Autographa californica multiple nucleopolyhedrovirus ORF 23 null mutant produces occlusion-derived virions with fewer nucleocapsids

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Two envelope fusion protein gene homologues have been identified in the baculovirus Autographa californica multiple nucleopolyhedrovirus (AcMNPV). AcMNPV GP64 protein is fusogenic and essential for propagation and pathogenicity. The F homologue (Ac23) is not essential, is fusion-incompetent in standard assays, but contributes to faster host death. Here, we show that occlusion bodies (OBs) from Ac23null mutants and control viruses do not differ significantly in size and the number of occlusion-derived virions (ODVs) contained; however, Ac23null OBs had a much higher percentage of ODVs with a single nucleocapsid (44.6%) than the near-isogenic control (11.3%). Infection of Sf9 cells with Ac23–green fluorescent protein (gfp)-expressing recombinant viruses showed Ac23–gfp fluorescence overlapping perinuclear DAPI staining at later times, a pattern not observed with GP64. These results suggest that F proteins have evolved functions beyond envelope fusion and play a different role from that of GP64 in viruses that contain both proteins.

The baculovirus Autographa californica multiple nucleopolyhedrovirus (AcMNPV) produces two virion phenotypes with distinctively different roles in the same cell (Miller, 1997; Theilmann & Blissard, 2008). Occlusion-derived viruses (ODVs) contain one or more nucleocapsids, and are encapsulated within the nucleus by polyhedrin proteins to form environmentally stable occlusion bodies (OBs; Slack & Arif, 2006). Upon ingestion of OBs by susceptible hosts, OB disassembly and ODV release occurs within the host’s midgut after which a second virion phenotype, budded viruses (BVs), spread the infection systemically (Granados & Lawler, 1981; Keddie & Volkman, 1985; Keddie et al., 1989; Engelhard et al., 1994).

Of the two different envelope fusion protein genes identified in baculoviruses, F is more widely distributed than gp64, being found in both viruses with and without gp64 genes (Pearson & Rohrmann, 2002). F proteins from several gp64-minus baculoviruses have membrane fusion activity and contain conserved features of viral fusion proteins (Ijkel et al., 2000; Pearson et al., 2000, 2002b; Westenberg et al., 2002; Yin et al., 2008; Long et al., 2008). In contrast, F proteins from baculoviruses with gp64 genes such as AcMNPV’s Ac23 and Orgyia pseudotsugata MNPV’s Op21 have no detectable membrane fusion activity and lack common features of functional viral fusion proteins such as proteolytic cleavage site, fusion peptide and heptad repeat regions (Pearson et al., 2000; Lung et al., 2003; Long et al., 2008); GP64 serves as the viral fusion protein in these viruses (Blissard & Wenz, 1992; Monsma et al., 1996).

Characterization of an Ac23null AcMNPV showed that Ac23 is a pathogenicity factor that is not essential for viral replication and infectivity (Lung et al., 2003). Animals infected with mutant viruses survived ~24 h longer than animals infected with control viruses (Lung et al., 2003). Ac23 is associated with both BVs (Lung et al., 2003; Braunagel et al., 2003) and ODVs (Braunagel et al., 2003). Existing evidence suggests Ac23’s role in BV infectivity and production can increase the rate of kill (Lung et al., 2003; Wang et al., 2008). However, the impact of Ac23 on OBs and ODVs has not been characterized. This study was undertaken to determine if deletion of Ac23 has an effect on OB size and composition.

Genetic differences between the Ac23null mutant, control and Ac23–green fluorescent protein (gfp)–expressing viruses used in this study are summarized in Fig. 1. Generation of mutant and control viruses and OB preparation were described previously (Lung et al., 2003). Since OBs are polyhedral crystals, the average of the two largest perpendicular length measurements was used as a measure of OB size. Scanning electron microscopy (SEM) analysis showed
significant size differences between wild-type (AcMNPV E2) OBs and OBs from all three bacmid-derived viruses (ANOVA and Tukey test, \( P<0.0001 \)). Wild-type OBs \((n=160, \text{mean} \pm \text{SD}, 2.48 \pm 0.60 \mu m)\) are generally larger than OBs of Ac23null-repair \((n=162,1.62 \pm 0.44 \mu m)\), Abcabmid \((n=154,1.74 \pm 0.44 \mu m)\) and Ac23null mutant \((n=113,1.75 \pm 0.54 \mu m)\) viruses. However, there was no significant difference between the sizes of OBs from the two bacmid-derived controls \((Ac23null-repair \text{and\ Abcabmid})\) and between the bacmid-derived controls and the Ac23null mutant. The sizes of OBs were also determined by measuring the widest OB cross section found in transmission electron microscopy (TEM) serial sections of purified agarose-embedded OBs. TEM was performed as described previously by Hong et al. (1994). Again, statistically significant size differences \((P<0.0001)\) were observed only between wild-type OBs and OBs of the two bacmid-derived viruses \((Ac23null\text{ and\ Abcabmid})\) analysed in this study. The average size of the widest section of wild-type OBs \((n=74,2.39 \pm 0.65 \mu m)\) is greater than that of Abcabmid \((n=64,1.87 \pm 0.41 \mu m)\) and the Ac23null mutant \((n=49,1.71 \pm 0.46 \mu m)\). Thus, both SEM and TEM analysis indicate that bacmid-derived OBs were on average significantly smaller than those from wild-type virus. However, the sizes of Ac23null OBs are not significantly different from the size of OBs from bacmid-derived controls. Since the bacmid is derived from the AcMNPV E2 variant (Luckow et al., 1993), the smaller size of bacmid-derived OBs is likely the consequence of changes in expression of polyhedrin and/or flanking ORF603 and ORF1629 genes. These changes in gene expression could be the result of insertion of non-baculovirus sequences such as a kanamycin resistance marker, mini-F replicon and attTn7 into the polyhedrin locus during construction of the bacmid.

To examine whether the number of occluded ODVs within OBs differed between Ac23null and control viruses, the widest sections of 47 wild-type, 37 Abcabmid and 26 Ac23null OBs were identified from serial sections and used to determine the number of ODVs present. The range and average number of ODVs in these sections were similar among the three viruses \((Ac23null: 18.6 \text{ ODVs per section, } \text{SD}=12.5, \text{range}=0–54; \text{Abcabmid: 18.7 \text{ ODVs per section, } SD=10.5, range=0–45; wild-type: 20.0 \text{ ODVs per section, } SD=11.08, \text{range=0–53)}\). The number of ODV particles within the widest section is positively correlated with the size of the widest section for all three viruses \((Ac23null: r=0.50, P=0.007; \text{Abcabmid: } r=0.37, P=0.024; \text{wild-type: } r=0.51, P=0.0002)\). The size of the widest OB section was a significant and positive predictor of ODV number in Ac23null \((\text{mean} \pm \text{SEM}, \beta=14.74 \pm 5.06, \text{Ac23null} (\beta=10.23 \pm 4.28)\) and wild-type \((\beta=8.94 \pm 2.24)\) virus. However, the proportion of variance \((R^2)\) accounted for by the regression model was only 25.4% for Ac23null, 14.0% for Abcabmid and 26.2% for wild-type. Determining the exact number of ODVs present in an entire OB by examination of our serial section series was not possible because the Formvar and carbon films necessary for serial section support reduced resolution and contrast. However, we consistently find that ODV abundance is comparable throughout the serial section series of any particular OB. This observation suggests that the ODV number calculated in the widest OB section is a good indicator of the abundance of ODVs in an OB, and that there are likely no significant differences on average between the quantities of ODVs embedded within Ac23null and control OBs. Extreme examples of the widest section of two different OBs with dramatically different ODV numbers are shown in Fig. 2(a).

**Fig. 1.** Genetic differences between the Ac23null mutant, control and Ac23–gfp-expressing (Abcabmid/Ac23–gfp) viruses used in this study. Type I transfer vector used to construct the Ac23null and Abcabmid virus contained a GUS reporter gene under the control of a P6.9 promoter and a polyhedrin gene under the control of its own promoter. Type II transfer vector used to generate the Ac23null-repair virus contained an Ac23 gene under the control of its own promoter in addition to genes in the type I vector. The type III transfer vector used to generate the Abcabmid/Ac23–gfp virus is identical to the type II vector except that the Ac23 gene was replaced by an Ac23–gfp fusion gene. polyh, Polyhedrin promoter; Gm', gentamicin resistance gene; Kan', kanamycin resistance gene; attTn7, Tn7 attachment site.
Thus, the data suggest that only ODVs with one to three nucleocapsids, respectively.

Transverse sections of 337 wild-type, 376 Abacmid, 326 Ac23-null-repair and 289 Ac23null ODVs were analysed to determine if the number of nucleocapsids enclosed within them were different. The results show a significantly higher percentage of Ac23null ODV with a single nucleocapsid (44.6 %), when compared with the controls (Ac23null-repair: 11.3 %; Abacmid: 21.8 %; wild-type: 13.6 %). The opposite trend was observed when the percentages of ODVs containing five or more nucleocapsids were compared (Ac23null: 16.9 %; Ac23null-repair: 49.7 %; Abacmid: 39.4 %; wild-type: 55 %). ODVs with up to 15 clearly distinguishable nucleocapsids were observed in all three controls, while the maximum number of clearly distinguishable nucleocapsids in Ac23null ODVs on our micrographs was seven. Consistent with the result that the majority of Ac23null ODVs contained one or two nucleocapsids, a higher percentage (43 %) of Ac23null OB sections examined contained only ODVs with three nucleocapsids or less (n=57). In contrast, 23.4 %, 31 % and 14.6 % of the Ac23null-repair (n=155), Abacmid (n=126) and wild-type OB (n=144) sections examined contained only ODVs with one to three nucleocapsids, respectively. Thus, the data suggest that Ac23null OBs contain significantly fewer nucleocapsids per OB than Ac23-containing control OBs.

Examination of a large number of ODV cross sections shows that nucleocapsids in Ac23null and control ODVs are arranged in a similar pattern. The majority of ODVs with fewer than 11 nucleocapsids are arranged in one or two distinct patterns, examples of prevalent patterns are shown in Fig. 2(b).

The hallmark of multiple nucleopolyhedrovirus (MNPV) is the presence of multiple nucleocapsids within each ODV. For AcMNPV, when multiple ODV nucleocapsids are delivered to host midgut cells, an alternate pathway of infection that accelerates the progression of infection by many hours may occur (Adams et al., 1977; Granados & Lawler, 1981). Thus, the results presented here suggest that Ac23null mutant’s slower rate of kill may also be due to Ac23null ODVs having fewer nucleocapsids for the faster alternative pathway of infection. Packaging of multiple nucleocapsids has been suggested to offer a selective advantage over baculovirus that package a single nucleocapsid per virion (SNPV) (Washburn et al., 1999, 2003). The biological basis for multiple nucleocapsid envelopment has not been determined; however, Ac142 was recently shown to be essential for ODV nucleocapsid envelopment (McCarthy et al., 2008). The observations that Ac23null ODVs with high nucleocapsid counts are present and that the percentage of Ac23null ODVs with two to four nucleocapsids are comparable to the controls (data not shown) suggest that Ac23 somehow facilitates, but is not essential for multiple nucleocapsid envelopment. Interestingly, deletion of Sj29 from Spodoptera frugiperda MNPV reduces ODV numbers in OBs, but had no apparent affect on ODV nucleocapsid content (Simón et al., 2008).

Viruses expressing Ac23 with a C-terminal gfp fusion were used to examine Ac23 localization in infected Sf9 cells by confocal microscopy (Fig. 3). At later times (48 h post-infection, h p.i.) Ac23–gfp fluorescence frequently overlapped with the peripheral ring of DAPI staining, suggesting that Ac23–gfp may localize to the inner nuclear membrane (INM). This localization was not observed at earlier time points (e.g. 12 and 18 h p.i.). Ac23–gfp fluorescence was also detected in the cytoplasm and the plasma membrane, but not in the nucleoplasm. The spatial changes in Ac23–gfp fluorescence over time suggest that Ac23 produced early is primarily targeted to BVs via the secretory pathway, while some Ac23 produced later are diverted to ODVs via the INM. Ac23 contains both early and late promoter sequences (Pearson et al., 2002a). Ac23–gfp’s perinuclear localization is consistent with Ac23 being involved in ODV envelopment as ODV envelopes are suggested to be derived from either the nuclear membrane or by de novo synthesis within the nucleus (Hong et al., 1994, 1997).

A putative nuclear localization signal (NLS), PKKKFNF, is present at position 513–519 of the Ac23 protein. Transport
of proteins to the INM relies on mechanisms that include NLS-dependent nuclear pore complex (NPC)-mediated active transport (Zuleger et al., 2008). NLS has been identified in the majority of INM proteins (Lusk et al., 2007), and deletion of the NLS from the INM protein Heh2 results in its exclusion from the INM (King et al., 2006). Lusk et al. (2007) proposed that steady-state distribution of INM proteins to the INM and outer nuclear membrane/endoplasmic reticulum (ER) depend on factors such as the size of the extraluminal domain, the affinity of the NLS for karyopherin-\(\xi\), the presence of INM retention factors, interaction with nuclear architecture, as well as the protein’s biophysical properties. Thus Ac23’s broad distribution to the outer nuclear membrane/ER and INM could be due to a low-affinity NLS insufficient to confer exclusive INM localization. Putative NLSs are also present in F homologues from other baculoviruses. For example, the F protein of Bombyx mori MNPV has the same NLS as Ac23, while a different NLS is present in Op21 (PRRRFNY) and SeF (PKYKRGK). In contrast to Ac23, Pearson et al. (2001) showed by Western blot analysis that Op21 is not associated with ODVs, and by immunocytochemistry that Op21 localizes to the plasma membrane, but not to the nuclear membrane. These discrepancies could be due to differences in NLSs, differences in host or other viral factors, and an amino acid identity of only 47.9\% between mature Ac23 and Op21. Alternatively, differences in experimental methodologies, such as the lack of HgCl\(_2\) for protease inhibition during Orgyia pseudotsugata MNPV ODV purification, may have prevented the detection of Op21 in ODVs. The use of non-cell permeabilizing protocols for immunostaining of Op21 may also have prevented detection of Op21 at the nuclear membrane. Since Ac23 is also associated with BVs (Lung et al., 2003), and since most BVs contain single nucleocapsids, factors in the cytosol/plasma membrane or BV nucleocapsid may inhibit Ac23 promotion of multiple capsid packaging in BV. Alternatively, unidentified factors found exclusively in the nucleus or ODV nucleocapsids could be enabling Ac23 to promote envelopment of multiple nucleocapsids into the ODV. In SNPVs, the F homologues and/or their interaction partners may have enough sequence divergence to prevent the envelopment of multiple capsids into a single ODV. The long cytoplasmic tail domain of Op21 and other F proteins relative to GP64 has been suggested to mediate the interaction with the nucleocapsid (Pearson et al., 2001). Thus the identification of host and viral interacting partners of F protein’s cytoplasmic domain will help elucidate the similarities and differences in the function of these proteins. Analysis performed in cultured cells indicate Ac23null mutant BVs have retarded growth and infection kinetics, when compared with control viruses (Lung et al., 2003). This phenotype, however, may be caused by deficiencies in viral attachment or entry. Recently, Wang et al. (2008) showed the production of a SeF-pseudotyped Ac23 and gp64 double mutant was three orders of magnitude lower than SeF-pseudotyped gp64null viruses, suggesting that Ac23 has a significant effect on BV production. Together, these results suggest Ac23’s affects on both ODVs and BVs impact the rate of kill.

Ac23’s distinct roles in ODVs and in BVs could be due to different post-translational modifications resulting from Ac23 being targeted to the INM and to the plasma membrane via the secretory pathway, respectively. Consistent with this hypothesis, Ac23 from purified BVs had a higher \(M_t\) than Ac23 from purified ODVs on Western blots (Braunagel et al., 2003). Generation of ODVs with higher nucleocapsid numbers could facilitate rapid establishment of primary infection, while facilitating BV infection and production promotes systemic infection and OB formation. In contrast, GP64, the BV envelope fusion protein, is not associated with ODVs. These intriguing observations further support the contention that the F protein plays a different role from that of GP64 in viruses that contain both proteins. \(f\) is likely to have been acquired from a host, and has been evolving in baculovirus genomes over a longer period of time than the more recently acquired \(gp64\) (Pearson & Rohrmann, 2002; Lung & Blissard, 2005). Thus it is not surprising to discover that F proteins may have evolved pleiotrophic functions beyond envelope fusion. Existing data suggest that, although Ac23 is not essential, the multiple auxiliary functions that it performs offer selective advantages to AcMNPV.
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