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New insights into the induction of the heat shock proteins in baculovirus infected insect cells

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ABSTRACT

Eight members of the HSP/HSC70 family were identified in *Spodoptera frugiperda* Sf9 cells infected with *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) by 2D electrophoresis followed by mass spectrometry (MALDI/TOF) and a Mascot search. The family includes five HSP70s induced by AcMNPV-infection and three constitutive cognate HSC70s that remained abundant in infected cells. Confocal microscopy revealed dynamic changes in subcellular localization of HSP/HSC70s in the course of infection. At the early stages (4 to 10 hpi), a fraction of HSPs is localized in distinct speckles in cytoplasm. The speckles contained ubiquitinylated proteins suggesting that they may be aggresomes where proteins targeted by ubiquitin are sequestered or processed for proteolysis. *S. frugiperda* HSP90 was identified in the 2D gels by Western blotting. Its amount was unchanged during infection. A selective inhibitor of HSP90, 17-AAG, decreased the rate of viral DNA synthesis in infected cells suggesting a supportive role of HSP90 in virus replication.

Introduction

Baculoviruses contain a circular double-stranded DNA genome of 80 to 180 kbp and infect insects of orders Diptera, Hymenoptera and Lepidoptera. The best studied baculovirus, *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), possesses a 134-kbp genome that encodes about 150 proteins including factors essential for viral DNA synthesis. During infection, baculoviruses interrupt cell cycle, down-regulate most host genes while inducing synthesis of viral products necessary for replication of viral genomes and for the assembly of viral particles. In infected cells, baculoviruses produce two types of progeny viruses, budded virions (BV) and occlusion derived virions (ODV), and in permissive infections eventually kill the infected cells and their host (for review see Rohrmann, 2011). Baculovirus infection is recognized as a stress factor in host cells and this can lead to the activation of various cellular pathways including apoptosis. In order to prevent premature death of infected cells and ensure productive infection, baculoviruses use a dual strategy. First, baculoviruses inhibit the apoptotic pathway by expressing viral factors, such as p35 and other anti-apoptotic proteins (Clem, 2007; Clem et al., 1991). Second, baculoviruses activate prosurvival pathways mediated by the signal kinases such as MAPKs, ERK, and JNK, and the phosphatidylinositol 3-kinase (PI3K)–Akt pathway. The inhibition of these pathways significantly reduces virus production in infected cells (Chen et al., 2009; Katsuma et al., 2007; Schultz and Friesen, 2009; Xiao et al., 2009). A hallmark of universal cellular defense reactions to various environmental and pharmacological stresses is the activation of the heat shock response (HSR). It is characterized by drastic up-regulation of members of the ubiquitous chaperone family of heat shock proteins (HSPs) with molecular masses of approximately 70 kDa. HSP70s and the cognate heat shock proteins (HSC70s) play a central role in protein homeostasis and protection against proteotoxic stresses by preventing protein misfolding and aggregation, or by directing damaged proteins to the ubiquitin–proteasome system for degradation. The ATP-dependent chaperoning activity of HSP/HSC70s and another key chaperone, HSP90, is regulated by a battery of co-chaperones, which specify the function of HSPs and regulate their interaction with client proteins. HSP70s and HSP90s are involved in the replicative cycles of various viruses regulating gene expression *via* interaction with viral proteins and participating in capsid assembly and disassembly (Burch and Weller, 2004, 2005; Couturier et al., 2010; Dutta et al., 2009; Lahaye et al., 2009; Livingston et al.,

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2008, 2009; Mayer, 2005; Nagy et al., 2011; Song et al., 2010; Weeks et al., 2010; Yu et al., 2009; Zhao et al., 2009).

Little is known about function of HSPs in the infection cycle of baculoviruses. The cognate HSC70 was found associated with occlusion derived virus (ODV) of the *Bombyx mori* NPV (BmNPV) (Liu et al., 2008) suggesting the possible involvement of HSPs in the assembly of virions. Although expression of the HSP genes has been analyzed during the course of baculovirus infection by several researchers, different approaches and diverse viral and cellular strains used in these experiments prevent the formulation of a unified understanding of their role during infection. In the case of *Spodoptera frugiperda* cell lines commonly used for propagation of AcMNPV, the analysis was further hampered by the absence of the host cell genome sequence. In an early report, Ooi and Miller demonstrated by Northern blot analysis the substantial reduction of mRNA levels of actin, histone, and HSP70 from 12 to 18 h following infection of Sf21 cells with AcMNPV (Ooi and Miller, 1988). Nobiron and coworkers used a differential display approach and confirmed the down regulation of several selected Sf9 genes from 12 h post infection with AcMNPV. One transiently up-regulated host transcript was encoded by the *hsc70* gene. Its mRNA level peaked at 6 hpi and dropped markedly from 12 to 24 hpi (Nobiron et al., 2003). Expression of the silkworm *B. mori hsc70* ortholog was increased by 1.6-fold in NIAS-Bm-oyanagi2 cells 24 hpi after infection with BmNPV, but no increase was evident at 12 hpi (Sagisaka et al., 2010). A microarray assay based on ESTs validated by qRT-PCR was used by Salem et al. (2011) for the host transcriptome analysis of Sf21 cells during AcMNPV infection. In contrast to the majority of host genes that were down regulated in the course of infection, a few genes were up regulated. Among the most highly up-regulated genes were two representatives of the *hsp70* family.

In our previous report, five members of the HSP/HSC70 family were detected in Sf9 cells infected with AcMNPV by using Western blotting and monoclonal antibody to Drosophila HSP70s (Lyupina et al., 2010). Three of HSP70s were induced or highly stimulated by virus infection. The cellular HSP/HSC70 content increased moderately in AcMNPV-infected cells under standard conditions, whereas the infection markedly potentiated the response to heat shock boosting the HSP/HSC70s content in infected cells in comparison with uninfected cells. The inhibition of the HSR by KNK437 decreased markedly the rate of viral DNA synthesis in infected cells indicating the importance of HSR for baculovirus replication. In this report we describe a continuation of the study of HSPs in Sf9 cells infected with AcMNPV. The proteome analysis enabled the identification of eight members of the HSP/HSC70 family and suggested possible roles that they may play during the course of baculovirus infection. The possible cooperation of HSP/HSC70s with the ubiquitin–proteasome system in infected cells was revealed by confocal microscopy.

Results

S. frugiperda HSP/HSP70s

In order to identify members of the HSP/HSC70 family in Sf9 cells, proteomic analysis was applied to a fraction of cellular proteins that were recognized by a monoclonal antibody and to several other proteins having molecular masses and pI values similar to HSP70s. Proteins for the analysis were obtained from uninfected cells and from AcMNPV-infected cells heated at 37 °C for 2 h at 22 hpi. As shown in the previous report, moderate heating markedly augments expression of HSP70s induced by AcMNPV (Lyupina et al., 2010). Proteins were subjected to 2D electrophoresis as previously described (Lyupina et al., 2010). One gel for each sample was stained with Coomassie blue (Figs. 1A and B), and the other was processed for Western-blot analysis with the anti-HSP/HSC70 antibody 7.10.3 and with anti-Drosophila HSP90 antibody 16F1. The blot derived from mock infected cells is shown in Fig. 1C, whereas Figs. 1D1 and D2 are from infected cells and show different exposures of an area delimited by the large arrows. The areas of the gel

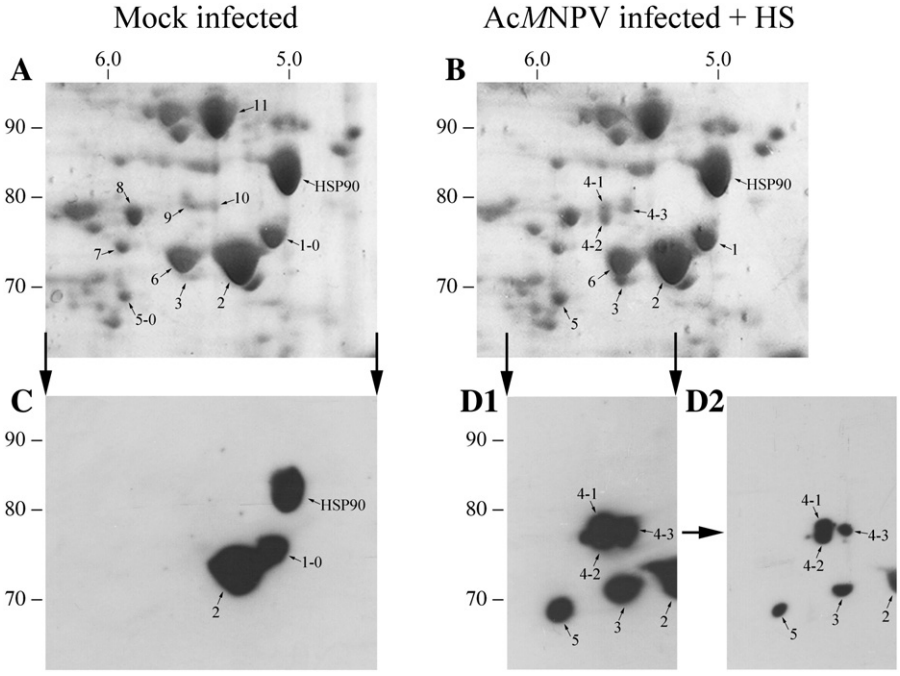

Fig. 1. Changes in the HSP/HSC70 pattern in Sf9 cells after infection with AcMNPV and heating analyzed by two-dimensional PAGE followed by Coomassie staining and Western blotting. Extracts were prepared from uninfected cells (A, C) and cells infected with AcMNPV and heated at 37 °C for 2 h at 22 hpi (B, D1 and D2). Electrophoresis was performed by the isoelectric focusing in the first dimension (horizontal) and then by SDS-8% PAGE in the second dimension (vertical). Each sample was analyzed in duplicate. One gel was stained with Coomassie blue (A, B), and another one was blotted onto a membrane for antigen visualization (C, D1, and D2). The membranes were probed sequentially with the rat mAb 7.10.3 to HSP/HSC70s and then with the rat anti-HSP90 mAb. Panels D1 and D2 show different exposures (long and short, respectively) of a portion of the membrane corresponding to the gel area delimited by the large arrows. Protein spots indicated by small arrows and numbers were cut from the Coomassie stained gels and subjected to mass spectrometry (MALDI/TOF) followed by Mascot software analysis.

containing individual proteins in the Coomassie stained gels were removed and digested with trypsin, and analyzed by mass-spectrometry (MALDI/TOF) followed by a search for homologs in the NCBI database using Mascot software (www.matrixscience.com). A total of fifteen proteins (7 from the mock-infected cells and 8 from the AcMNPV-infected and heated cells) were isolated for the analysis (Figs. 1A and B). Eleven proteins produced scores greater than 84 under the *in silico* search, and they were considered as positively identified. Four proteins (spots no. 5–0, 7, 8, and 5) had no significant homologs in the database and presumably represent yet unknown proteins in *S. frugiperda* cells. The identified proteins and one unidentified but having limited homology to HSPs (spot no. 5) are listed in Table 1. Up to three homologs with the highest scores and some selected species marked by asterisks are shown for each spot. The analysis revealed eight different putative members of the HSP/HSC70 family in Sf9 cells that were referenced in this report as indicated in the last column of the Table. Numbers in brackets correspond to those used in the previous report (Lyupina et al., 2010). All of them except HSC70(6) (spot no. 6) were recognized by the antibody 7.10.3 (Figs. 1C, D1, and D1). Three other identified proteins represent homologs of the CbiG protein (spot no. 9), an oxidoreductase (spot no. 10), and transitional endoplasmic reticulum ATPase (spot no. 11). A single member of the HSP90 family in Sf9 cells was identified by using monoclonal antibody to Drosophila HSP90 (16F1) (Figs. 1A and C). A unique gene encoding *Sf*HSP90 was described earlier (Landais et al., 2001), and the corresponding protein spot was not subjected to the *in silico* analysis.

Heat shock cognate protein HSC70(1) (protein acc. no. gi|27260894) (Nobiron et al., 2003) was identified in both the mock-infected (spot no. 1–0, E-value 6.4e−26) and AcMNPV-infected (spot no. 1, E-value 2e−16) Sf9 cells (Table 1). It was second or third in abundance among HSP/HSC70s in Sf9 cells based on Coomassie staining (Figs. 1A and B). A corresponding gene *hsc70* was the only one sequenced so far for members of the HSP/HSC70 family in *S. frugiperda* cells (Nobiron et al., 2003). By using a differential display approach, it was shown that this gene was transiently up-regulated in Sf9 cells in the course of infection with AcMNPV. Its mRNA level peaked at 6 hpi and dropped markedly from 12 to 24 hpi (Nobiron et al., 2003). Several HSC70(1) orthologs have been identified in other Lepidoptera species. Expression of the silkworm *Bombyx mori* HSC70 (protein acc. no. gi|112984012, E-value 1e−06) gene increased by 1.6-fold in NIAS-Bm-oyanagi2 cells 24 hpi after infection with BmNPV, but did not increase at 12 hpi (Sagisaka et al., 2010).

Table 1
Proteins from *Spodoptera frugiperda* Sf9 cells identified by *in silico* analysis using Mascot software and the NCBI database.

Spot no.	Accession no.		Mass	Score[a]	No. peptides[b]	E-value[c]	Putative protein[d]	Reference in the report
	NCBI	GenBank						
1	gi|27260894	AAN86047	73,064	228	21	2e−16	Heat shock cognate 70 protein [*Spodoptera frugiperda*]	HSC70(1)
	gi|111380717	ABH09734	73,063	205	20	4e−14	Heat shock cognate 70 protein [*Trichoplusia ni*]	
	gi|111380719	ABH09735	73,093	205	20	4e−14	Heat shock cognate 70 protein [Trichoplusia ni]	
	*gi|112984012	NP_001036837	73,052	131	15	1e−06	Heat shock 70 kD protein cognate [*Bombyx mori*]	
	*gi|170034715	XP_001845218	72,263	110	13	0.00013	8 kDa glucose-regulated protein [*Culex quinquefasciatus*]	
1-0	gi|27260894	AAN86047	73,064	323	25	6.4e−26	Heat shock cognate 70 protein [*Spodoptera frugiperda*]	HSC70(1)
	gi|111380717	ABH09734	73,063	294	24	5.1e−23	Heat shock cognate 70 protein [*Trichoplusia ni*]	
	gi|111380719	ABH09735	73,093	294	24	5.1e−23	Heat shock cognate 70 protein [*Trichoplusia ni*]	
	*gi|112984012	NP_001036837	73,052	198	19	2e−13	Heat shock 70 kD protein cognate [*Bombyx mori*]	
	*gi|170034715	XP_001845218	72,263	169	17	1.6e−10	78 kDa glucose-regulated protein [*Culex quinquefasciatus*]	
	*gi|24641402	NP_727563	72,216	118	13	2e−05	Heat shock protein cognate 3, isoform A [*Drosophila melanogaster*]	
2	gi|164422265	ABY55233	71,445	373	31	6.4e−31	HSP70 [*Mythimna separata*]	HSC70(2)
	gi|305693941	ADM66138	71,463	373	31	6.4e−31	70 kDa heat shock protein [*Spodoptera litura*]	
	gi|12585261	Q9U639	71,387	354	30	5.1e−29	Heat shock 70 kDa protein cognate 4 [*Manduca sexta*]	
	*gi|256862214	ACV32641	70,112	319	28	1.6e−25	Heat shock protein 70 cognate [*Helicoverpa zea*]	
	*gi|112982828	NP_001036892	71,131	301	27	1e−23	Heat shock cognate protein [*Bombyx mori*]	
	*gi|219671577	ACL31668	71,573	291	26	1e−22	Heat shock protein 70 [*Helicoverpa armigera*]	
3	gi|224999283	ACN78407	72,456	205	18	4e−14	HSP70 [*Spodoptera exigua*]	HSP70(3)
	gi|229562184	ACQ78180	72,490	205	18	4e−14	Heat shock protein 70 [*Spodoptera exigua*]	
	gi|317135488	ADV03160	70,606	193	17	6.4e−13	Heat shock protein 70 [*Spodoptera litura*]	
	*gi|167077413	ABZ10939	70,159	77	9	0.28	Heat shock protein 70 [*Sesamia nonagrioides*]	
4-1	gi|224999283	ACN78407	72,456	219	17	1.6e−15	HSP70 [*Spodoptera exigua*]	HSP70(4-1)
	gi|229562184	ACQ78180	72,490	219	17	1.6e−15	Heat shock protein 70 [*Spodoptera exigua*]	
	gi|317135488	ADV03160	70,606	203	16	6.4e−14	Heat shock protein 70 [*Spodoptera litura*]	
	*gi|167077413	ABZ10939	70,159	74	8	0.53	Heat shock protein 70 [*Sesamia nonagrioides*]	
4-2	gi|224999283	ACN78407	72,456	202	18	8e−14	HSP70 [*Spodoptera exigua*]	HSP70(4-2)
	gi|229562184	ACQ78180	72,490	202	18	8e−14	Heat shock protein 70 [*Spodoptera exigua*]	
	gi|317135488	ADV03160	70,606	191	17	1e−12	Heat shock protein 70 [*Spodoptera litura*]	
	*gi|167077413	ABZ10939	70,159	90	10	0.014	Heat shock protein 70 [*Sesamia nonagrioides*]	
4-3	gi|317135488	ADV03160	70,606	103	14	0.00064	Heat shock protein 70 [*Spodoptera litura*]	HSP70(4-3)
	gi|224999283	ACN78407	72,456	97	14	0.0025	HSP70 [*Spodoptera exigua*]	
	gi|229562184	ACQ78180	72,490	97	14	0.0025	Heat shock protein 70 [Spodoptera exigua]	
5	gi|254495295	ZP_05108219	27,177	66	7	3	Glycosyl transferase family 2 [*Polaribacter* sp. MED152]	HSP70(5)
	*gi|167077413	ABZ10939	70,159	58	9	20	Heat shock protein 70 [Sesamia nonagrioides]	
	*gi|310893431	ADP37711	69,939	57	9	23	Heat shock protein 70 [Helicoverpa armigera]	
6	gi|223036830	ACM78945	74,812	258	25	2e−19	Heat shock protein 70 [Spodoptera exigua]	HSC70(6)
9	gi|255280038	ZP_05344593	37,916	92	11	0.0089	CbiG protein [*Bryantella formatexigens* DSM 14469]	
10	gi|38679387	AAR26515	77,489	171	23	1.1e−10	Antennal oxidoreductase [*Mamestra brassicae*]	
	gi|323530562	ADX95746	77,713	148	23	2.2e−08	NADPH cytochrome P450 reductase [*Spodoptera exigua*]	
	gi|300510884	ADK25060	77,402	121	18	1.1e−05	NADPH cytochrome P450 reductase [*Helicoverpa armigera*]	
11	gi|112983322	NP_001037003	89,096	248	30	2e−18	Transitional endoplasmic reticulum ATPase TER94 [*Bombyx mori*]	

[a] Protein scores greater than 84 are significant ($p<0.05$).
[b] This is the number of peptide sequences identified by Mascot that contributed to the protein score.
[c] E-values generated by Mascot.
[d] Putative proteins with the highest scores and selected proteins marked by asterisks.

The *Helicoverpa zea* ortholog was transiently up-regulated in HzAM1 cells after addition of 15 μM prostaglandin E1 (by 1.5-fold at 12 h and down-regulated to 42% at 24 h) (Stanley et al., 2008). Interestingly, the glucose regulated proteins of 78 kDa (GRP78) that serve as master regulators of the unfolded protein response (UPR) in the endoplasmic reticulum were found among homologs of *Sf*HSC70(1), namely GRP78 from *Culex quinquefas* (protein acc. no. gi|170034715, E-values 0.00013 and 1.6e−10 for spots no. 1 and no. 1-0, respectively) and the heat shock cognate 3, isoform A (protein acc. no. gi|24641402, E-value 2e−05) from *Drosophila melanogaster* (Table 1). It is possible that HSC70(1) functions as GRP78 and regulates UPR in *S. frugiperda* cells. HSC70(1) is an abundant protein in both mock-infected and AcMNPV-infected cells (Figs. 1A and B). Optical densitometry of the respective Coomassie stained spots showed that the total increase in the HSC70(1) amount in Sf9 cells after the combined stress by AcMNPV-infection and heat shock (HS) at 22 hpi did not exceed 10–15% (the values normalized to actin in the same gels). It means that transitional up regulation of the HSC70(1) gene in Sf9 cells after infection with AcMNPV only modestly enhanced the cellular content of this protein. The results suggest that HSC70(1) is a constitutive member of the HSP/HSC70 family in insect cells that might be additionally up-regulated by different stresses including baculovirus infection.

The most abundant heat shock cognate protein HSC70 in Sf9 cells corresponded to spot no. 2 in the 2D-gels (HSC70(2)) (Figs. 1A and B). The cellular content of HSC70(2) was not changed after the combined stress of AcMNPV-infection and HS. The protein from the AcMNPV-infected cells (spot no. 2, Fig. 1B) was subjected to *in silico* analysis and showed a high homology to HSC70s from other Lepidoptera species (Table 1). The orthologs are strongly expressed in insect cells and may be induced by different stresses. The *Helicoverpa zea* HSC70 (gi|256862214, E-value 1.6e−25) was transiently up-regulated in *H. zea* (HzAM1) cells after addition of 15 μM prostaglandin E1 by 1.8-fold at 12 h and down-regulated to 88% at 24 h (Stanley et al., 2008). The *Heliothis virescens* ortholog of *Helicoverpa armigera* HSP70 (protein acc. no. gi|219671577, E-value 1e−22) was up-regulated by 5- to 7-fold under infection of *H. virescens* (Hv-AM1) cells with AcMNPV (Popham et al., 2010). However, the latter value should be considered with caution. Two proteins with masses in a range from 20 to 25 kDa were used for estimation of the HSP70 content in Hv-AM1 cells (Popham et al., 2010). These fragments might reflect induction of proteolysis in infected cells instead of the HSP70 level. The function of HSC70(2) in cells infected with baculoviruses is unknown. Some data suggests that HSC70(2) may be important for production of infectious viruses. The *Bombyx mori* ortholog of HSC70(2) (gi|112982828, E-value 1e−23) was identified as a component of ODV (Liu et al., 2008). The presence in ODV suggests possible involvement of the HSC70(2) in the assembly of baculoviruses.

Spots no. 3, 4, and 5 in the 2D-gels corresponded to inducible members of the HSP/HSC70 family in Sf9 cells. The respective immunoreactive bands appeared or were highly enlarged in extracts from Sf9 cells after AcMNPV-infection and HS (Lyupina et al., 2010). In the infected and heated cells, spot no. 4 produced a strong fluorescence signal comparable with that generated by HSC70(2) (spot no. 2). At lower exposure, the spot no. 4 was shown to contain at least three overlapping immunoreactive bands referenced as 4–1, 4–2, and 4–3 (compare Figs. 1D1 and D2). The respective bands from the Coomassie stained gel (Fig. 1B) were separately subjected to the *in silico* analysis. Proteins HSP70(3), HSP70 (4–1), HSP70(4–2), and HSP70(4–3) that correspond to the spots no. 3, 4–1, 4–2, and 4–3, respectively, were positively identified as members of the HSP/HSC70 family. These proteins exhibited a high homology to the same set of HSP70s from other *Spodoptera* species including *S. exigua* HSP70, protein acc. no. gi|229562184 (Table 1). Two genes of the *hsp70* family, protein acc. no. gi|229562184 and gi|256862212, were among the most highly up-regulated in Sf21 cells infected with AcMNPV (Salem et al., 2011). There was a dramatic increase in transcripts of these two genes at 6 hpi. Transcripts remained up relative to controls at 12 and 24 hpi, but declined in magnitude. Expression of the *S. exigua* gene (protein acc. no. gi|224999283) that represents a close homolog of *S. exigua* HSP70 with acc. no. gi|229562184 or its isoform was up regulated during heat and cold shock and differentially regulated in development of *S. exigua* (Xu et al., 2011). The second highly up-regulated *hsp70* gene presumable encodes an ortholog of *Sesamia nonagrioides* HSP70 (Salem et al., 2011). Limited homology to *S. nonagrioides* HSP70 (protein access no. gi|167077413) was exhibited by all inducible members of *Sf*HSP70 (Table 1). In the case of HSP70(4–2), the homology to *S. nonagrioides* HSP70 was significant (E-value 0.014).

A Mascot search for homologs of the spot no. 5 protein in the NCBI database did not reveal anything significant. However, a list of 20 proteins with the highest scores contained two HSP70s including the *S. nonagrioides* HSP70 (protein access no. gi|167077413) (Table 1). We suggested that spot no. 5 represents an unknown member of the HSP/HSC70 family referenced here as HSP70(5). It probably retains a specific epitope domain of HSP70s but has highly diverged sequences in other domains. Another factor that might decrease the score of HSP70(5) is overlapping in the gels with unknown host protein marked as 5–0 (Fig. 1A). The latter protein did not react with 7.10.3 antibody, and the list of 20 proteins with the highest homology to the 5–0 protein did not contain any HSP70s. Overlapping with the spot no. 9 protein might also decrease the score for orthologs of HSP70(4–3) although they remained above the significance threshold.

The spot no. 6 protein did not react with the monoclonal antibody 7.10.3 to drosophila HSP/HSC70s (Fig. 1). However, the Mascot search identified unambiguously this protein as a member of the HSP/HSC70 family (Table 1). This second to third in abundance cognate HSP70 was referenced in this report as HSC70(6). The ortholog of HSC70(6) in *Spodoptera exigua* cells (gi|223036830, E-value 2e−19) was called *Sex*HSP74 (Xu et al., 2011). Expression of the corresponding gene was up-regulated during HS and cooling and it was also differentially regulated during development (Xu et al., 2011). The cellular HSC70(6) content was not changed in Sf9 cells after infection with AcMNPV and HS at 22 hpi (Figs. 1A and B).

S. frugiperda HSP90

The *S. frugiperda* chaperone HSP90 with an expected molecular mass of ~83 kDa (Landais et al., 2001) was identified in Sf9 cells by Western blotting with monoclonal anti-HSP90 antibody 16F1 (Fig. 1C). The amount of HSP90 was not significantly altered after the infection with AcMNPV and HS at 22 hpi (Figs. 1A and B). In animal cells, HSP90 is essential for regulation of signal pathways under normal physiological conditions (for review see (Hahn, 2009; Taipale et al., 2010; Usmani et al., 2009)), and has also been shown to participate in the infection cycles of several different viruses (Dutta et al., 2011; Nagy et al., 2011; Radhakrishnan et al., 2010; Shim et al., 2011; Smith et al., 2010; Vozzolo et al., 2010; Wen and Damania, 2010; Zhang et al., 2011). In order to address a possible function of HSP90 in the replication of AcMNPV, we compared amplification of the viral genome in Sf9 cells in the presence or absence of a specific HSP90 inhibitor, a derivative of geldanamycin — 17-AAG (Fig. 2). 17-AAG was added to Sf9 cells after infection with AcMNPV at zero time at a concentration of 2.5 μM that is high enough to completely inhibit the ATPase activity of HSP90 (Usmani et al., 2009). DNA was extracted from the cells at 4-h intervals from 10 to 22 hpi and the relative amount of viral DNA (vDNA) sequences was measured by real time PCR (Lyupina et al., 2010). The doubling time of vDNA during 10 to 22 hpi in the presence of 17-AAG was approximately 1.3-fold lower than that in the control infected cells in the absence of 17-AAG. At the terminal stage of vDNA replication at 22 hpi, the amount of vDNA in infected cells incubated with 17-AAG was 2.2-fold lower

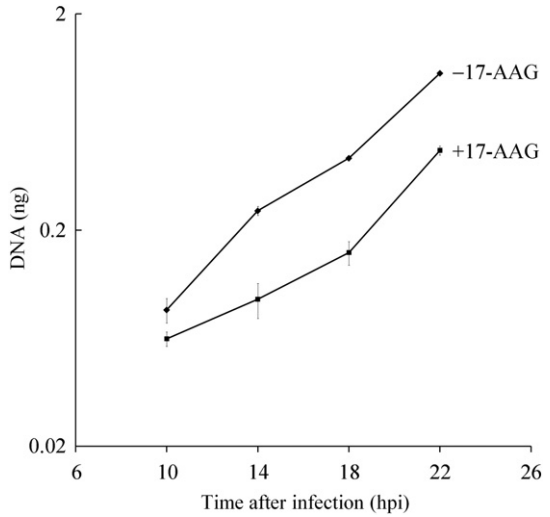

Fig. 2. vDNA synthesis in Sf9 cells infected with AcMNPV and then incubated in the presence of 2.5 mM 17-AAG or in its absence. The vDNA content was determined by RT-PCR in triplicate probes for each time point. The error bars represent the standard deviation.

than that in the control cells. Therefore, 17-AAG inhibited the rate of vDNA synthesis and affected vDNA accumulation in infected cells. HSP90 appeared to play a supportive role in AcMNPV genome replication. However, the inhibitory effect of 17-AAG on vDNA synthesis was lower than in the case when the heat shock response (HSR) was suppressed by KNK437 (Lyupina et al., 2010). In those experiments, KNK437 inhibited the rate of vDNA synthesis in AcMNPV-infected cells by one order of magnitude suggesting that HSP90 function apparently did not play a key role in the stimulatory effect of HSR on virus replication.

Subcellular localization of HSP/HSC70s in AcMNPV-infected cells

The localization of HSP/HSC70s in Sf9 cells was analyzed by confocal microscopy using the monoclonal antibody 7.10.3. The HSP distribution showed dynamic changes in the course of AcMNPV infection (Fig. 3). In uninfected cells, HSP70s (predominantly the cognate members) were localized in both, nucleus and cytoplasm, displaying a diffuse pattern. The first change in the pattern was observed early in infection, at 4 to 10 hpi. At these stages, HSP70s formed distinct speckles in the cytoplasm. The number of speckles varied averaging 2.8 ± 2.7 (STDEV) per infected cell (22 cells at 4 and 10 hpi). Contrary to the infected cells, the cytoplasmic speckles of HSPs in uninfected cells were rare with average number of 0.6 ± 1.2 (STDEV) per cell (46 cells). In the course of infection, the large speckles mostly disappeared by the time at which vDNA synthesis was terminated (24 hpi), when the HSP distribution appeared to be more uniform. The next change in the HSP pattern was observed at 48 hpi, when HSP70s were concentrated mostly in nuclei in granules of different sizes. The accumulation of HSPs in nucleus may be linked to the assembly of viral particles especially taking into account the presence of the abundant HSC70(2) in ODV, as shown for BmNPV (Liu et al., 2008). On the other hand, the cytoplasmic HSP speckles that appeared early in infection may represent aggresomes that were generated as a defense reaction by host cells to virus infection. In order to clarify the origin of the HSP speckles generated at the early stages in infection, we compared the subcellular localization of HSP70s with that of ubiquitinated proteins that serve as hallmarks for the aggresomes (Lahaye et al., 2009). The Sf9 cells were infected at a MOI of 10, fixed at 4 hpi, and treated at first with rat antibody 7.10.3 to HSP70s and then with mouse monoclonal antibody to ubiquitin (FK2) that recognizes mono- and polyubiquitinylated proteins but not free ubiquitin. The primary antibodies were visualized by specific second fluorescent antibodies with different emission spectra (Fig. 4). The absence of cross-reactivity between the heterologous first and second antibodies was confirmed by control experiments (data not shown). The ubiquitinylated proteins showed a subcellular distribution that was non-identical to the distribution of HSPs, but closely coincided with it. Although, the ubiquitinylated proteins were localized mostly in the nuclei and showed a diffuse distribution there, the cytoplasmic fraction was present preferentially in the speckles that colocalized with the HSP speckles. In order to confirm colocalization of the speckles in cytoplasm, a 3D stack of 25 sections was constructed for the nucleus shown in the upper portion of the left-most panels in Fig. 4. The orthogonal projections of the 3D stack with rotation by 70° (Rotation 1) and 90° (Rotation 2) are shown on the right-most panels in Fig. 4. Clear-cut colocalization of the ubiquitinylated proteins with HSP70s confirmed a relation of the cytoplasmic speckles to the aggresomes. These structures were generated in Sf9 cells early in infection with AcMNPV and mostly disappeared at 24 and 48 hpi (Fig. 3).

Discussion

The proteome analysis described in this report allowed the identification of eight members of the HSP/HSC70 family in Sf9 cells including three cognate HSC70s, numbered 1, 2, and 6, and five inducible HSP70s, numbered 3, 4–1, 4–2, 4–3, and 5. Initial identification of *Sf*HSP70s in our previous report was performed by Western blotting with the antibody 7.10.3 to Drosophila HSP/HSC70s (Lyupina et al., 2010). However, HSC70(6) was not detected because it does not react with the antibody. Three HSP70s, 4–1, 4–2, and 4–3, that formed overlapping immunoreactive bands were previously considered as a single HSP70(4). Although Western blotting revealed the increase by approximately 40% in total amount of HSP/HSC70s in Sf9 cells infected with AcMNPV and a several-fold increase after the combined

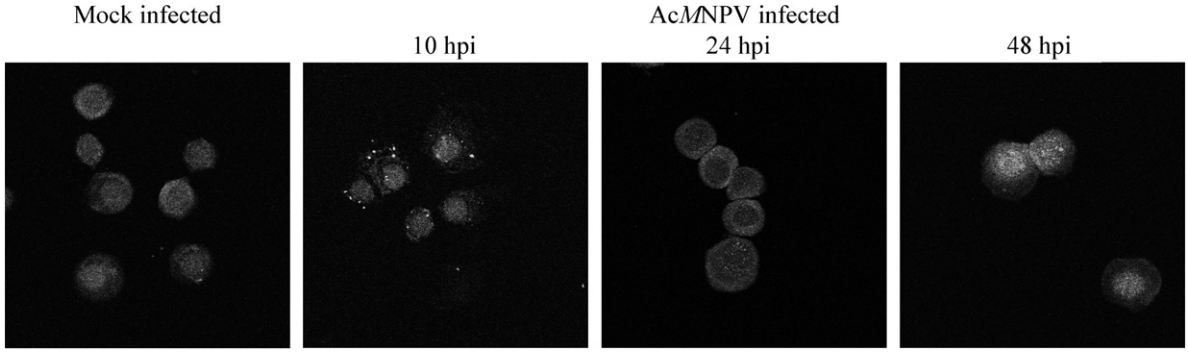

Fig. 3. Subcellular localization of HSP/HSC70s in Sf9 cells mock-infected and AcMNPV-infected and collected at 10, 24, and 48 hpi. The images of immunostained HSPs were obtained by using monoclonal antibody 7.10.3.

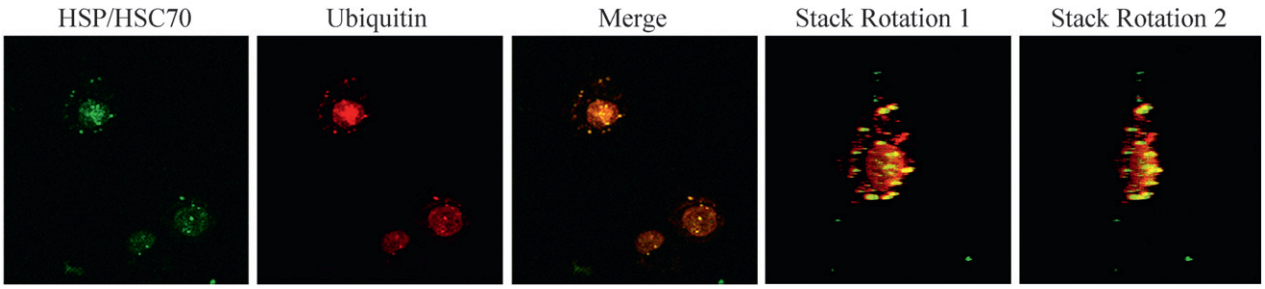

Fig. 4. Subcellular localization of HSP/HSC70s and ubiquitinylated proteins in Sf9 cells infected with AcMNPV and collected at 4 hpi. The cells were stained first with rat monoclonal antibody 7.10.3 to HSPs (green) and then with mouse monoclonal antibody FK2 to ubiquitinylated proteins (red). 25 merge images were taken for construction of 3D stack for one cell. Orthogonal projections of the 3D stack were obtained with rotation by 70° (Rotation 1) and by 90° (Rotation 2).

stress by AcMNPV-infection and moderate heating at 22 hpi (Lyupina et al., 2010), the actual increase in the quantity of HSP70 appeared to be lower when analyzed by Coomassie staining (Fig. 1). The amount of cognate HSC70(1) increased only by 10–15% under the combined stress, the amount of HSC70(2) and HSC70(6) was not changed, whereas the inducible HSP70s gave a total amount less than that of any cognate HSC70. Overestimation of the HSP content in infected and heated cells obtained by the Western blotting resulted from different affinities of the cognate HSC70s and inducible HSP70s to the antibody 7.10.3. Although HSC70(1) and HSC70(2) do interact with the antibody 7.10.3, these proteins showed much lower reactivity in comparison with that of the inducible HSP70s.

The major change in the HSP/HSC70 pattern in AcMNPV-infected Sf9 cells was the induction of five HSP70s. Four of HSP70s numbered as 3, 4–1, 4–2, and 4–3 were highly homologous, though their interrelationship remains unclear. Three HSP70(4) species formed closely located bands in the 2D-gels, and they were induced by AcMNPV-infection or by HS in apparently coordinative manner but differently from HSP70(3) and HSP70(5) (Lyupina et al., 2010). These three coordinately induced HSP70(4)s may be encoded by a single cluster of tightly regulated homologous genes. Connection of HSP70(3) to the presumptive cluster of HSP70(4)s is uncertain. HSP70(3) was induced by stresses differently from HSP70(4)s and had a mass by 6–7 kDa lower. It is known that isoforms of HSP70s may be generated in insect cells as shown for the Drosophila species (Evgen'ev et al., 2004). In the absence of direct genomic information, the five inducible *S. frugiperda* HSP70s may be regarded as individual members of the HSP/HSC70 family.

The identification of the eight proteins of the HSP/HSC70 family and single HSP90 in Sf9 cells allowed for the reevaluation of the dispersed published data on induction of HSPs in the course of baculovirus infection and make a few preliminary conclusions: (i) Baculoviruses induce an early defense response in infected cells that is accompanied with transcriptional up-regulation of *hsp70* genes and selected *hsc70* genes and with the accumulation of HSP/HSC70 proteins. The heat shock response (HSR) in Sf cells infected with AcMNPV is observed as early as 3 to 6 hpi (Lyupina et al., 2010; Nobiron et al., 2003; Salem et al., 2011); (ii) The cognate HSC70s remain abundant species of the HSP/HSC70 family in cells infected with baculoviruses. Transient up-regulation of some *hsc70* genes at the early stages in infection that was shown for *hsc70(1)* (Nobiron et al., 2003; Sagisaka et al., 2010) and suggested for *hsc70 (2)* (Popham et al., 2010) does not dramatically change the cellular content of HSC70s. Although the specific functions of HSC70s in infected cells are unknown, some data suggest the involvement of HSC70(1) in regulation of the unfolded protein response in the endoplasmic reticulum (Table 1) and participation of HSC70(2) in the assembly of viral particles (Liu et al., 2008); (iii) The inducible *hsp70* genes belong to a group that is mostly up-regulated in the course of infection (Salem et al., 2011). Although two different transcripts of *hsp70* genes were reported to be strongly up-regulated in the infected Sf21 cells (Salem et al., 2011), the correspondent homologues mRNAs may encode several HSP70s (presumably five in Sf9 cells) (Table 1). The cellular content of inducible HSP70s increased markedly in infected cells (Lyupina et al., 2010), but they appear to represent a minor fraction of cellular HSPs in comparison to the cognate HSC70s. The importance of the heat shock response (HSR) in Sf9 cells for replication of AcMNPV (Lyupina et al., 2010) suggests that inducible HSP70s may play specific roles in baculovirus replication despite their relatively low cellular content; (iv) Colocalization of HSP70s and ubiquitinylated proteins in the speckles in the cytoplasm of AcMNPV-infected cells (Fig. 4) suggests generation of the aggresomes-like structures that may contain proteins targeted with ubiquitin for digestion and/or sequestration during infection. These data suggest close collaboration of HSPs with the ubiquitin–proteasome system that may participate in baculovirus infection cycle as suggested recently (Katsuma et al., 2011); (v) The moderate inhibitory effect of 17-AAG on viral DNA synthesis (Fig. 2) suggests a supportive role for HSP90 in baculovirus replication; (vi) The published data suggest that the peculiarities in the induction of HSP/HSC70s depend strongly on the origin of cells and the different baculoviruses examined and the details of experimental protocols.

Due to the multiple and highly diverse functions of HSPs in cellular metabolism, the HSP/HSC70 chaperone system serves as an important factor in host cell response to baculovirus infection. The defense reaction featured by HSR may help the cells to survive the stress caused by infection on one hand and on the other hand appears to support the productive replication cycle of baculoviruses.

Materials and methods

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 AcMNPV at the MOI of 10. For the heat shock, the flasks were immersed in a water bath at 37 °C for 2 h. 17-(Allylamino)-17-demethoxygeldanamycin (17-AAG) obtained from Sigma-Aldrich was dissolved in DMSO. The final concentration of DMSO in the culture medium was 0.25% (v/v). The rat mAb 7.10.3 to HSP/HSC70s of *Drosophila melanogaster* was from Lindquist Lab. The rat anti-HSP90 mAb (16F1) and the anti-ubiquitin mouse mAb (FK2) were from Enzo Life Sciences.

MALDI-TOF mass spectrometry

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 Ultraflex II BRUKER (Germany), equipped with UV laser (Nd) and reflectron at the Human Proteome Shared Facility Center in Institute of Biomedical Chemistry (Moscow, Russia). Briefly, proteins of interest in pieces (2×2 mm) from a 2D polyacrylamide gel were washed twice in 100 μl 40% acetonitrile in 0.1 M

NH_4HCO_3, once in 100 μl of acetonitrile and were hydrolyzed with 4 μl of the modified trypsin (Promega) (15 μg/ml in 0.05 M NH_4HCO_3) at 37 °C for 18 h. After mixing with 7 μl 0.5% trifluoroacetic acid (TFA) in 10% acetonitrile, portion of 0.5 μl from the sample was mixed with 0.5 μl 2,5-dihydroxy benzoic acid (Aldrich), 20 mg/ml in 20% acetonitrile and 0.5% TFA, spotted on a MALDI plate and air dried. Mass of the tryptic peptides as positive ions was measured with accuracy of 0.007% (70 ppm). 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.

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 7% FBS and 0.3% Triton X-100 in PBS for 1 h, and then subjected to antibody treatments. Antigen localization was determined by incubation of the cells with rat mAb 7.10.3 to HSP/HSC70s (1:200 dilution with 5% FBS and 0.1% Triton X-100 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, FITC-conjugated goat anti-rat IgG (1:100 dilution, Santa Cruz Biotechnology) for 2 h. After four washes with PBS (10 min per wash), the slides were mounted with the Mowiol. For double staining, the cells were firstly treated for visualization of HSPs as described above. After four washes with PBS (10 min per wash), the cells were incubated with other primary antibody, mouse anti-ubiquitin mAb (FK2) (1:800 dilution with 5% FBS and 0.1% Triton X-100 in PBS) overnight at room temperature. The cells were washed four times (5 min per wash) with PBS and then treated with the secondary antibody, Alexa-633 goat anti-mouse IgG (1:1000 dilution, Invitrogen) 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. For the HSP70/Ub dual-immunostained cells, orthogonal optical plane images were collected into a *z*-stack to confirm colocalization or apposition. The stacks consisted images taken through a *z*-axis at 0.50–0.80-μm step size using the acquisition software of the microscope. 3D reconstructions of the image stacks were analyzed by the ImageJ (Version 1.44 p) software. Control experiments were performed by omitting primary or secondary antibodies.

Other methods

Two-dimensional PAGE was carried out by method of O'Farrell (O'Farrell et al., 1977) as previously described (Lyupina et al., 2010; Ulmasov et al., 1992; Zatsepina et al., 2001). For Western blotting, proteins were transferred on Hybond-ECL membrane (Amersham) and probed with rat sequentially with the rat mAb 7.10.3 to HSP/HSC70s and then with the rat anti-HSP90 mAb (16F1). The anti-rat IgG conjugated to horseradish peroxidase and the ECL detection reagents were from Amersham. 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).

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