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Short communication

Egress of budded virions of *Autographa californica* nucleopolyhedrovirus does not require activity of *Spodoptera frugiperda* HSP/HSC70 chaperones

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ABSTRACT

The induction of heat shock proteins in baculovirus infected cells is well documented. However a role of these chaperones in infection cycle remains unknown. The observation that HSP70s are associated with virions of different baculoviruses reported by several researchers suggests that HSPs might be structural components of viruses or involved in virion assembly. These hypotheses were examined by using a novel inhibitor of the ATPase and chaperoning activity of HSP/HSC70s, VER-155008. When VER-155008 was added early in infection, the synthesis of viral proteins, genome replication and the production of budded virions (BV) were markedly inhibited indicating the dependence of virus reproduction on host chaperones. However, BV production was unaffected when VER-155008 was added in the mid-replication phase which is after accumulation of products required for completion of the viral DNA replication. These results suggest that the final stages in assembly of BV and their egress from cells do not depend on chaperone activity of host HSP/HSC70s.

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The *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) serves as a model for the study of a baculovirus infection cycle in insect cells. AcMNPV contains a 134-kbp double-stranded DNA genome that encodes approximately 150 proteins. Replication of viral DNA (vDNA) and the assembly of viral capsids occur in cell nuclei and yield two types of mature viruses, the budded virions (BV) and occlusion-derived virions (ODV) (for review see Rohrmann, 2013). Baculoviruses cause stress of infected cells that is 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 et al., 2013; Mitchell and Friesen, 2012), the oxidative (Wang et al., 2001) and proteotoxic (Lyupina et al., 2013, 2011) stress, and the heat shock response (Breitenbach and Popham, 2013; Iwanaga et al., 2014; Lyupina et al., 2010, 2011). Induction of HSP/HSC70s in infected cells is well-documented at both, the mRNA (Breitenbach and Popham, 2013; Breitenbach et al., 2011; Choi et al., 2012; Nguyen et al., 2013, 2012; Nobiron et al., 2003; Sagisaka et al., 2010; Salem et al., 2011; Xue et al., 2012) and protein level (Carinhas et al., 2011; Lyupina et al., 2010, 2011; Popham et al., 2010). Suppression of the heat shock response markedly decreases synthesis of vDNA and viral proteins and disturbs the progression of the infection cycle (Iwanaga et al., 2014; Lyupina et al., 2010). Major role in guarding the cellular proteome is played by chaperones of the HSP/HSC70 family that are ubiquitous proteins that assist folding of nascent proteins, support intracellular trafficking and regulate proteolysis. In *Spodoptera frugiperda* cells, AcMNPV infection increased expression of several members of the HSP/HSC70 family, one cognate protein HSC70(1) and three or four inducible HSP70s (Lyupina et al., 2011). HSC70(1) has sequence homology to GRP78, a factor that associates with the endoplasmic reticulum and regulates the unfolded protein response in cytoplasm. Therefore, this chaperone may protect infected cells from proteotoxicity caused by infection. The role of other HSP/HSC70s in the baculovirus infection cycle remains unknown. The observation that HSP70s are associated with some baculoviruses (Braconi et al., 2014; Hou et al., 2013; Iwanaga et al., 2014; Liu et al., 2008) suggests that these chaperones may be structural components of

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viruses or they may assist in the assembly of viral structures. In this report, we attempted to verify these hypotheses by using VER-155008, a specific inhibitor of the ATPase and chaperoning activity of HSP70s and HSC70s (Massey et al., 2010).

S. 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 AcMNPV at the MOI of 10. VER-155008 from Tocris Bioscience was dissolved in DMSO. The following antibodies (Abs) were used: rat mAb 7.10.3 to HSP/HSC70s of *Drosophila melanogaster* from Lindquist Lab.; polyclonal Ab to Human SQSTM1/p62 from Abcam (ab91526); polyclonal Ab to BmNPV DBP (Okano et al., 1999). Peroxidase-conjugated anti-rat IgG, anti-rabbit IgG, and ECL reagents were purchased from GE Healthcare Life Sciences. Viability of Sf9 cells was examined by the trypan blue exclusion. BV titer was determined by the endpoint dilution assay (Reed and Muench, 1938) as described (Langfield et al., 2011). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to 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. Measurement of viral DNA content in AcMNPV-infected cells by real-time PCR (RT-PCR) was carried out by method of Rosinski et al. (2002) as described (Lyupina et al., 2010).

Two concentrations of VER-155008, low (20 μM) and high (100 μM), were tested in experiments with uninfected Sf9 cells. The inhibitor produced a dose-dependent cytostatic effect on proliferation of Sf9 cells and decreased the cell viability (Fig. 1). These data confirmed the cytotoxicity of VER-155008 for insect cells as was observed previously for human cell lines (Massey et al., 2010). Whereas the low concentration slightly affected the Sf9 cells, the 100-μM concentration of VER-155008 efficiently blocked the proliferation and decreased the viability by approximately 20%. Addition of VER-155008 to AcMNPV-infected cells caused a dose-dependent inhibition of vDNA replication (Fig. 2A). Despite potent cytotoxicity, the 100-μM VER-155008 did not completely block synthesis of vDNA although it decreased the rate by approximately one-order of magnitude. Inhibition of the ATPase activity of HSP90 by 2.5 μM of 17-AAG additionally suppressed the vDNA synthesis in infected cells incubated with 100 μM of VER-155008 (Fig. 2B). This result confirmed the sensitivity of baculovirus replication to 17-AAG that was observed in the earlier paper (Lyupina et al., 2011) and is in agreement with the specific affinity of VER-155008 for the active site of HSP70 in comparison to that of HSP90 reported by Massey et al. (2010). In parallel with the assay of vDNA replication, the effect of VER-155008 on expression of selected host and viral proteins was analyzed (Fig. 2C). VER-155008 at 100-μM inhibited the induction of HSP/HSP70s in infected cells that is typical for a heat-shock response (Lyupina et al., 2010) and markedly inhibited production of late DNA-binding protein (DBP) and the very late protein polyhedrin.

The addition of 100-μM VER-155008 to AcMNPV-infected cells at 0 hpi blocked production of BV at 24, 32 and 48 hpi (Fig. 3A). This result was expected considering that 100-μM VER-155008 inhibited vDNA amplification (Fig. 2A) and suppressed viral products that was observed at 24 and 48 hpi in the cells used for titration of BV (Fig. 3B). When VER-155008 was added at 6 hpi, i.e. after the initiation of viral genome replication, a less efficient although high inhibition of BV and viral protein production was observed (Fig. 3A and B). The induction of host HSP/HSC70s was also incomplete similar to that observed when VER-155008 was added at 0 hpi (Fig. 3B). In the next experiment, VER-155008 was added at 24 hpi and BV production was determined at 24, 32 and 48 hpi (Fig. 3C). Synthesis of late viral proteins was nearly completed at 24 hpi as confirmed by the accumulation of DBP (Fig. 3D). However, induction of the very late proteins in the infection cycle was blocked by VER-155008 as shown for polyhedrin (Fig. 3D). Surprisingly, the addition of 100-μM VER-155008 at 24 hpi did not affect the release of BV at 32 and 48 hpi. Microscopic examination showed that the efficient egress of BV in the presence of VER-155008 was not caused by disintegration of infected cells in the presence of inhibitor. Although the morphology of the infected cells incubated with VER-155008 for 24 h was altered, most of these cells showed structural integrity and retained apparently intact cellular membranes (Fig. 3E). To examine the role of the host HSP/HSC70s in the egress of BV in more detail, we analyzed the dynamics of BV release from 20 to 32 hpi at 4-h intervals (Fig. 3F). In these experiments, 100-μM VER-155008 was added at 16 hpi, at the mid-phase in the virus replication cycle. The dynamics of BV production was essentially the same in infected cells incubated in the presence or absence of VER-155008.

The inhibitory effect of 100-μM VER-155008 on the viral infection cycle was significant, when the inhibitor was added early in infection (0 or 6 hpi). These data confirmed that the early stages in the virus infection cycle directly or indirectly depend on the ATPase activity of cellular HSP/HSC70s. VER-155008 strongly inhibited the synthesis of viral proteins (Figs. 2 and 3). This allowed connecting the VER-induced inhibition with depletion of viral products required for the genome replication and BV assembly. However, VER-155008 did not affect the BV production when added to

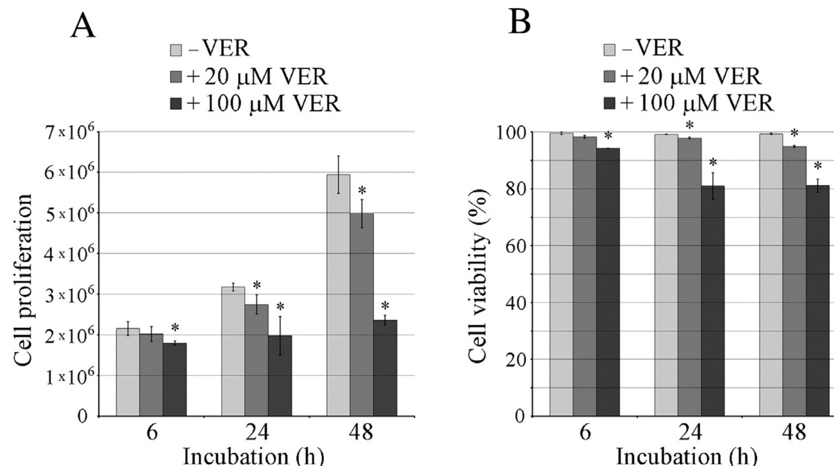

Fig. 1. Effect of VER-155008 on proliferation (A) and viability (B) of Sf9 cells. The quantity and percent of viable cells were determined in triplicate at 6, 24, and 48 h after the addition of VER-155008 (VER) to the media as indicated. Pairwise statistical comparisons to the DMSO group were performed using Student's *t* test (*$P < 0.05$). The bars indicate the standard deviation.

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