The BRO proteins of *Bombyx mori* nucleopolyhedrovirus are nucleocytoplasmic shuttling proteins that utilize the CRM1-mediated nuclear export pathway

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Abstract

The BRO proteins of *Bombyx mori* nucleopolyhedrovirus (BmNPV) display a biphasic pattern of intracellular localization during infection. At early times, they reside in the nucleus but then show both cytoplasmic and nuclear localization as the infection proceeds. Therefore, we examined the possibility of nuclear export. Using inhibitors, we reveal that BmNPV BRO proteins shuttle between the nucleus and cytoplasm. Mutations on the leucine-rich region of BRO proteins resulted in nuclear accumulation of transiently expressed proteins, suggesting that this region functions as a CRM1-dependent nuclear export signal (NES). On the contrary, mutant BRO-D with an altered NES did not show nuclear accumulation in infected cells, although protein production seemed to be reduced. RT-PCR analysis showed that the lower level of protein production was due to a reduction in RNA synthesis. Taken together, our results suggest that BRO proteins are nucleocytoplasmic shuttling proteins that utilize the CRM1-mediated nuclear export pathway.

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Keywords: Baculovirus; BmNPV; BRO; Nuclear export; CRM1

Introduction

*Bombyx mori* nucleopolyhedrovirus (BmNPV) is a member of the Baculoviridae, a diverse family of viruses with circular double-stranded (ds) DNA genomes that are pathogenic for invertebrates, particularly insects of the order Lepidoptera. BmNPV has a genome of 128 kb that potentially encodes 136 genes (Gomi et al., 1997). Among these ORFs, five genes (*bro-a, bro-b, bro-c, bro-d, and bro-e*) were found to belong to a unique baculovirus multigene family (*baculovirus repeated orf; bro*) (Gomi et al., 1997; Kang et al., 1999). Multiple members of the *bro* gene family also have been reported in the genomes of other baculoviruses (Ahrens et al., 1997; Chen et al., 2001; Hayakawa et al., 1999; Kuzio et al., 1999; Nakai et al., 2003; Pang et al., 2001). Furthermore, homologues of the *bro* gene have been identified in other insect dsDNA viruses. It was shown that *Amsacta moorei* entomopoxvirus (EPV) and *Melanoplus sanguinipes* EPV contain homologues that were referred to as ALI family (Afonso et al., 1999; Bawden et al., 2000). They have been also reported in iridovirus and ascovirus (Jacob et al., 2001; Bideshi et al., 2003). These findings suggest that *bro* genes may be widespread among insect dsDNA viruses. Although the conservation among the dsDNA viruses suggests that *bro* genes serve an important role in infection, the function of this gene family remains unclear. It has been previously reported that all *bro* genes of BmNPV are actively transcribed as delayed early genes since *bro* genes require IE1 for their expression, and that BRO proteins are produced at high levels between 8 and 14 h postinfection (p.i.) (Kang et al., 1999). It has been also reported that one of the BmNPV *bro* genes (*bro-d*) is essential for viral infection, and that *bro-a* and *bro-c* may functionally complement each other (Kang et al., 1999). In addition, Zemskov et al. (2000) have shown that BmNPV BRO proteins have nucleic acid binding activity and are involved in nucleosome organization in infected cells.
Eukaryotic cells possess a double nuclear membrane containing nuclear pores that regulate bidirectional transport of proteins and RNAs between the nucleus and the cytoplasm. The nuclear export of proteins and RNA is a signal-dependent process mediated by soluble receptors called exportins. The best characterized nuclear export signal (NES) was first described in the human immunodeficiency virus Rev protein (Fischer et al., 1995). NESs are generally short stretches of amino acids characterized by multiple hydrophobic residues with a conserved motif \( \Phi - X_2 - \Phi - X_2 - \Phi - X - \Phi \), where \( \Phi \) indicates hydrophobic residues such as leucine, isoleucine, methionine, valine, or phenylalanine and \( X \) is any amino acid) that interacts with the export receptor (Kutay and Güttinger, 2005). The exportin CRM1 recognizes NES on cargo molecules targeted for export (Fornerod et al., 1997; Fukuda et al., 1997). As a specific inhibitor of CRM1 function, the cytotoxin Leptomycin B (LMB) provides a useful reagent for studying nuclear export in cells. LMB inhibits CRM1-mediated nuclear export of a range of proteins and RNAs by binding to the central domain of CRM1 to disrupt its interaction with the NES (Fornerod et al., 1997; Kudo et al., 1999; Nishi et al., 1994).

Baculovirus replication occurs in the host cell nucleus, consequently, the newly synthesized viral mRNAs in the nucleus must be exported to the cytoplasm, while some viral proteins produced in the cytoplasm are of necessity imported into the nucleus. However, our understanding of the nucleocytoplasmic transport of baculoviral products remains limited. At the subcellular level, BRO proteins of BmNPV are nuclear proteins during the early stage of infection such as 4 hpi but distribute to both the nucleus and the cytoplasm as the infection proceeds (Kang et al., 1999). This double localization suggests the possibility that BRO proteins undergo nuclear export. In this report, we examined this possibility and show that BRO proteins are indeed nucleocytoplasmic shuttling proteins that utilize the CRM1 mediated nuclear export pathway.

Results

**BmNPV BRO proteins shuttle between the nucleus and the cytoplasm**

We have previously reported that BmNPV BRO proteins distributed to the nucleus at 4 hpi and then localized both in the nucleus and the cytoplasm as the infection proceeds (Kang et al., 1999). Since this double localization suggested the possibility for nuclear export, we treated BmN cells with Leptomycin B (LMB), an inhibitor for CRM1-mediated nuclear export to test whether BRO proteins are exported by this general export receptor (Fornerod et al., 1997). If CRM1 is involved in BRO export, treatment with LMB should restrain the protein to the nucleus. BmN cells infected with BmNPV were treated with 10 nM LMB at 1 hpi and then analyzed by immunofluorescence staining 5 h later (6 hpi). In untreated cells, BRO localized both to the nucleus and the cytoplasm (Fig. 1, panels a and c). In contrast, cells treated with LMB showed a dramatic change in BRO distribution,

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**Fig. 1.** Intracellular localization of BmNPV BRO and IE1 in the presence of inhibitors. BmN cells infected with BmNPV were incubated in the absence of any inhibitor (a–c, j) or presence of LMB (d–f, k) or ActD and CH (g–i, l). Panels a, d, g in the left show BRO immunofluorescence images using anti-BRO, and the panels b, e, h are differential interface contrast images of the same fields as the left ones. These were merged in the panels c, f, i. Panels j–l show IE1 immunofluorescence images using anti-IE1. The scale bar is 10 \( \mu \)m.
with most cells showing nuclear accumulation of BRO (Fig. 1, panels d and f). These results indicate that LMB treatment inhibits the nuclear export of BRO.

In this immunofluorescence study, cycloheximide (CH) and actinomycin D (ActD) were also added to inhibit new protein synthesis and RNA polymerase II transcription, respectively. It has been reported that ActD also inhibits the import of some rapidly shuttling proteins (Chen et al., 2002; Pinol-Rama and Dreyfuss, 1992). In untreated cells, BRO localized both to the nucleus and the cytoplasm (Fig. 1, panels a and c). Nuclear localization suggests the import of BRO to the nucleus. In the presence of ActD and CH, BRO proteins distributed throughout the cell (Fig. 1, panels g and i), and this was similar with the distribution in untreated cells. The BRO proteins were not predominantly cytoplasmic, suggesting that the presence of ActD may not inhibit the import of BRO, or alternatively that import of BRO into the nucleus does not occur as rapidly. Therefore, the localization of another BmNPV protein was examined in the presence of these inhibitors. BmNPV IE1 localizes to specific nuclear sites where virus replication and transcription occur (Okano et al., 1999). In the presence of ActD and CH, IE1 distributed throughout the cell as BRO did but localized to the nucleus following LMB treatment (Fig. 1, panels j to l). The addition of ActD resulted in the cytoplasmic accumulation of the nuclear protein IE1, although nuclear localization was also observed, thereby supporting the possibilities described above. Taken together, our results demonstrated that BmNPV BRO proteins shuttle between the nucleus and the cytoplasm.

**BRO possesses a functional CRM1-dependent nuclear export signal**

The nuclear accumulation by treatment with LMB suggested the presence of nuclear export signals (NESs) in BRO proteins since NESs mediate the nuclear export of proteins via interaction with CRM1. CRM1-dependent NESs are characterized as leucine-rich sequences, Φ-X₂₋₃Φ-X₂₋₃Φ-X₋Φ (Φ = leucine, isoleucine, methionine, valine, or phenylalanine; X is any amino acid) (Kutay and Güttinger, 2005). BmNPV BRO proteins contain such hydrophobic leucine-rich sequences in their N-terminal regions (Fig. 2A). To determine if this region confers nuclear export, we introduced mutations into BRO-A, BRO-C, and BRO-D such that three hydrophobic residues were replaced with glutamine or arginine residues (Fig. 2A) and assessed the localization of the mutant BRO proteins following coexpression with ie1 because the expression of bro genes requires IE1 (Kang et al., 1999). A HA-tag was fused to the C-terminus of BRO-D (BRO-D-HA) since the anti-BRO antibodies react very poorly with BRO-D. Immunofluorescence assays using either anti-BRO or anti-HA revealed nuclear accumulation of the mutant (mt) BRO proteins compared with the results from wild-type (wt) proteins (Fig. 2B). This result demonstrated that the hydrophobic rich sequences in BRO proteins function as an NES, confirming that BRO proteins are exported via the CRM1-mediated pathway.

**Nuclear export of mutant BRO-D-HA with altered NES is not inhibited in infected cells.**

The bro-d gene has been shown to be essential for viral growth in BmN cells (Kang et al., 1999). To examine whether an altered NES sequence affects this function of BRO-D, we constructed recombinant BmNPVs. First, bro-d regions containing wt or mt bro-d fused with HA-tag (used in Fig. 2) were
inserted into the upstream region of the polyhedrin locus. Then the authentic bro-d was replaced with a β-galactosidase gene cassette expressed from the Drosophila heat shock promoter. Recombinant viruses were isolated by identification of plaques expressing β-galactosidase. The deletion of authentic bro-d gene was confirmed by PCR and Southern hybridization (data not shown). We were able to obtain recombinant virus carrying the wt BRO-D-HA and lacking authentic BRO-D, however, were unable to knock out authentic bro-d in recombinant virus carrying the mt BRO-D-HA, although several attempts were made. This suggests that alteration on hydrophobic residues in the NES of BRO-D prevented virus replication.

Viral growth curves of three recombinant viruses, carrying both authentic bro-d and wt bro-d-HA (WT), carrying wt bro-d-HA only (WT(Δbro-d)), or carrying both authentic bro-d and mt bro-d-HA (MT), showed that they have similar ability to produce BV as a wild-type virus (data not shown). We then assessed the localization of BRO-D-HA in BmN cells infected with each recombinant virus. Unexpectedly, mt BRO-D-HA showed both nuclear and cytoplasmic localizations, suggesting that the nuclear export of the mt protein occurs normally in infected cells (Fig. 3A). We therefore examined whether this nuclear export is CRM1-dependent by treating BmN cells with LMB. Both wt and mt BRO-D-HA proteins localized to the nucleus and cytoplasm in untreated cells but accumulated in the nucleus following treatment, indicating that their distribution is also sensitive to LMB (Fig. 3B). It was clear from the previous set of experiments that the nuclear export of BRO-D was inhibited by the altered NES following transient expression (Fig. 2). Therefore, it appears that some baculoviral factor might complement the inhibition of nuclear export of BRO-D due to altered NES in infected cells, and that this pathway is also CRM1-dependent.

Low levels in protein synthesis result from a reduction in the RNA synthesis of BRO-D-HA with an altered NES.

The protein expression level of mt BRO-D-HA with an altered NES seemed lower than that of wt BRO-D-HA at 6 hpi (Fig. 3A). Therefore, we analyzed protein production in infected cells. BmN cells infected with wt or three recombinant BmNPVs were harvested at 6 hpi and subjected to Western...
analysis using anti-HA, anti-BRO, and antibody to a BmNPV DNA binding protein (anti-DBP) as a control. The protein level of mt BRO-D-HA was slightly lower than that of wt ones, however, there was no difference in the expression of other proteins such as authentic BRO or DBP (Fig. 4A).

RT-PCR analysis was then performed to examine the level of RNA synthesis (Fig. 4B). The RNA level of mutant bro-d-HA was slightly less than that of wt bro-d-HA, suggesting that the lower level of protein production was due to a reduction in RNA synthesis. In addition, it was shown that the RNA synthesis of bro genes was inhibited by LMB treatment (Fig. 4C) although protein accumulation of BRO does not seem to be affected (Fig. 4A, lanes 1 and 2).

Discussion

BmNPV BRO proteins display a biphasic pattern of intracellular localization during infection. During the early stage of infection such as 4 hpi, they reside in the nucleus where virus replication occurs, but after that, they are localized in both the cytoplasm and nucleus (Kang et al., 1999). This double localization suggested the possibility for nuclear export. Here, we describe that BmNPV BRO proteins are nucleocytoplasmic shuttling proteins that utilize the CRM1-mediated nuclear export pathway.

Using inhibitors, we examined the possibility that the BRO proteins undergo nuclear export as was suggested by the double localization of BRO (Fig. 1). We first tested whether BRO proteins are exported from the nucleus via the general export receptor, CRM1, by using LMB. LMB inhibits the CRM1-mediated nuclear export of a range of proteins and RNAs by covalently modifying a specific cysteine residue in CRM1 (Kudo et al., 1999). Following LMB treatment, BmNPV BRO proteins were retained in the nucleus of infected cells, strongly suggesting that BmNPV BRO proteins do indeed undergo nuclear export. We then used ActD since it has been shown to inhibit the import of some shuttling proteins (Chen et al., 2002; Pinol-Rama and Dreyfuss, 1992). BRO distributed throughout the cell in the presence of ActD and CH, implying that ActD may not impede the import of BRO from the cytoplasm or that import into the nucleus does not appear to occur as rapidly. Thus, we examined the localization of BmNPV IE1 that is known to localize to specific sites in the nucleus of infected BmN cells (Okano et al., 1999). The presence of ActD resulted in the cytoplasmic accumulation and diffused nuclear localization of IE1, suggesting that import of viral proteins into the nucleus does not occur as rapidly in the presence of ActD. Taken together, we concluded that BmNPV BRO proteins shuttle between the nucleus and the cytoplasm.

We also demonstrated that the N-terminal region containing the hydrophobic leucine-rich sequences in BRO-A, BRO-C, and BRO-D function as NESs by introducing mutations into this region. Identification of a functional NES in BRO further supports the CRM1-dependent nuclear export of BRO. The NESs of BmNPV BRO proteins are canonical with the consensus NES regarding a high content of hydrophobic residues and typical spacing. We have not yet identified nuclear localization signal (NLS), however, the BRO proteins may be small enough to freely pass through the nuclear pores since they are between 36 and 40 kDa. Alternatively, BRO may contain an as of yet undetermined NLS. It has been shown that phosphorylation in the vicinity of an NLS or NES may play a role in the intracellular distribution of proteins (Jans and Hubner, 1996). Zemskov et al. (2000) reported that BmNPV BRO proteins are phosphorylated. Therefore, the phosphorylation of BRO could have an effect on the nuclear import if BRO does contain an NLS-like sequence.

The localization of mt BRO-D-HA showed difference between transient expression and infected cells, suggesting that some other BmNPV protein(s) may facilitate the nuclear export of the mutant protein by compensating for the altered NES. This pathway is also CRM1-dependent since mt BRO-D-HA was retained in the nucleus following LMB treatment. The recombinant virus carrying the mt bro-d-HA still contains other bro genes including an authentic bro-d, therefore, it may be possible that the other BRO proteins mediate the nuclear export of the mt BRO-D-HA.

Although the nuclear export of the mutant BRO-D-HA appears to occur smoothly, protein synthesis seemed to be
decreased. RT-PCR analysis showed that a decreased level in RNA synthesis resulted in the reduced level of the protein. In addition, LMB treatment also affected the RNA synthesis of the bro genes. Therefore, it is possible that the delay in the expression of the mtbro-d-HA results from the inhibited nuclear export. We do not presently know if this delay in gene expression caused by the mutated NES (or LMB) is a result of blocking RNA export or some other proteins. However, this raises the possibility that BRO proteins might mediate RNA export including their own RNA directly or indirectly. This could explain why we were unable to knock out authentic bro-d gene from a recombinant virus containing mt BRO-D-HA with altered NES. Regardless of whether this is true, this RNA export pathway should be limited in early stage of infection since the gene expression of mutant bro-d-HA appears to have recovered by 13 hpi, which was suggested by the lack of significant differences in the brightness of immunofluorescence between WT and MT (Fig. 3A). We also found that the RNA synthesis of some BmNPV genes including bro genes is reduced by LMB in the early stage of infection but that this reduction has completely recovered by 13 hpi using a real-time RT-PCR analysis (unpublished data). Baculovirus mRNAs are structurally very similar to cellular mRNA except that they are not spliced since most of the genes are intronless. How are viral mRNAs identified and selectively exported from the nucleus? One possibility proposed by Soliman and Silversren (2000) is that the activity of viral gene products shuts down cellular mRNA export pathways, while virus mRNAs are able to access a separate pathway. They suggested that herpesvirus (HSV) ICP27 may possess dual functions that inhibit the splicing of cellular mRNAs by binding to the core of spliceosome and assist intronless viral RNAs in export from the nucleus. Another possibility is that the newly synthesized viral mRNAs have exclusive access to the cellular mRNA export pathways. Ornelles and Shenk (1991) proposed that limiting the cellular factors recruited to the sites of viral RNA transcription might promote viral mRNA export while at the same time making them unavailable to promote cellular mRNA export. Furthermore, Chen et al. (2002) showed that HSV ICP27 interacts with and is able to recruit, the exon-junction complex protein Aly/REF to sites of viral transcription. To investigate whether this is the case for baculovirus RNA export and whether the BRO proteins play similar roles with HSV ICP27, it should be determined whether nuclear export of BmNPV mRNAs is dependent on BRO proteins.

### Materials and methods

**Viruses, cell line, infection, and transfection**

The BmN-4 (BmN) cell line was maintained in TC-100 with 10% fetal bovine serum as described previously (Maeda, 1989a, 1989b). Table 1

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<td>pSK-BRO-A</td>
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<td>EcoEex9’</td>
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<tr>
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* Solid underlines indicate the recognition sites for EcoRI and PstI, respectively. Kang et al. (1999).
1989b). The BmNPV T3 isolate (Maeda et al., 1985) and recombinant viruses were propagated on BmN cells as described (Maeda, 1995). BmN cells were infected with wt BmNPV or recombinant viruses at an MOI of 10. For transient expression, 1 μg of plasmid DNA described below was transfected into BmN cells using Lipofectin by following the instruction provided by the manufacturer (GibcoBRL). Recombinant viruses were constructed by cotransfection of plasmids described below with BmNPV-abb DNA (Ko et al., 2000) into BmN cells using Lipofectin (GibcoBRL) and isolated by identification of plaques containing polyhedron. The genomic DNA of these recombinant viruses was then cotransfected with a plasmid containing the β-galactosidase gene inserted into the bro-d gene (Kang et al., 1999) to knock-out authentic bro-d. The resulting mutant virus was isolated by identification of plaques expressing β-galactosidase as described (Kang et al., 1999). The construction of recombinant viruses was confirmed by PCR, Southern hybridization, and Western analysis.

Plasmids

For transient expression of bro genes, DNA fragments containing promoter, coding region, and 3’ untranslated region of bro-a, bro-c, and bro-d were subcloned. Briefly, these DNA fragments were amplified by PCR using a BmNPV genomic library and the primers (Table 1), digested with EcoRI (bro-a) or PsI (bro-c and bro-d), inserted into the EcoRI (Pst I), and EcoRV sites of pBluescript SK+, and designated pSK-BRO-A, pSK-BRO-C, pSK-BRO-D, respectively. To obtain HA-tagged BRO-D and mutants with an altered NES, site-specific substitutions were introduced by overlapping PCR (Higuchi, 1990) using the corresponding mutagenic primers and template shown in Table 1 and flanking pBluescript SK+ specific primers (T3 and T7). The resulting PCR products were digested with the same restriction enzymes used for the above pSK series constructions and inserted into the corresponding sites of pBluescript SK+. The DNA fragments containing HA-tagged BRO-D were also inserted into pBhEPS1 (Kang et al., 1998) to construct transfer vectors for recombinant viruses. pSK-ie1 was described in Kang et al. (1999). The nucleotide sequences of all the constructs were confirmed using an ABI sequencer.

Immunofluorescent microscopy and Western blotting

Immunohistochemistry and confocal microscopy were performed as described by Kang et al. (1999). Briefly, BmN cells grown on cover slips were transfected or infected with BmNPV T3 or mutant viruses as indicated in each figure. Leptomycin B (LMB; 10 ng/ml) (Sigma) was added for 5 h at 1 hpi and cycloheximide (CH; 100 μg/ml) and actinomycin D (ActD; 12.5 μg/ml) were added for 2 h at 4 hpi infected or transfected (at 48 h posttransfection) cells were then fixed and subjected to immunohistochemistry. For this, anti-BRO serum (1:200 dilution) (Kang et al., 1999), anti-IE1 (1:200 dilution, a generous gift from L. Guarino, Texas A and M University), or anti-HA tag polyclonal antibody (1:100 dilution; Clontech) was used with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:500 dilution; Cappel). SDS-PAGE was performed in 10% polyacrylamide gels as described by Laemmli (1970) with Precision Plus Protein Standards (Bio-Rad) as size markers. For Western blotting, BmN cells infected with wt or mutant viruses (MOI = 10) were harvested at 6 hpi. The whole extracts were subjected to protein assay (Bio-Rad), and 20 μg of protein from each sample was loaded. The Western blotting protocol was described previously (Kang et al., 1999). Antibodies against DBP were described in Mikhailov et al. (1998).

RT-PCR analysis

RT-PCR analysis was performed as described (Suzuki et al., 2003). Briefly, total RNA was extracted from BmN cells using EASYPrep RNA (TaKaRa Bio Inc.) and treated with RNase-free DNase I to remove contamination of DNA (TaKaRa Bio Inc.) for 15 min at room temperature. Poly(A)+ RNA was purified from 500 μg of total RNA with the Micro-FastTrack 2.0. kit (Invitrogen) using poly(A) binding resins as described by the manufacturer. cDNA was synthesized from 200 ng of poly(A)+ RNA using MV RT (TaKaRa Bio Inc.) and oligo(dT) primer. RT-PCR was performed using cDNAs, and the specific primers were listed in Table 1. PCR using an RNA template without RT was also performed to confirm the absence of DNA contamination (data not shown).

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