

Heterologous expression of *Nosema bombycis* hexokinase in the baculovirus-Sf9 insect cell system confirms its accumulation in the host nuclei and secretion by the microsporidian parasite

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Summary

Hexokinase is one of the most interesting proteins secreted by entomopathogenic microsporidia into an infected cell. Its accumulation in the insect host nuclei suggests that the hexose-phosphorylating enzyme may also be involved in the regulation of transcription of host genes. In this study, a hexokinase of microsporidia *Nosema bombycis* was expressed in Sf9 lepidopteran cells using the baculovirus system. In contrast to green fluorescence protein and gluten-hydrolyzing proteinase of Sunn pest *Eurygaster integriceps*, expressed as the control proteins, the microsporidian recombinant enzyme without N-terminal signal peptide was accumulating in the nuclei of insect cells during heterologous expression. This result one more time confirms the nuclear localization of hexokinases secreted by microsporidia into the insect host cells and suggests that a model used is a convenient one to study an impact of *N. bombycis* hexokinase on the lepidopteran cells. Surprisingly, despite the elimination of signal peptide, the parasite enzyme was effectively secreted into the culture medium by Sf9 cells. Previously, we expressed several secretory proteins with their own or baculoviral glycoprotein gp67 signal peptides, but none of them was secreted as effectively as *N. bombycis* hexokinase. This unexpected result raises the question about signal sequences and mechanisms of protein secretion in microsporidia.

Key words: Microsporidia, *Nosema bombycis*, hexokinase, Sf9 insect cells, baculovirus, heterologous expression, nuclear localization, protein secretion

Introduction

Microsporidia is a large group of fungi-related obligate intracellular parasites infecting almost all phyla of the animal kingdom. Some genera have

been recognized as the pathogens in humans with weakened immunity (Didier and Weiss, 2008). Being widespread insect parasites, microsporidia are important natural and introduced pest control agents on the one hand. On the other one, entomopatho-

genic microsporidia cause destructive epizootics of beneficial and domesticated insects (Bjornson and Oi, 2014). The long adaptation of microsporidia to intracellular lifestyle was accompanied by unique minimization of parasite cell machinery (Katinka et al., 2001), increasing their dependence on the host metabolic system, and the acquisition of effective mechanisms for its exploitation using unique membrane transporters (Heinz et al., 2014; Dean et al., 2018). These data, as well as the fact that the most microsporidian species develop in direct contact with the host cytoplasm, suggest their ability to control the metabolic status of infected host cells. Although the mechanisms for such influence have not been studied well, some interesting information has been accumulated about microsporidian hexokinase (HK) enzyme. Several years ago it was shown that HK of the microsporidium *Nematocida parisii* behaved differently than other glycolytic enzymes (Cuomo et al., 2012) being highly expressed at the early stages of intracellular development. In the same paper, it was shown, that N-terminal signal peptides (SPs) of six microsporidian HKs fused with the reporter enzyme directed its secretion by the yeast *Saccharomyces cerevisiae* cells. The antibodies (Abs) against microsporidia *Paranosema* (*Antonospora*) *locustae* HK, which was expressed in *Escherichia coli*, showed a high rate of secretion of the enzyme and its accumulation in the host cell nuclei (Senderskiy et al., 2014). The secretion of HK and a high probability of its transport into the host nuclei was also demonstrated for two microsporidian parasites of the nematode *Caenorhabditis elegans* (Reinke et al., 2017). In the case of HK of a dangerous silkworm pathogen *Nosema bombycis*, the enzyme secretion and its accumulation in the nuclei of infected cells was confirmed by immunofluorescent microscopy (IFA) (Huang et al., 2018). However, the nuclear localization of secreted HKs of microsporidia may be a feature of the parasites of insects and other invertebrates. Secretion of two HKs of the microsporidium *Trachipleistophora hominis* into rabbit kidney RK13 cells was not accompanied by their transport into mammalian nuclei (Ferguson and Lucocq, 2018).

Another interesting question is related to the functional role of microsporidian HKs in the infected host cell. On the one hand, metabolic activity of *T. hominis* HKs was indirectly confirmed using 2-NBDG fluorescent analog of glucose (Ferguson and Lucocq, 2018). Recently, we overexpressed active *Vairimorpha* (*Nosema*) *ceranae* and *N. bom-*

bomyis HKs in *E. coli* and demonstrated that their kinetic characteristics are comparable with those of HKs from other organisms (Dolgikh et al., 2019). On the other hand, secretion of the enzyme by many microsporidian species and its nuclear localization suggest a regulatory role in the host-parasite relations.

An effective instrument for the functional analysis of microsporidia HKs should be their expression in the heterologous systems imitating the host cell. For insect microsporidia, it may be lepidopteran cell lines widely used for heterologous expression of many proteins. Previously we demonstrated that heterologous expression of *P. locustae* HK without N-terminal SP in lepidopteran Sf9 cells was accompanied by its accumulation in the insect cell nuclei (Timofeev et al., 2017). However, *N. bombycis* HK (NbHK) fused with DsRed fluorescent protein and expressed in BmN cells of silkworm *Bombyx mori* was not found in the insect nuclei (Huang et al., 2018) even though this species is the natural parasite of the silkworm.

In this study, NbHK without N-terminal 22-amino acid (a/a) SP and without any additional recombinant tags was expressed in lepidopteran Sf9 cells using the baculovirus system. To localize NbHK in the insect cells, its fragment was overexpressed in *E. coli* and polyclonal Abs against the recombinant protein were produced. IFA with anti-NbHK Abs confirmed the accumulation of the microsporidian enzyme in the nuclei of insect cells during its heterologous expression. At the same time, immunoblotting of the cells infected with recombinant baculoviruses and the medium after their growth showed an effective secretion of NbHK despite the removal of N-terminal SP.

Material and methods

PLASMID CONSTRUCTS

To express NbHK in the baculovirus system, we amplified 1221 bp DNA fragment encoding the enzyme without 22-a/a SP. NbHK gene copy, previously cloned in pOPE101 vector (Dolgikh et al., 2019), forward primer ccaaGAATTCATGGAATTAATTAAGACATTGGGAA (*EcoRI* site is underlined) and reverse primer gtcaCTGCAGTTAATAAATAATTCGATGTAAAGT (*PstI* site and stop codon are underlined), as well as Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher

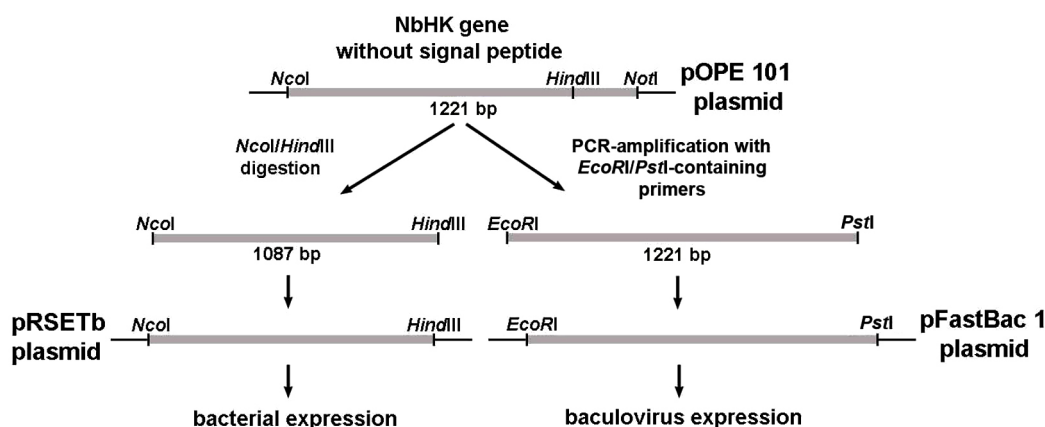


Fig. 1. The scheme illustrating the construction of plasmids for NbHK expression in the baculovirus system and for expression of its fragment in bacteria *E. coli*.

Scientific, MA), were used for PCR. The amplified gene copy of 1221 bp was inserted into the pFastBac 1 vector (Thermo Fisher Scientific) at *EcoRI*/*PstI* sites (Fig. 1).

To express gluten-hydrolyzing proteinase of the Sunn pest *Eurygaster integriceps* GHP3 (Konarev et al., 2011) as a control protein (EiGHP3), the sequence encoding full-size protein was amplified using the gene copy previously cloned in pPIC3.5 vector (Dolgikh et al., 2014), forward primer ccatAG ATCTATGCGGTGTACATTGGTACTGGT (*BglII* site is underlined) and reverse primer agtcGAA TTCTTACTTCGATTCTTTGACATC (*EcoRI* site and stop codon are underlined). For expression of enhanced green fluorescent protein (eGFP) in insect cells, its encoding sequence was PCR-amplified with forward primer ccatGAATTCATGGTGA GCAAGGGCGAGGAGCTG (*EcoRI* site is underlined) and reverse primer gtcaCTCGAGTTACTTG TACAGCTCGTCCATGCCGA (*XhoI* site and stop codon are underlined) using pEGFP-N3 plasmid (Clontech Lab Inc., CA) as a matrix. The PCR products were gel purified, digested with the restriction enzymes, and inserted into vector pFastBac 1 linearized by the *BamHI*/*EcoRI* or *EcoRI*/*XhoI* enzymes respectively. The constructed plasmids were used to transform competent *E. coli* DH10Bac cells containing the baculovirus genome in bacmid form.

To overexpress NbHK fragment in *E. coli* for production of specific Abs, the part of the gene, previously cloned in pOPE101 plasmid and flanked by *NcoI* and *HindIII* sites (1087 bp), was re-cloned in pRSETb expression vector (Thermo Fisher Scientific) (Fig. 1).

HETEROLOGOUS EXPRESSION OF NBHK FRAGMENT IN *E. COLI*, RECOMBINANT PROTEIN ISOLATION, AND PRODUCTION OF POLYCLONAL ABS

E. coli BL21(DE3)-derived C41 cells (Miroux and Walker, 1996) were electroporated by pRSETb plasmid carrying 1087 bp fragment of NbHK gene at 1700 V using Electroporator 2510 (Eppendorf, Germany). Bacterial colonies from agar plates with Luria Broth (LB) medium containing 0.15 mg/ml ampicillin were inoculated into flasks with 100 ml of the same liquid medium. The cultures were grown to OD₆₀₀ 0.6, then NbHK expression was induced by the addition of 0.7 mM IPTG (final concentration) with the following incubation at 37 °C for 15 h. After cultivation, bacterial cells were pelleted by centrifugation at 4000 g for 10 min and sonicated in 4 ml of 50 mM Tris-HCl buffer solution (TB, pH 7.5). The bacterial homogenate was centrifuged at 14000 g for 15 min and 5 µl of supernatant and the pellet resuspended in TB to homogenate volume were analyzed by SDS-PAGE in 12% gel (Laemmli, 1970). To purify the recombinant NbHK fragment, the upper layer of the pellet (bacterial membranes) was removed by careful pipetting. The lower white layer of inclusion bodies (IBs) significantly enriched in the recombinant protein was washed in TB, resuspended in TB with 8 M urea and incubated 10 min at room temperature. After centrifugation at 14000 g for 5 min, 5 µl of supernatant and the pellet resuspended in TB to the supernatant volume were also analyzed by SDS-PAGE. The recombinant protein dissolved in the presence of 8M urea was used for rabbit immunization, production and

purification of specific anti-NbHK polyclonal Abs using previously described methods (Dolgikh et al., 2009).

RECOMBINANT BACMID PREPARATION

DH10Bac chemical competent cells were transformed with 1 ng of pFastBac1 plasmid carrying NbHK gene and plated on LB agar containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml X-gal and 40 µg/ml IPTG. The plates were incubated for 48 h at 37 °C. White colonies, which included recombinant bacmid *E. coli* clones, were picked and analyzed by PCR with pUC/M13 primers (Bac-to-Bac Baculovirus Expression System Protocol, Thermo Fisher Scientific) and DreamTaq Green PCR Master Mix (Thermo Fisher Scientific).

To isolate a recombinant bacmid, a single white PCR-positive colony was inoculated into 50 ml Super Optimal Complete Broth (SOC) supplemented with the aforementioned antibiotics and grown at 37 °C to stationary phase (up to 48 hours). Pelleted at 14000 g for 1 min cells were resuspended in 5 ml solution containing 20 mM Tris-HCl (pH 8.0), 1 mM EDTA. After mixing with 8 ml solution of 0.2 M NaOH, 1% sodium lauroylsarcosine, the tube was incubated at room temperature for 5 min. After gentle addition of 10 ml 3 M potassium acetate (pH 5.5) the sample was kept at –20 °C for 20 min. Bacterial proteins and genomic DNA were pelleted at 14000 g for 10 min and the supernatant was transferred to another tube with 15 ml isopropanol. Mixed by gentle inverting the tube was centrifuged at 14000 g for 15 min at room temperature. Dried pellet was dissolved in 500 µl TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA), 20 µg/ml RNAase, incubated at 25 °C for 10 min and purified successively with 500 µl phenol, 500 µl phenol:chloroform:isoamyl alcohol (25:24:1) and the same volume of chloroform. Bacmids were precipitated at 14000 g for 10 min after the addition of 50 µl of 3 M potassium acetate (pH 5.5) and 1 ml of 96% ethanol. Washed with 80 % ethanol pellet was dried, dissolved in 150 µl sterile water and stored at +4 °C.

INSECT CELLS TRANSFECTION AND PRODUCTION OF RECOMBINANT PROTEINS

Spodoptera frugiperda (fall armyworm) Sf9 cell line was obtained from European Collection of Authenticated Cell Cultures (ECACC, UK), General collection, Catalog No: 89070101. Cells were

cultured in Sf900III serum-free medium (SFM) (Thermo Fisher Scientific) and routinely maintained according to the manufacturer's instructions. Cells were transfected in the mid-log phase growth with viability over 90%. Transfection procedure was carried out in a well of 6-well tissue culture plate (Eppendorf) with 8×10⁵ adhesive Sf9 cells in 2 ml SFM. 2 µg of recombinant bacmid DNA and 8 µl of Cellfectin II reagent (Thermo Fisher Scientific) were diluted in 200 µl of SFM, incubated 15 min and added to the cells. Infected cells were cultivated 5 days to amplify the next generation of recombinant baculoviruses with an increased titer of viral particles. To gain baculovirus stocks 5 passages were made. Production of each recombinant protein was carried out in a well of 6-well tissue culture plate with 2×10⁶ Sf9 cells infected by 100 µl of corresponding viral stock with total volume 2 ml SFM.

ANALYSIS OF NBHK EXPRESSION IN Sf9 CELLS

Western blot analysis of recombinant proteins in infected Sf9 cells and culture medium with anti-NbHK or previously produced anti-EiGHP (Dolgikh et al., 2014) Abs was performed on 3, 5 and 7 days post infection (dpi) as previously described (Dolgikh et al., 2009). Cells were dislodged from surface of well, the suspension was centrifuged at 500 g for 5 min and supernatant (culture medium) was concentrated tenfold in Amicon 10 kDa centrifugal concentrators (Merck, Germany). Cell pellet was resuspended in fresh SFM to the volume of concentrated medium and 5 µl of both samples were analyzed by immunoblotting.

Immunofluorescence assay (IFA) of Sf9 cells also was previously described (Timofeev et al., 2017). The cell suspension 3 dpi was fixed in an equal volume of 8% paraformaldehyde (Serva, Germany) prepared in phosphate-buffered saline. The preparations in Prolong glass antifade mountant (Thermo Fisher Scientific) were observed with Carl Zeiss Axio Imager M1 fluorescent microscope.

Results and discussion

OVEREXPRESSION OF NBHK FRAGMENT IN *E. COLI*

To express the microsporidian enzyme in insect cells while keeping its natural, native conformation, we did not add any additional tag or label to the NbHK sequence. Therefore, we had to express it also in bacteria for production of specific Abs and

Fig. 2. The SDS-PAGE analysis of NbHK fragment expressed in *E. coli*. A – Bacteria were grown to OD₆₀₀ 0.6 and incubated with (lanes 3, 4) or without (lanes 1, 2) 0.7 mM IPTG at 37 °C for 15 h. After cultivation bacteria were sonicated in 50 mM Tris-HCl buffer solution (TB, pH 7.5) and centrifuged at 14000 g for 15 min. Supernatants (lanes 1, 3) and the pellets resuspended in TB to supernatant volume (lanes 2, 4), were analyzed by SDS-PAGE. B – Isolated IBs were resuspended in TB containing 8 M urea, incubated for 10 min and centrifuged at 14000 g for 5 min. Re-extraction of the pellet with the same solution was followed by SDS-PAGE analysis of both supernatants (lanes 1, 2) and the final pellet resuspended in TB to the volume of supernatants (lane 3). Gels were stained with Coomassie Brilliant Blue R-250. The recombinant NbHK fragment is indicated by *arrows*.

immunodetection. The gene fragment of 1087 bp, encoding 362 a/a polypeptide, was cloned in the pRSETb expression vector. Transformation of C41 *E. coli* cells by the constructed plasmid, heterologous expression, sonication of bacteria and centrifugation of the homogenate followed by SDS-PAGE analysis demonstrated a high level of the recombinant protein synthesis in the form of IBs (Fig. 2, A, lane 4). Removal of supernatant and upper pellet layer of bacterial membranes enabled us to get white fraction of IBs significantly enriched in the recombinant protein. Treatment of isolated IBs with 8M urea showed not very effective solubilization of

Fig. 3. Immunoblotting of culture medium (lanes M) and Sf9 cells (lanes C) cultivated after their infection with recombinant baculoviruses carrying the genes of *Nosema bombycis* hexokinase (NbHK) or Sunn pest *Eurygaster integriceps* proteinase (EiGHP, control protein).

the recombinant protein in the presence of this chaotropic agent (Fig. 2, B). However, due to a very high level of NbHK expression in bacteria, the amount of antigen solubilized with this procedure was sufficient for immunization of animals and production of specific Abs. Polyclonal NbHK-specific Abs were raised in rabbits, purified against immobilized recombinant protein and used for localization of the parasite enzyme in insect cells.

NbHK EXPRESSION IN Sf9 CELLS IS FOLLOWED BY ITS EFFECTIVE SECRETION INTO THE CULTURE MEDIUM

Infection of Sf9 culture with recombinant baculoviruses carrying the microsporidian gene followed by its cultivation up to 7 days and by the immunoblotting, showed that the produced polyclonal Abs were specific against NbHK. They recognized a single protein band about 46 kDa in size, which was identified both in cells and in the culture medium (Fig. 3). The protein size approximately corresponded to the predicted molecular weight of NbHK (47 kDa). Since the volume of concentrated medium after NbHK expression and the volume of cells resuspended in fresh SFM, were the same, the intensity of protein band staining on M and C lanes of Fig. 3 reflects the ratio of intracellular and secreted forms of the enzyme. The results of immunoblotting showed that NbHK amounts accumulated in cells and secreted into the medium were approximately the same on the third day after infection. Further cultivation led to an increasing accumulation of the enzyme outside of Sf9 cells.

The effective NbHK secretion in the baculovirus expression system was surprising, because, as in our previous experiments on HK of the microsporidium *P. locustae* (Timofeev et al., 2017), we intentionally

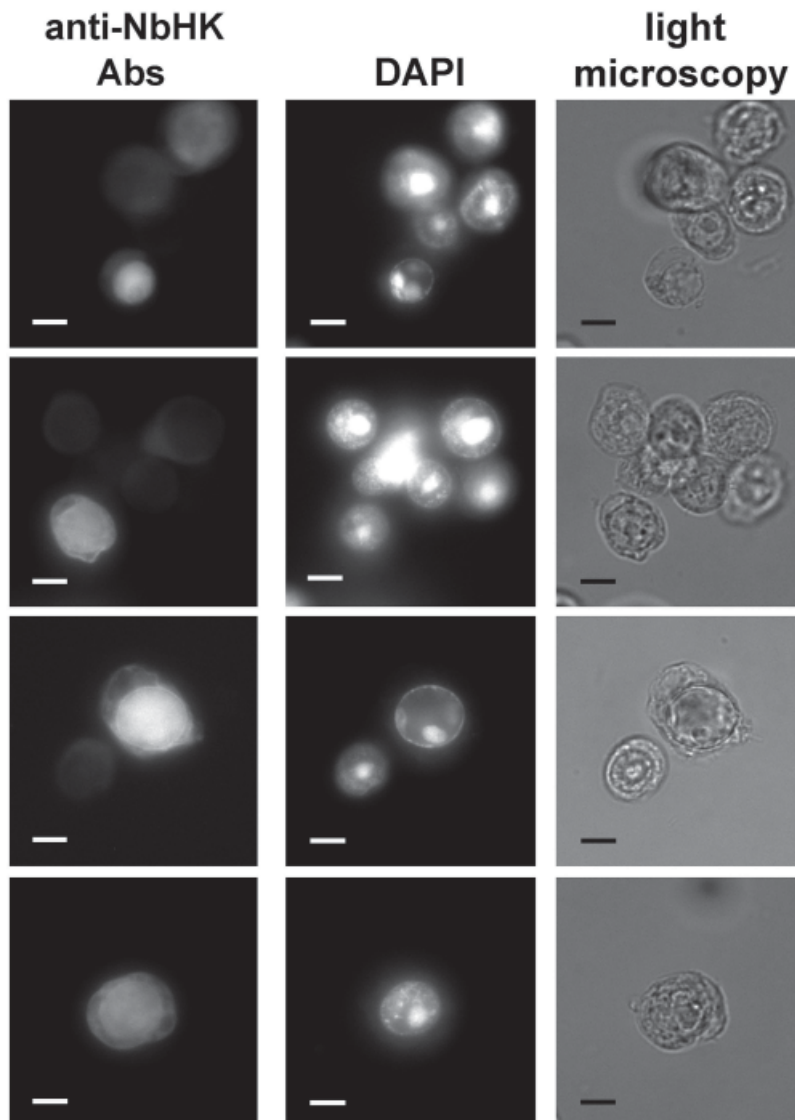


Fig. 4. Immunolocalization of *Nosema bombycis* hexokinase expressed in *Spodoptera frugiperda* Sf9 cells using the baculovirus system. Staining of the cells with anti-NbHK Abs and DAPI demonstrated localization of the microsporidian protein within the insect cell nuclei. Scale bars: 10 μ m.

removed the N-terminal 22-a/a SP from NbHK to prevent its entry into the secretory pathway and to imitate the process of accumulation of the parasitic protein in the cytoplasm of an infected host cell. Previously, we expressed secretory proteins with their own or baculoviral glycoprotein gp67 SPs. In this study, gluten-hydrolyzing proteinase EiGHP was expressed in parallel as the control protein (Fig. 3). However, none of them was secreted as effectively as NbHK. This unexpected finding suggests that NbHK secretion may occur through some unconventional pathway independent of protein translocation into the lumen of the endoplasmic reticulum (ER). The ER-independent intracellular transport was

reported for some secreted and membrane proteins in mammals (Rubartelli, 1990; Mignatti et al., 1992; Lindstedt et al., 1993), viruses (Chang et al., 1997; Lecellier et al., 2002), and protozoa (Denny et al., 2000). These proteins lack the SP responsible for their entry into the ER lumen (Nickel, 2003). Interestingly, although we identified the N-terminal SP in NbHK using SignalP 5.0, TargetP 1.1, and TargetP 2.0 servers with a probability of 0.63, 0.62 and 0.85 respectively, it was not predicted by SignalP 3.0, SignalP 4.1, PrediSi, Signal-3L programs. The latter result supports to some extent the idea that NbHK may be secreted by some ER-independent, still unexplored mechanism.

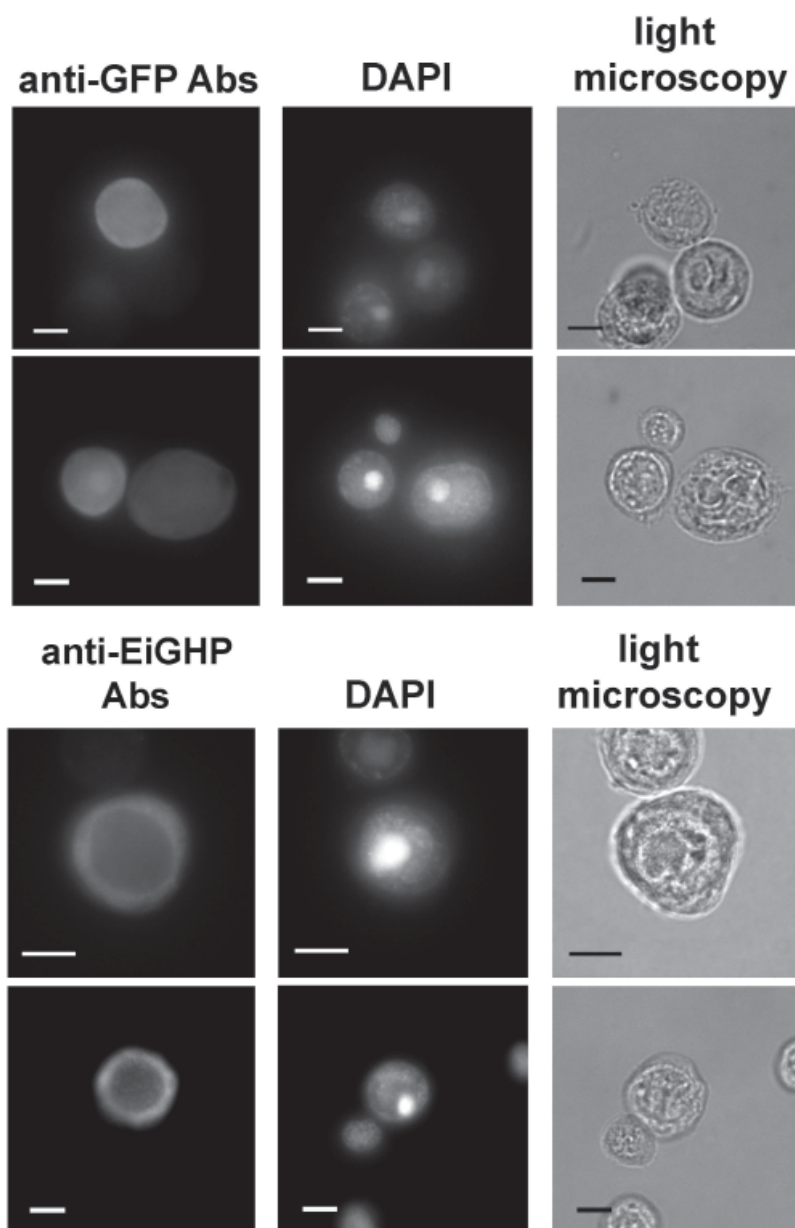


Fig. 5. Heterologous expression of eGFP (top two rows) and Sunn pest *Eurygaster integriceps* proteinase GHP3 (EiGHP, two lower rows) in Sf9 cells using baculovirus system demonstrated localization of both control proteins outside of the nuclear zone stained with DAPI. Scale bars: 10 μ m.

NUCLEAR LOCALIZATION OF NbHK EXPRESSED IN Sf9 CELLS

Considering the strong effect that baculoviruses have on insect cells, we set two controls at once, infecting the cell culture with three recombinant viruses carrying NbHK gene, eGFP gene and the sequence encoding EiGHP. IFA of the cells cultivated 3 days post baculovirus infection demonstrated that the microsporidian enzyme was accumulated in the nuclei of insect cells during its heterologous

expression (Fig. 4). Neither eGFP nor proteinase EiGHP expressed in the baculovirus system as control proteins, showed nuclear localization (Fig. 5). In both cases, the cytoplasm of infected cells was brighter than the nucleus area. Taking into account this result, as well as the data from previous experiments on the expression of tag-free (Timofeev et al., 2017) and fluorescently labeled (Huang et al., 2018) HK forms, we can suggest that fusion of a microsporidia enzyme with any additional protein may abolish its transport to the nucleus in the insect

cells. Anyway, the nuclear localization of NbHK expressed in the baculovirus-lepidopteran cell line system without any tag, offers a convenient model to explore the impact of this parasite enzyme on the insect cells.

Previously, HK was discovered as a bifunctional protein in the yeast (Petit et al., 2000; Moreno and Herrero, 2002), cancer cells (Neary and Pastorino, 2010) and plants (Moore et al., 2003) demonstrating dual nucleocytoplasmic cellular localization. In infected insect cells, microsporidian HKs may be involved in upregulation of uptake of exogenous glucose. It could intensify the processes of microsporidia replication and sporogenesis, as well as ATP production by host mitochondria (Hacker et al., 2014). Large amounts of carbohydrates are required to form microsporidian spore, which has a thick, chitin-rich wall and highly glycosylated polar tube (Xu et al., 2004, Beznoussenko et al., 2007). This hypothesis is also supported by observations on yeasts, in which HK2 diminished expression of high-affinity hexose carriers and elevated expression of low-affinity transporters in the presence of high glucose concentrations (Petit et al., 2000). Further analysis of gene transcription of hexose transporters and glucose uptake in NbHK-expressing lepidopteran cells should verify this hypothesis.

Acknowledgments

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