Baculovirus IE2 functions as a transregulator and is also involved in viral DNA replication. However, the mechanism for these functions remains unknown. It has previously been reported that Bombyx mori nucleopolyhedrovirus (BmNPV) IE2 has a ubiquitin ligase activity that is dependent on the RING finger domain and that IE2 can oligomerize through its C-terminal coiled-coil region. Here, confocal microscopy analysis demonstrated that IE2 formed nuclear foci only during the early phase of infection (2–6 h post-infection). Therefore, it was determined whether the IE2 functional regions described above could affect this characteristic distribution. Transient expression of ie2 also showed focus formation, suggesting that IE2 does not require any other viral factors. IE2 mutants lacking the C-terminal coiled-coil region did not form foci, while a mutant of the RING finger domain showed nuclear foci that appeared larger and brighter than those formed by wild-type IE2. In addition, IE2 exhibited enlarged foci in infected cells following treatment with a proteasome inhibitor, suggesting that foci enlargement resulted from accumulation of IE2 due to inhibition of the ubiquitin-proteasome pathway. These results suggest that BmNPV IE2 oligomerization and ubiquitin ligase activity functional domains regulate nuclear foci formation.

INTRODUCTION

DNA viruses replicate their genomes in the nucleus of the host cell during infection. To do this, the virus reconstructs nuclear structures and regulates nuclear domains. The best-characterized nuclear domains are the promyelocytic leukaemia protein (PML) bodies (also referred to as nuclear domain 10 or PML oncogenic domains) (Maul, 1998). PML bodies are proposed to be: (i) active sites for enzymic modification of proteins, in particular ubiquitylation, sumoylation and acetylation; (ii) sites for transient accumulation of proteins such as transcription factors, co-activators or co-repressors to be sequestered; and (iii) sites for degradation of misfolded proteins or for tagging such proteins (Takahashi et al., 2004). Viruses utilize PML bodies as a marker site to deposit their viral genomes for replication and transcription, although it is still unclear how and why viruses choose this site (Maul, 1998). It has also been indicated that nuclear domains are likewise important for baculoviruses. Okano et al. (1999) reported that for DNA replication of Bombyx mori nucleopolyhedrovirus (BmNPV) nuclear domains are occupied by IE1, LEF3 and DBP. On the other hand, it was shown that IE2 of Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV) formed nuclear foci in host cells (Krappa et al., 1995). At these foci, IE2 co-localized with DBP and LEF3 and sometimes with transiently expressed human PML (Mainz et al., 2002). These reports imply that IE2 may function in virus replication by localizing at specific domains such as PML body-like domains of the host insect cell.

Baculovirus ie2 encodes a protein that functions as a transregulator for transcription of viral genes (Carson et al., 1988, 1991; Theilmann & Stewart, 1992; Yoo & Guarino, 1994a, b) and is also involved in viral DNA replication (Gomi et al., 1997; Kool et al., 1994; Lu & Miller, 1995; Prikhod’ko et al., 1999). In spite of these important functions, it is not well understood how IE2 works. Previously, we showed using a yeast two-hybrid system and a cross-linking assay that BmNPV IE2 interacts with itself (Imai et al., 2000). The 80 aa C-terminal coiled-coil region was identified as an interacting region. IE2 of Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV) formed nuclear foci in host cells (Krappa et al., 1995). At these foci, IE2 co-localized with DBP and LEF3 and sometimes with transiently expressed human PML (Mainz et al., 2002). These reports imply that IE2 may function in virus replication by localizing at specific domains such as PML body-like domains of the host insect cell.

Analysis of BmNPV IE2 distribution in BmN cells showed that IE2 formed nuclear foci during infection. In this study, we showed that BmNPV IE2 localization in such
characteristic domains did not require any other viral factors. Furthermore, we demonstrated that IE2 focus formation was regulated by two functional regions involved in oligomerization and ubiquitin ligase activity.

**METHODS**

**Virus, cell line, infection and transfection.** The BmN-4 (BmN) cell line was maintained in TC-100 medium with 10% fetal bovine serum as described previously (Maeda, 1989). The BmNPV T3 isolate (Maeda *et al*., 1985) was propagated on BmN cells as described previously (Maeda, 1995). BmN cells were infected with wild-type (wt) BmNPV T3 at an m.o.i. of 10. For transient expression, 1 μg plasmid DNA (described below) was transfected into BmN cells using Lipofectin by following the instructions provided by the manufacturer (GibcoBRL). Transfected cells were subjected to immunohistochemistry (IHC) at 24 or 72 h post-transfection (p.t.).

**Construction of ie2 derivatives.** To express ie2 derivatives transiently in BmN cells, we produced an expression vector, phspMC, containing the *Drosophila melanogaster* heat-shock protein (hsp) 70 promoter (GenBank accession no. J01104). The hsp fragment of the LacZ gene cassette (Gomi *et al*., 1997) was inserted into the BglII and HindIII sites of pEGFP-1 (Clontech) and the EGF region was then removed by digestion with AgeI and NotI and self-ligation. To generate phsp-IE2wt, the ie2-coding region was amplified by PCR using pPC-ie2 (Imai *et al*., 2000) as a template, which encodes the full-length ie2 gene, and primers pPC-1 (5'-GATTAAGTGGGAC-ATCATCATC-3') and pPC-2 (5'-GATAAATTCTGGCAAGGTTAGA-C-3'). The amplified product was digested using *SalI* and inserted into the *SalI* and *Smal* sites of phspMC. Plasmids phsp-IE2N1, phsp-IE2N3 and phsp-IE2C3 were likewise constructed by PCR using pPC-ie2N1, pPC-ie2N3 and pPC-ie2C3 as templates, respectively (Imai *et al*., 2000). Two other plasmids containing predicted nuclear localization signal (NLS) regions (phsp-IE2NLS-C1 and phsp-IE2NLS-C2) were constructed by truncation of the ie2 internal region using PCR and the primers pNLS-C1f (5'-GACGGAGTTGGTGTGGTACGTG-3') and pNLS-C1r (complement of pNLS-C1f, and pNLS-C2f (5'-GACGGAGTGCTAGGTAGACTCAATGAAGGAGGACGATCTCATCTCA-3') and pNLS-C2r (complement of pNLS-C2f). Plasmid phsp-IE2C243S was generated by site-specific mutagenesis PCR using the primer 5'-GACGGAGGTGTGTGGTACGTG-3' and its complement (Imai *et al*., 2003).

**Antibody production and immunodetection.** The BmNPV ie2 gene was amplified by PCR using an upstream primer (5'-GACTGGATGGTTAAGATGTCG GAATTCTGGCAAGGTTAGA-C-3') incorporating an Nhel site (underlined) and a downstream primer (5'-GAATTCTGGCAAGGTTAGA CT CATCTCAATGAAGGAGGACGATCTCATCTCA-3') with a Xhol site (underlined). The amplified fragment was digested with *Nhel* and *Xhol* and fused in frame with a hexa-histidine tag (His-tag) in the pET-28c (+) vector (Novagen). After expression of His-tagged IE2 in *Escherichia coli* recombinant IE2 protein was purified using His Bind Resin (Novagen) and used to raise polyclonal antibodies in rats. Infected cells were harvested at designated times and subjected to Western blot analysis and IHC. Subcellular fractionation and immunoblotting were performed as described previously (Jarvis *et al*., 1991; Zemskov *et al*., 2000). IHC and confocal microscopy were performed as described by Kang *et al*. (1999) and Okano *et al*., (1999). Each analysis was repeated independently two to five times. For IHC, anti-ie2 antiserum (1:200 dilution) and Cy-5-conjugated goat anti-rabbit IgG (1:200 dilution; Amersham Life Sciences). To quantify total intensity of immunofluorescence in foci or the nucleus, the scanned confocal image was analysed using the TCS NT v. 1.6 (Leica).

**Proteasome inhibition assay.** BmN cells infected with BmNPV at an m.o.i. of 10 were treated with MG132 (20 μM; Calbiochem) at 0 h post-infection (p.i.). Cells were incubated in the presence of MG132 until they were fixed or harvested at 4 h p.i. This assay was repeated three times.

**RESULTS**

**BmNPV ie2 is expressed as an immediate-early gene**

To confirm that BmNPV ie2 was expressed as an immediate-early gene, we performed Western blot analysis using nuclear and cytosolic fractions of infected BmN cells and an anti-IE2 polyclonal antiserum. While two very close immunoreactive bands that migrated at 50 kDa were detected in the nuclear fraction, no band was observed in cytosolic fractions at any point during the time course of the experiment (Fig. 1). Both bands from the nuclear fraction probably corresponded to IE2, since the predicted molecular mass of BmNPV IE2 is 48 783 Da. Post-translational modification such as phosphorylation might result in two bands, which has also been suggested to occur for AcMNPV IE2 (Yoo & Guarino, 1994b). The synthesis of IE2 was readily apparent at 2 h p.i., but its expression gradually decreased and it had disappeared by 24 h p.i., suggesting that BmNPV ie2 is indeed an immediate-early gene.

**BmNPV IE2 forms foci in the nucleus**

The cellular localization of IE2 in BmNPV-infected BmN cells was visualized by confocal microscopy analysis using an anti-IE2 polyclonal antiserum. As shown in Fig. 2, IE2 formed several foci in the nucleus at 2 h p.i. and then showed dispersed granular localization throughout the nucleus at 4 h p.i. The foci gradually diminished after 6 h p.i. and the dispersed granular localization was not detected at 24 h p.i.
BmNPV IE1 has been shown to localize in nuclear domains where viral DNA replication occurs (Okano et al., 1999). Since the formation of IE2 foci appeared similar to that of IE1, we analysed whether IE2 co-localized with IE1 during infection. Double immunostaining demonstrated that IE1 was occasionally associated with IE2 foci at 2–4 h p.i. (Fig. 3). It was also shown that IE2 formed foci earlier than IE1, at 2 h p.i., and that there were more IE2 than IE1 foci. The IE2 foci disappeared at 12 h p.i. when the DNA replication foci shown by IE1 enlarged.

**Fig. 2.** Distribution of IE2 in BmNPV-infected BmN cells. Upper panels show IE2 immunofluorescence images (IE2) and lower panels show differential interface contrast images (DIC) of the upper images. BmN cells were mock infected or infected with BmNPV for 2, 4, 6, 12 and 24 h and subjected to IHC using a polyclonal anti-IE2 antiserum. Bar, 10 μm.

**Fig. 3.** Double staining of BmNPV-infected BmN cells with anti-IE2 and -IE1. BmN cells were infected with BmNPV for 2, 4, 6 and 12 h and subjected to IHC using a polyclonal anti-IE2 antiserum and a polyclonal anti-IE1 antiserum. Upper and middle panels show IE2 and IE1 immunofluorescence images, respectively. These were merged in the bottom panels. White arrowheads in merged panels indicate adjacent localization of IE2 and IE1. Bar, 10 μm.
A self-interacting region of IE2 is involved in focus formation

Because IE2 was specifically localized in the nucleus of infected cells, we examined whether IE2 was able to form foci when transiently expressed. A clone containing the intact ORF for ie2 under the control of the Drosophila hsp70 promoter showed several foci and granular distribution throughout the nucleus at 72 h p.t. [Fig. 4b(ii)]. This was very similar to the pattern of IE2 localization in infected cells at 4 h p.i. [Fig. 4b(i)], suggesting that IE2 did not need any other viral factor for focus formation.

Previously, we reported the intermolecular interaction of IE2 through a coiled-coil domain at its C terminus using a yeast two-hybrid method (Imai et al., 2000). Therefore, we assessed the localization of truncated ie2 derivatives driven by the Drosophila hsp70 promoter in transient expression. Fig. 4a shows the scheme for the clones used. In the yeast two-hybrid, IE2C3 (lacking the N-terminal 342 aa) showed self-interaction while the clones IE2N1 (containing the N-terminal 178 aa) and IE2N3 (containing the N-terminal 349 aa) did not (Imai et al., 2000). When these clones were transiently expressed in BmN cells, IE2N1 and IE2N3...
localized to the nucleus but did not show focus formation [Fig. 4b(iii) and (iv)]. In addition, IE2C3 expression was observed at low levels in only a few cells, but showed cytoplasmic localization [Fig. 4b(v)]. This low expression may have been due to loss of the region reacting with the antiserum. As shown in Fig. 4(a), there are two predicted NLSs in IE2. Since IE2C3 did not contain either, we constructed IE2NLS-C1 (lacking aa 76–185) and IE2NLS-C2 (lacking aa 76–293), which retained both NLSs. As expected, these two clones showed nuclear localization and focus formation [Fig. 4b(vi) and (vii)]. Taken together, we concluded that interaction between IE2 molecules is necessary for focus formation.

However, IE2NLS-C1 and IE2NLS-C2 exhibited very different localization characteristics. IE2NLS-C1 formed small foci and seemed to gather on the nuclear membrane, while IE2NLS-C2 showed enlarged foci [Fig. 4b(vi) and (vii), respectively]. This difference may be due to the region between aa 186 and 294 where another coiled-coil domain and a RING finger domain are contained (Fig. 4a). IE2NLS-C1 contained both regions, while IE2NLS-C2 had neither, suggesting that this region is also involved in focus formation.

Formation of IE2 foci is also regulated by the ubiquitin ligase activity of IE2

We further investigated the possibility that the different localization characteristics of IE2NLS-C1 and IE2NLS-C2 were due to the region containing the coiled-coil domain and RING finger domain. Involvement of the RING finger domain was tested first, since we have previously shown that the activity of BmNPV IE2 as a ubiquitin ligase E3 is dependent on the RING finger domain (Imai et al., 2003). IE2C243S contained a single amino acid change (Cys-243→Ser), which resulted in disruption of the RING finger structure (Fig. 4a). Transient expression of IE2C243S showed focus formation in the nucleus. However, the foci appeared larger and brighter than those of wt IE2 at 24 and 72 h p.t. (Fig. 5a). This localization was very similar to that of IE2NLS-C2 [Fig. 4b(vii)]. Since there was only a single amino acid change in IE2C243S, we concluded that the RING finger domain is required for focus formation.

The brightness of the IE2C243S foci was compared with that of wt IE2 by quantifying the total intensity of immunofluorescence in each nucleus and subjecting to IHC at 24 and 72 h p.t. To confirm this, we treated BmNPV-infected cells with the proteasome inhibitor MG132 and analysed IE2 localization. As expected, IE2 foci were enlarged and brighter in the presence of MG132 (Fig. 5b). In addition, Western blot analysis of BmNPV-infected cells treated with MG132 showed IE2 accumulation with several slower-migrating bands (data not shown), supporting the suggestion that IE2 is actively degraded by proteasome. These

IE2 accumulates in foci by inhibiting the proteasome pathway

It has been reported that some ubiquitin ligase E3s are auto-ubiquitylated and then degraded by the proteasome as a means of controlling E3 levels (Fang et al., 2000; Honda & Yasuda, 1999; Nuber et al., 1998; Yang et al., 2000). Therefore, we speculated that IE2 was also being auto-ubiquitylated and degraded during infection, and that the brightness and enlargement of foci shown by IE2C243S and IE2NLS-C2 were caused by the accumulation of IE2 in foci. To confirm this, we treated BmNPV-infected cells with the proteasome inhibitor MG132 and analysed IE2 localization. As expected, IE2 foci were enlarged and brighter in the presence of MG132 (Fig. 5b). In addition, Western blot analysis of BmNPV-infected cells treated with MG132 showed IE2 accumulation with several slower-migrating bands (data not shown), supporting the suggestion that IE2 is actively degraded by proteasome. These

**Fig. 5.** Involvement of the ubiquitin-proteasome pathway in focus formation. (a) Distribution of IE2C243S. BmN cells were transfected with a plasmid expressing wt IE2 or a mutant containing a single point mutation in the RING finger domain and subjected to IHC at 24 and 72 h p.t. (b) Treatment with proteasome inhibitor. BmN cells infected with BmNPV were incubated in the presence (+) or absence (−) of the proteasome inhibitor MG132. After 4 h of treatment, cells were subjected to IHC. Bar, 10 μm.
results suggested that the protein level of IE2 is controlled by its own E3 activity.

**DISCUSSION**

In this study, we demonstrated that BmNPV IE2 localized to specific foci in the host cell nucleus and that this focus formation was regulated by two functional regions of IE2. In addition, we showed that IE2 occasionally localized adjacent to IE1 during the early stage of infection. BmNPV IE1 has been demonstrated to localize to sites of virus replication (Okano et al., 1999). The cellular localization of AcMNPV IE2 has been also investigated and was reported to show a punctate nuclear distribution in TN-368 cells during the early phase of infection (Krappa et al., 1995; Murges et al., 2001). In addition, Mainz et al. (2002) reported that IE2 foci of AcMNPV co-localized with sites of viral DNA replication and also with some other nuclear domains such as PML bodies. These and our data further support the suggestion that baculovirus IE2 may play an important role in virus replication by localizing at nuclear functional domains, including sites for viral DNA replication.

We showed that two regions of BmNPV IE2, the RING finger domain and the C-terminal coiled-coil region, were involved in IE2 focus formation. IE2 mutants lacking the C-terminal coiled-coil region did not form foci, although they localized to the nucleus. Due to a lack of NLSs, we were not able to determine whether foci were formed only by the C-terminal coiled-coil region. However, foci were observed when the region containing NLSs was included, suggesting that the C-terminal region is necessary. We have previously shown that IE2 oligomerizes through this C-terminal coiled-coil region (Imai et al., 2000). Therefore, we concluded that IE2 self-interaction is required for focus formation. Using Western blot analysis, Yoo & Guarino (1994b) showed that full-length AcMNPV IE2 also localized to the nucleus. They also found that the transactivation activity of C-terminal deletion mutants was reduced or lost, and suggested that reduction of transactivation might be due to dispersion of IE2 to the cytosol or loss of its activity domain. Our results indicated that C-terminal deletion mutants of BmNPV IE2 are not able to localize to specific sites in the nucleus correctly. Therefore, this suggests that similar mislocalization of AcMNPV IE2 mutants results in the reduction of transactivation. However, we did not examine the transactivation activities of BmNPV IE2 or its derivatives.

Deletion of a central region containing another coiled-coil domain and the RING finger domain showed enlarged and brightened foci. Previously, we reported that BmNPV IE2 could function as a ubiquitin ligase E3 dependent on this RING finger domain (Imai et al., 2003). Therefore, we examined focus formation of an IE2 mutant containing a mutation of the RING finger domain and found that foci of this mutant were also enlarged and brightened. Quantification of immunofluorescence showed that foci of this mutant were 10 times brighter than those of wt IE2. In addition, treatment of infected cells with a proteasome inhibitor resulted in a similar increase in size and brightness of foci. These results indicated that loss of E3 ligase activity and/or inhibition of proteasomal degradation result in brighter and larger foci due to an accumulation of IE2. This proteasomal degradation by auto-ubiquitylation may explain how the IE2 expression level is regulated. Our Western blot analysis showed that BmNPV IE2 exists only during the early phase of infection, which is similar to the expression profile of AcMNPV IE2 (Krappa et al., 1995). However, it has been reported that the BmNPV ie2 transcript is present throughout infection (2–72 h p.i.) (Katsuma et al., 2004). This suggests that BmNPV IE2 expression is post-translationally regulated by proteasomal degradation.

The ICP0 of Herpes simplex virus type 1 (HSV-1) is also known to be a RING-type viral E3 (Boutell et al., 2002; Hagglund & Roizman, 2002; Hagglund et al., 2002; Van Sant et al., 2001). In addition, it has been shown that ICP0 forms nuclear foci associated with PML bodies (Everett & Maul, 1994; Maul & Everett, 1994; Maul et al., 1993). ICP0 targets and disrupts PML bodies by inducing the degradation of PML body components such as PML and Sp100 through the proteasome pathway (Boutell et al., 2003; Chelbi-Alix & de The, 1999; Everett et al., 1998; Muller & Dejean, 1999; Parkinson & Everett, 2000). Moreover, the disruption of PML bodies by ICP0 is dependent on the RING finger domain (Everett & Maul, 1994; Maul & Everett, 1994; Maul et al., 1993). Therefore, it is likely that PML bodies are regulated by the ubiquitylation ability of ICP0 during infection and that PML-related protein(s) might be ubiquitylated by ICP0. On the other hand, it has been shown that AcMNPV IE2 occasionally co-localizes with transiently expressed human PML (Mainz et al., 2002). IE2 foci could be the PML body-like domains of insect cells, suggesting that BmNPV IE2 localizes to foci in order to prepare the sites for replication by targeting and ubiquitylating host protein(s), as is the case with HSV-1 ICP0. This may explain why IE2 forms foci earlier than IE1. Based on our data, we propose the following working model for IE2. Immediately after infection occurs, IE2 is transported to specific sites in the cell nucleus and forms foci there. During this time, IE2 oligomerization is required for anchoring of IE2 at these specific nuclear sites. At these foci, IE2 ubiquitylates its substrate(s) to support infection of BmNPV and then is gradually removed by auto-ubiquitylation and the proteasomal degradation system. Further analysis, including identification of the proteins interacting with IE2, will help to validate this model.

**ACKNOWLEDGEMENTS**

We thank J. J. Hull for critical reading of the manuscript, L. A. Guarino for the gift of anti-IE1 antiserum and M. Kurihara for providing BmN cells. This research was supported by grants from the Bioarchitect program of the Science and Technology Agency of Japan, Grant-in-Aid for Young Scientists (B) (14760034) (N. I.) and Grant-in-Aid for
Scientific Research (C) (15580044) (W.K.) from The Ministry of Education, Culture, Sports, Science and Technology, and a special postdoctoral fellowship from RIKEN (N.I.).

REFERENCES


Maul, G. G. & Everett, R. D. (1994). The nuclear location of PML, a cellular member of the C3HC4 zinc-binding domain protein family, is rearranged during herpes simplex virus infection by the C3HC4 viral protein ICP0. J Gen Virol 75, 1223–1233.


