2015) and members of the RING-family of ubiquitin ligases (Imai et al., 2003; Katsuma et al., 2008) which were shown recently to participate in the maturation of viral nucleocapsids and their egress from cells (Biswas et al., 2018). All these data suggest that VCP/p97 functions upstream of proteasomes in the UPS and might play an important role in the infection cycle of baculoviruses. In this report, this prediction was confirmed in experiments with NMS-873, the most potent and specific inhibitor of the VCP/p97 ATPase activity identified to date.

2. Materials and methods

2.1. Cells and reagents

Spodoptera frugiperda Sf9 cells were cultured in SF-900 II SFM media (Invitrogen) supplemented with 10% fetal bovine serum (FBS) in the flasks at 27 °C. The cells were infected with the Autographa californica multiple nucleopolyhedrovirus (AcMNPV, the family *Baculoviridae*) at the MOI of 10. NMS-873 and Bortezomib (PS-341) from Sigma were dissolved in DMSO. The following antibodies (Abs) were used: polyclonal Ab to human VCP protein from Cell Signalling Technology (2648S); polyclonal Ab to BmNPV DBP (Okano et al., 1999); mouse mAb (FK2) to mono- and polyubiquitinated conjugates from Enzo Life Sciences (BML-PW8810; mouse mAb to beta-actin from Santa Cruz Biotechnology. Peroxidase-conjugated anti-rabbit IgG and ECL reagents were purchased from GE Healthcare Life Sciences.

2.2. Real-time PCR

Measurement of viral DNA content in AcMNPV-infected cells by real-time PCR (RT-PCR) was carried out by method of Rosinski et al. (Rosinski et al., 2002) as described (Lyupina et al., 2010) by using Syntol R-442 Reagent Kit (www.sintol.ru) and the Applied Biosystems 7500 Real-Time PCR System. DNA was purified from AcMNPV-infected cells by using the Genomic DNA Purification Kit K0512 (Thermo Scientific). The forward primer was 5′-ATTAGCGTGGCGTGCTTTTAC-3′. The reverse primer was 5′-GGGTCAGGCTCCTCTTTGC-3′. Viral DNA purified from budded virions was used as a standard.

2.3. Immunohistochemistry and confocal microscopy

Virus- or mock-infected Sf9 cells were fixed for 15 min with 4% paraformaldehyde in phosphate-buffered saline (PBS), washed three times with PBS, and permeabilized for 2 min in cold acetone (−20 °C). The cells were rehydrated with PBS, treated with 1% SDS in PBS for 5 min at room temperature, washed three times with PBS for 5 min each, blocked with 5% FBS and 0.3% Triton X100 in PBS for 1 h, and then subjected to antibody treatments. Antigen localization was determined by incubation of the cells with mouse mAb (FK2) to mono- and polyubiquitinated conjugates (1:1000 dilution with 5% FBS and 0.3% Triton X100 in PBS) overnight at room temperature. After incubation with the primary antibody, cells were washed four times (5 min per wash) with PBS and then treated with the secondary antibody, Alexa 594-conjugated Donkey anti-mouse IgG (1:700 dilution, Invitrogen, USA) for 2 h. After four washes with PBS (10 min per wash), the slides were mounted with the Mowiol and analyzed under confocal microscope Leica SPE equipped with an Ar-Kr laser at the Core Facility on Cell Technologies and Optical Research Methods in Developmental Biology of IDB RAS. To ensure equal illumination for all treatments, the same intensity and filter settings were used throughout. Images were recorded at a resolution of 1024 × 1024 pixels and processed with the Leica LCS software. Control experiments were performed by omitting primary or secondary antibodies.

2.4. Determination of proteasome activity

Determination of proteasome activity in cell extract was performed as described (Lyupina et al., 2013). Cells for analysis were washed with PBS buffer and collected by centrifugation. Samples of 10^6 cells were allowed to swell for 30 min at 4 °C in 30 µl of a hypotonic buffer containing 5 mM Tris−HCl, pH 8.0, 2 mM EDTA, and 1 mM DTT, vortexed and disrupted by freezing (−80 °C)-thawing. The extracts were clarified by centrifugation at 10,000 × g for 30 min. The chymotrypsin-like proteasome activities was determined by hydrolysis of fluorogenic substrate Suc-LLVY-AMC. The activity was determined in portions of 0.5 to 10 µl from the extracts in final volume of 100 µl containing 30 mM Tris−HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, and 30 µM Suc-LLVY-AMC. Inhibitors were added to the reaction mixtures on ice. The reactions were carried out at 37°C for 20 min and terminated by the addition of 1% SDS and chilling. The digestion product was detected by using fluorimeter VersaFluor (Bio-Rad) with the excitation wavelength of 380 nm and the emission wavelength of 440 nm. The proteasome-independent activity was determined in the presence of 3 µM MG-132 (less than 10% in this report) and subtracted from the values obtained in the absence of MG-132.

2.5. Other methods

Electrophoresis in a native polyacrylamide gel followed by detection of proteasome activity in the gel was performed as described earlier (Lyupina et al., 2013). Two-dimensional PAGE was carried out by the method of O'Farrell (O'Farrell et al., 1977) as previously described (Lyupina et al., 2013). Mass spectra of the tryptic peptides of Sf9 proteins were obtained by using the matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass (TOF) spectrometer as described (Lyupina et al., 2016, 2011). Identification of Sf9 proteins was performed by using Mascot software (www.matrixscience.com) in the NCBI database taking into account possible oxidation of methionines and modification of cysteines by acrylamide. SDS-polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli (Laemmli, 1970). Polyhedrin was visualized by Coomassie staining. For Western blotting, proteins were transferred on Hybond-ECL membrane (Amersham) and probed with respective primary antibody. BV titer was determined by the endpoint dilution assay (Reed and Muench, 1938) as described (Langfield et al., 2011). Viability of Sf9 cells was examined by the trypan blue exclusion as described In Growth and Maintenance of Insect Cell Lines Version K July 12, 2002 25-0127 (www.invitrogen.comtech_service@invitrogen.com.Aus: http://wolfson.huji.ac.il/expression/insect/insect_man.pdf).

3. Results

3.1. Identification of S. frugiperda VCP/p97

The VCP/p97 protein was identified in *S. frugiperda* cells by mass spectrometry after fractionation of the Sf9 cell extracts in polyacrylamide gels by 2D electrophoresis (Fig. 1A) and by a native electrophoresis (Fig. 1B). A Mascot search using the NCBI database confirmed the protein homology to insect TER94 ATPases with the best score to the *Helicoverpa armigera* homolog (XP_021183018.1) (E value of 4.1e-16). Blast searches in the database for the lepidopteran genus Spodoptera, *spodobase* (http://bioweb.ensam.inra.fr/spodobase/) revealed several mRNAs encoded by the vcp/p97 gene. A complete sequence of *S. frugiperda* VCP/p97 was constructed from three partial overlapped mRNAs coding the N-terminus (Sf9LR126164-5-1-C1), the middle fragment (Sf9LR817849-5-1-C1) and the C-terminus