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Ubiquitins of *Bombyx mori* nucleopolyhedrovirus and *Helicoverpa armigera* nucleopolyhedrovirus show distinct subcellular localization in infected cells

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Summary. – Ubiquitin (UB) is a conserved protein that regulates a number of processes in eukaryotic cells. Nearly all lepidopteran baculoviruses encode UB homologs showing a partial sequence identity with human UB (Hu-UB). In this study, the sequence, predicted 3D-structure and subcellular localization of UB homologs encoded by two different nucleopolyhedroviruses of *Bombyx mori* (BmNPV) and *Helicoverpa armigera* (HaNPV) were compared. UBs of BmNPV and HaNPV (Bm-UB, Ha-UB, respectively) shared only 73% of sequence identity of the different aa in relation to Hu-UB being localized in non-conserved parts, namely in two heterogeneous regions of aa 15–32 and aa 53–60. Interestingly, Bm-UB and Ha-UB share the same seven lysines except for an additional Lys54 in Bm-UB. However, in spite of the sequence heterogeneity, Bm-UB and Ha-UB have a similar predicted 3D-structure. A difference in their subcellular localization during virus growth in insect cell lines was found in the late stage of formation of occlusion-derived virus (ODV). In particular Bm-UB was localized mainly and evenly in the nucleus, while Ha-UB on the nuclear membrane. These data suggest that (i) UBs, besides being engaged in various cellular processes, have a role in specific processes of virus growth, and (ii) Bm-UB and Ha-UB may show certain different activities associated with the virus growth.

Keywords: Bombyx mori nucleopolyhedrovirus ubiquitin; Helicoverpa armigera nucleopolyhedrovirus ubiquitin; sequence analysis; subcellular localization

Introduction

UB is a protein containing 76 aa found in all eukaryotic cells. Its sequence is extremely well-conserved ranging from protozoan to vertebrates. UB acts through its post-translational attachment (ubiquitination) to the proteins and takes part in many diverse cellular processes such as the protein degradation, receptor internalization, vesicle sorting, DNA repair, protein kinase activation, and gene silencing (Hurley *et al*., 2006). In addition, UB is also involved in some important viral processes (Boname and Lehner, 2008). For instance, human papilloma virus directs degradation of retinoblastoma protein pRb through the UB-proteasome pathway to facilitate the virus infection (Mammas *et al*., 2008; Isaacson and Ploegh, 2009). Moreover, UB is required for the budding of retroviruses from infected cells (Patnaik *et al*., 2000; Strack *et al*., 2000).

So far many sequenced baculoviruses encode UB homologues. Baculoviral UB is a structural protein being part of the budded virus (BV) particle and attached to the inner face of BV envelope by a covalently linked phospholipid anchor (Guarino *et al*., 1995). The disruption studies suggest that baculovirus UB is not essential to the viral DNA replication, but it does participate in the budding of virion and in the virus growth (Reilly and Guarino, 1996). It's supposed that it may function in the baculovirus replication to prevent degradation of the useful proteins required for virus infection by the host degradation pathway (Haas *et al*., 1996). Although some progress is made in the studies of baculovirus UB, its function remains elusive.

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Abbreviations: BmNPV = *Bombyx mori* nucleopolyhedrovirus; Bm-UB = BmNPV ubiquitin; BV = budded virus; EGFP = enhanced green fluorescence protein; HaNPV = *Helicoverpa armigera* nucleopolyhedrovirus; Ha-UB = HaNPV ubiquitin; Hu-UB = human ubiquitin; ODV = occlusion-derived virus; p.i. = post infection; UB = ubiquitin

Table 1. Primers used in this study

Primer designation	Primer sequence[a] (5'→3')
BmUB-F	T CTCGAG CT ATG CAA ATA TTC ATC AAA AC
BmUB-R	T CTGCAG TTA ATA CCC TCC TCG TAA TCG
HaUB-F	T CTCGAG CT ATG CAG ATA TTT GTT AAA AC
HaUB-R	T CTGCAG TCA GAA ACC AGT TCG AAT TG
BmV24623	C GAATTC TGAAAATCACACCACAGCCAAT
BmV25034	CTCCTCGCCCTTGCTCACCATTTTTACA CTATTACTTATAAATG
EGFP-F	ATG GTG AGC AAG GGC GAG GAG
EGFP-R	T CTGCAG TTA CTT GTA CAG CTC GTC CAT GC
HaV24523	T GAATTC GATACGTATTCGTCTGGATAG
HaV24924	CTCCTCGCCCTTGCTCACCATGATG TTAACTTTTGTAACGCAAG

[a]Restriction sites are underlined.

In this study, we compared (i) the sequences and predicted 3D-structures of Bm-UB and Ha-UB and (ii) their subcellular localization in virus-infected cells. Its results suggest that Bm-UB and Ha-UB show some common as well as distinct characteristics and may exert certain different activities associated with the virus growth.

Materials and Methods

Cells, bacterial strains, bacmids, plasmids, and primers. *Helicoverpa zea* cell line HzAM1 and *Bombyx mori* cell line BmN were cultured at 27°C in TNM-FH and TC-100 insect medium (Sigma-Aldrich) supplemented with 10% (v/v) heat-inactivated FBS (Gibco-BRL). The *Escherichia coli* strain BmDH10Bac containing the BmNPV genome bacmid and a helper plasmid pMON7124 encoding a transposase was provided by Dr. Enoch Y. Park (Motohashi *et al*., 2005). *E. coli* DH10B containing the HaNPV bacmid (HaBacHZ8) and pMON7124 was provided by Dr. Hua Ling Wang (Wang *et al*., 2003). All primers used in this study are listed in Table 1.

3D-structure modeling. Molecular models of Bm-UB and Ha-UB were generated by submitting the relevant protein sequences to Swiss-Model using the automated mode (Guex and Peitsch, 1997; Schwede *et al*., 2003; Arnold *et al*., 2006). Swiss-Model outputs were opened with the program RasMol (v2.7.5). The 3D-structure comparison of Bm-UB and Ha-UB was performed with MATRA program (Kawabata and Nishikawa, 2000; Kawabata, 2003).

Bm-UB and Ha-UB fused with enhanced green fluorescence protein (EGFP). To express Bm-UB fused with EGFP under the control of Bm-UB gene promoter, a splicing by overlapping extension PCR (SOE-PCR), with some modifications, was performed according to the procedure described by Warrens *et al*. (1997). Firstly, the Bm-UB-encoded region was amplified with primers BmUB-F and BmUB-R and cloned into *Xho*I and *Pst*I sites of plasmid pEGFP-C1 (BD Biosciences Clontech) to produce vector pEGFP-BmUB. Secondly, two DNA fragments were amplified by PCR with Pyrobest DNA polymerase (TaKaRa Bio). On e DNA fragment from the BmNPV genome was amplified with primer pair BmV24623/BmV25034, the other one from plasmid pEGFP-BmUB was amplified with the primers EGFP-F and BmUB-R. After being purified, the two DNA fragments were mixed for the third PCR reaction by rTaq DNA polymerase (TaKaRa Bio) with primers BmV24623 and BmUB-R to obtain a sequence expressing Bm-UB fused to the C-terminus of EGFP under the control of Bm-UB gene promoter. The final PCR product was cloned into pGEM-T Easy vector (Promega) and sequenced using T7 or SP6 primer. To construct a plasmid expressing Ha-UB fused with EGFP, the Ha-UB gene was amplified from HaNPV bacmid by PCR with the primers HaUB-F and HaUB-R. The primer pairs for SOE-PCR were designed as HaV24523/HaV24924, EGFP-F/HaUB-R, and HaV24523/HaUB-R for each step of PCR reaction. To construct a plasmid expressing EGFP alone, the primers EGFP-F and EGFP-R were applied.

To introduce EGFP and Bm-UB (or Ha-UB)-encoded sequence into the BmNPV (or HaNPV) bacmid, a donor plasmid was constructed as follows. Initially, the EGFP-Bm-UB- (or Ha-UB) encoded sequence was retrieved by digestion with *Eco*RI and *Pst*I from pGEM-T Easy vector and cloned into *Eco*RI-*Pst*I-digested plasmid pFast (Guo *et al*., 2010). The produced plasmids were named as pFast-EGFP-BmUB (or pFast-EGFP-HaUB) and transformed into *E. coli* BmDH10Bac (or DH10B), to obtain positive bacmids. *E. coli* extraction and transfection into insect cells of recombinant bacmids were performed according to the instruction manual of BAC-TO-BAC™ Baculovirus Expression Systems (Invitrogen Life Technologies), to obtain BVs. The BV titer was determined by the end-point dilution (King and Possee, 1992).

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         1        10        20        30        40        50        60        70    76
Bm-UB: MQIFIKTLTGKTITAETEPAETVADLKQKIADKEGVPVDQQRLIFAGKQLEDSKTMADYNIQKESTLHMVLRLRGG
Ha-UB: MQIFVKTLTGKTITVDVESSDSVETVKEKIAAKEGVPVDQQRLIYAGKQLEDSMTMNDYSIQKEATLHLVLRLRGG
Hu-UB: MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG
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Fig. 1

Sequences of Bm-UB, Ha-UB, and Hu-UB

aa sites (numbered), heterogeneous regions (shaded), lysines (K).

Confocal laser scanning microscopy. A monolayer (about 1.0×10^6, 70–80% confluence) of cells was infected with BV at the MOI of 20. At 18, 24, and 48 hrs post infection (p.i.), the infected cells were visualized using a Leica confocal laser scanning microscopy and scored for subcellular localization analysis.

Results

Sequence analysis and 3D-structure modeling of Bm-UB and Ha-UB

Search in the databases revealed that UB homologues were found in all of the sequenced lepidopteran baculoviruses except for *Leucania seperata* nucleopolyhedrovirus (Xiao and Qi, 2007). The Bm-UB was encoded by a group-I NPV, BmNPV, whereas the Ha-UB was expressed by a group-II NPV, HaNPV. They shared 73% sequence identity (Fig. 1). However, homolog modeling and 3D-structure comparison suggested that Bm-UB and Ha-UB shared similar 3D-structures to Hu-UB (data not shown). Several recognition sites essential for UB function are conserved on the structures, such as the hydrophobic patch including Leu8, Ile44, and Val70 that can interact with different proteins, the cluster containing Gln2, Phe4, and Thr12 that are responsible for endocytosis, the tail patch consisting of Leu73, Arg74, Gly75, and Gly76 that are important for UB conjugation and deubiquitination (Wilkinson *et al.*, 1995; Sloper-Mould *et al.*, 2001; Hurley *et al.*, 2006).

Baculovirus UBs have two interesting features that are different from the Hu-UB. First, baculovirus UBs are most distantly related to Hu-UB that only 51/76 aa are conserved from baculovirus to eukaryotic UB, compared with 73/76 aa conservation between yeast and animal UB. Bm-UB and Ha-UB had 76% sequence identity with Hu-UB. The most heterogeneous parts are the regions aa 15–32 and 53–60 (Fig. 1). Moreover, the Ha-UB carries seven conserved lysines, whose spatial locations on predicted 3D-structure are similar to those on Hu-UB 3D-structure. Besides these lysines, Bm-UB contains an additional Lys54 (Fig. 1), whose ε-amino group is fully exposed on the surface of predicted 3D-structure (data not shown).

Subcellular localization of Bm-UB and Ha-UB in virus-infected cells

The Bm-UB and Ha-UB fused with glutathione S-transferase were previously expressed in *E. coli* and used to generate a polyclonal antiserum. However, the produced polyclonal antibody to Bm-UB or Ha-UB was too nonspecific to be used in exact assays. Therefore, the expression of Bm-UB (or Ha-UB) fused to EGFP C-terminus under the control of self gene promoter was employed to investigate the subcellular distribution of Bm-UB and Ha-UB in the infected insect cells. Throughout the experiment, the expression of EGFP alone was evenly dispersed in whole cell (data not shown). The fluorescent cells could be observed at 14 hrs p.i. indicating that the Bm-UB and Ha-UB were lately expressed.

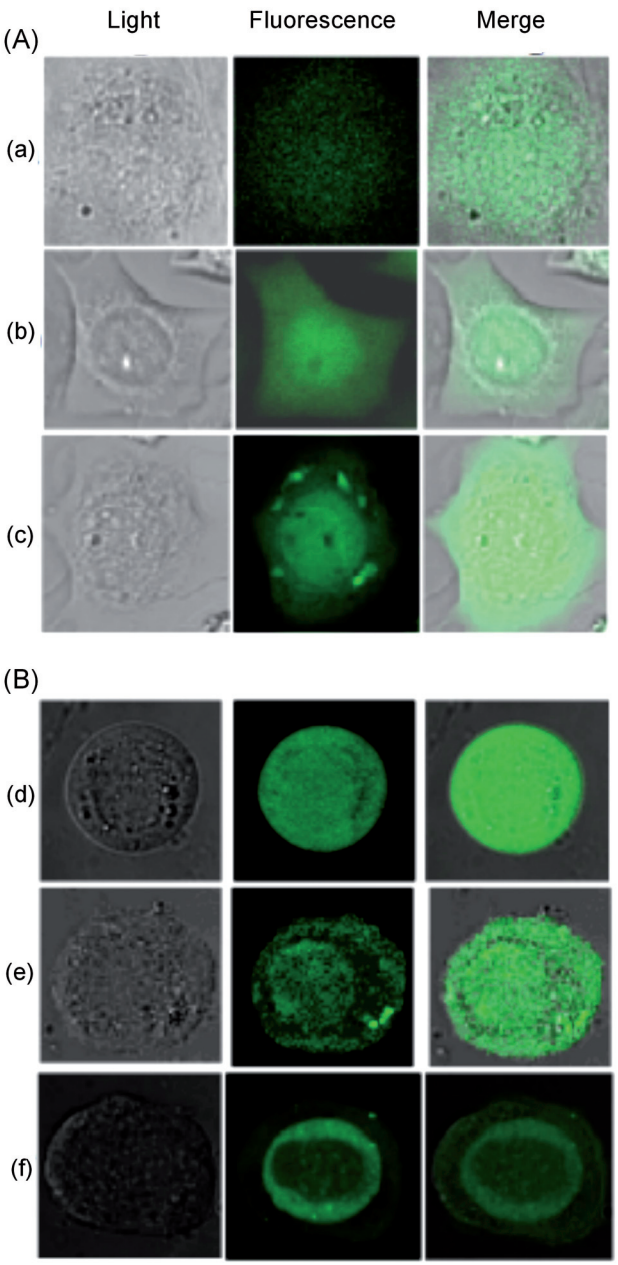

Fig. 2

Three forms of subcellular localization of Bm-UB and Ha-UB fused with EGFP in virus-infected cells

Cells infected with BVs harboring the gene EGFP-Bm-UB (A), EGFP-Ha-UB (B). Pancellular even dispersal (a, d), nucleus-dominated distribution (b, e), nucleus dispersal together with dot distribution in the cytoplasm (c), nuclear membrane distribution (f).

Table 2. Subcellular localization of Bm-UB and Ha-UB in infected BmN cells

p.i. (hrs)	Bm-UB†			Ha-UB†		
	a (%)	b (%)	c (%)	d (%)	e (%)	f (%)
18	37.1	61.6	1.3	23.2	74.8	2.0
24	23.2	62.3	14.5	19.3	68.9	11.8
48	3.9	30.3	65.8	2.5	7.0	90.5

†Each value refers to the percentage of each distribution and represents average of three independent infections. For each infection, 50–83 fluorescent cells were counted. The lowercase letters a, b, etc correspond to the panels shown in Fig. 2.

Three forms of the subcellular localization of Bm-UB and Ha-UB were observed in the individual viral life cycle (Fig. 2). At 18 and 24 hrs p.i., Bm-UB was distributed mainly in the nucleus (Fig. 2A, panel a, b; Table 2), similar to that observed with the expression of Ha-UB (Fig. 2B, panel d and e; Table 2). At 48 hrs p.i., Bm-UB was localized largely and evenly in the nucleus, though in the cytoplasm it was present in a dot distribution (Fig. 2A, panel c; Table 2). However, the protein displayed a nuclear membrane distribution, when analyzed with the subcellular localization of protein Ha-UB (Fig. 2B, panel f; Table 2).

Discussion

Search in the databases suggested that nearly all the lepidopteran baculoviruses encoded UB homologues that share 73%–86% sequence identity to Hu-UB. However, the predicted 3D-structures of these homologues were similar. Many conserved aa constitute recognition sites essential for the UB function. The most heterogeneous parts of UB concentrate on two regions aa 15–32 and aa 53–60. Compared to Hu-UB, the most interesting fact was that not only all seven lysines were conserved for Ha-UB, but also an additional Lys54 was inserted in the sequence of Bm-UB. As reported previously for eukaryotic UB, all seven lysines can be used as acceptors forming isopeptide bonds between the C-terminus of one UB and lysine of the next to target substrates for degradation (Peng *et al.,* 2003; Saeki *et al.*, 2009; Xu *et al.*, 2009). Poly-UB chains linked through these lysines may play versatile roles, since the chains formed through Lys63 generate multiple signals involved in the activation of protein kinases, DNA repair, endocytosis, multivesicular body sorting, and proteasomal degradation (Spence *et al.*, 1995; Mukhopadhyay and Riezman, 2007; Bohgaki *et al.*, 2008; Lauwers *et al.*, 2009; Saeki *et al.*, 2009). It is likely that Ha-UB has similar chain formations to Hu-UB for the seven lysines of Hu-UB and Ha-UB have similar spatial locations. Bm-UB carried additional one lysine, whose ε-amino group was fully exposed on the surface of predicted 3D structure suggesting that Bm-UB might form poly-UB chain at Lys54 and engage in some activities that Ha-UB did not.

The subcellular localization analysis showed that at 18 and 24 hrs p.i. Bm-UB and Ha-UB displayed similar subcellular distribution mainly in the nucleus. At 48 hrs p.i., Bm-UB was distributed largely and evenly in the nucleus, whereas the Ha-UB displayed a nuclear membrane distribution. It is well known now that the infection of baculovirus is characterized by a biphasic replication cycle yielding two structurally and functionally distinct virion phenotypes (Braunagel and Summers, 1994). One of them is BV produced by the budding of nucleocapsid from the surfaces of infected cells, the other one is ODV found within the occlusion bodies. As reported previously, baculovirus UB is attached to the inner face of BV envelope by a covalently linked phospholipid anchor (Guarino *et al.*, 1995). When the encoding sequence of UB is frame-shifted, the BV yield is decreased by 5- to 10-fold (Reilly and Guarino, 1996). These findings suggested that during BV formation stage, viral UB might be involved in the assembly of BV particles or budding of the nucleocapsid from infected cells, in favor of the BV yield. The results in this study enabled us to postulate that viral UB may be engaged in some activities in the process of ODV formation and to boost yield of ODV. Some of these activities associated with Bm-UB and Ha-UB may be different from each other.

Possibly baculovirus employs a variety of pathways associated with the viral or/and host UB. It is reported previously that BmNPV encodes IAP2, IE2, and PE38 proteins that function as ubiquitin ligase (E3) enzymes (Imai *et al.*, 2003). Hence, we recognize that the two pathways arisen, though their mechanisms remain unclear. One pathway regulates the process of viral DNA replication, since IE2 and PE38 act as stimulators for the DNA replication (Kool *et al.*, 1994). Another one is associated with a host insect cell apoptosis, because IAP2 acts as an inhibitor of apoptosis (Zeng *et al.*, 2009). Although much effort is paid to these processes, their mechanisms remain elusive. However, the attempts to investigate the role of baculovirus UB in these viral processes are essential and valuable.

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