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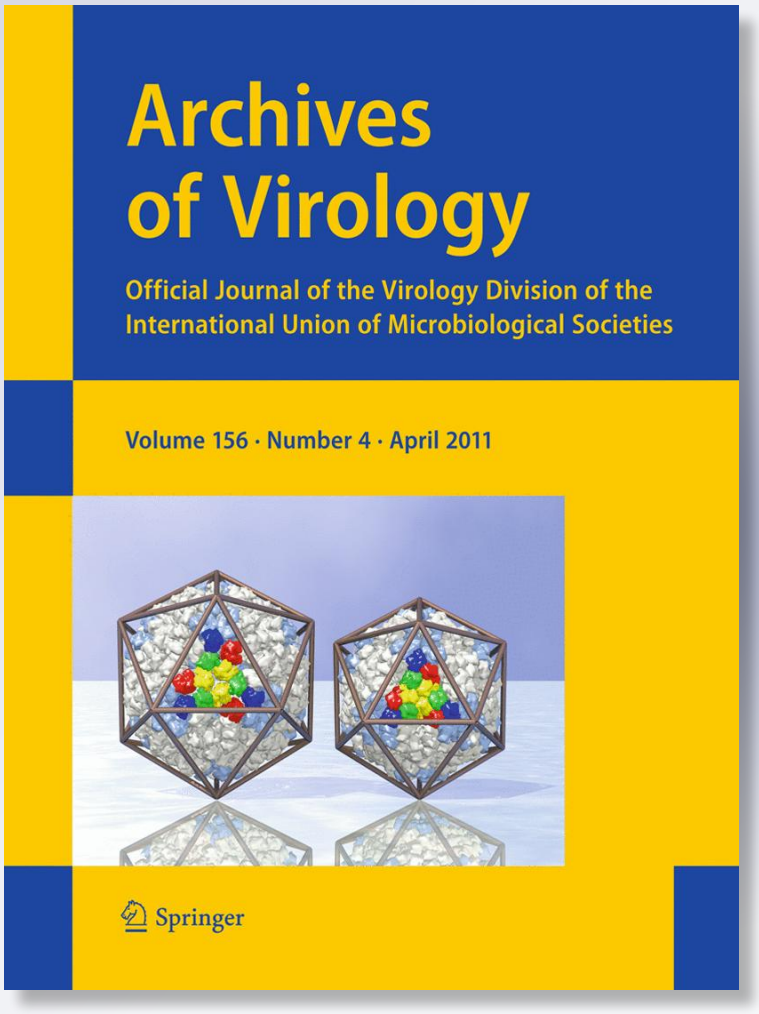

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BRIEF REPORT

Description of a novel single mutation in the *Ac*MNPV polyhedrin gene that results in abnormally large cubic polyhedra

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Abstract We describe a point mutation in the *Ac*MNPV polyhedrin gene that produces abnormally large cubic polyhedra in packaging cell lines. A polyhedrin mutant baculovirus in which the single change E44G was introduced confirmed that this mutation and no other alterations in the *Ac*MNPV genome was responsible for the abnormal phenotype. Although baculoviral VP39 protein was detected inside mutant polyhedra, electron microscopy demonstrated that only a proportion of the large crystals allow occlusion of virions. When compared with wild-type polyhedra, the mutant inoculum showed reduced oral infectivity for *Rachiplusia nu* larvae. Hence, the amino acid 44 substitution in the *Ac*MNPV polyhedrin protein alters polyhedrin assembly and affects viral occlusion and infectivity.

Keywords *Ac*MNPV polyhedrin · Mutant · Large polyhedra

Virions of a number of invertebrate viruses are specifically occluded within robust crystalline particles. The occlusion bodies (OBs) act as protective packages, allowing infectious virions to survive for long periods in harsh environments, protecting them from UV irradiation and desiccation. This unique mode of propagation within protein crystals is found only in two unrelated families of invertebrate viruses: *Baculoviridae* (baculovirus) and *Reoviridae* (cypovirus). This characteristic may have evolved to maintain the virus during periods when the larval population is too small, for example during diapause or seasonal hostile conditions [1, 2].

Baculoviruses of the genus *Alphabaculovirus* have several virus particles occluded in a polyhedrin crystalline matrix to form OBs or polyhedra. Feeding larvae become infected by ingesting OBs that dissolve in the alkaline environment of the midgut, releasing the occlusion-derived virus (ODV). Polyhedrin is one of the most conserved proteins of the virus. It has a molecular mass of around 29 kDa, and it is highly expressed in the late steps of infection in insect cells. How polyhedrin plays its role in the occlusion process is not yet well understood. It is believed that formation of OBs depends on interactions between polyhedrin and other proteins present on the envelope of the virion [3, 4], but some studies have demonstrated that it also depends on its own amino acid sequence [5, 6]. Three spontaneous mutant viruses with altered polyhedron morphology have been isolated and characterized in Autographa californica multiple nucleopolyhedrovirus (*Ac*MNPV). In each case, a single amino acid change in the polyhedrin sequence was responsible for the abnormal phenotype of the OBs. One of those mutants, bearing a substitution at position 118, produced large amounts of small particles instead of crystalline OBs [7]. The other reported mutants, which had a substitution at residue 59 [8] or 25 [9], produced a single cubic polyhedron in the nucleus of the infected cell. These polyhedron-deficient mutants have been classified by their different characteristics as class i and class ii, respectively [10]. This characterization is based on the location of point mutations in the polyhedrin secondary structure. According to these authors, class i mutations affect β sheet regions, in the

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so-called core, while those of class ii affect areas close to a region of disordered residues between amino acids 32 and 48.

In this work, we characterized a point mutation found in a clone of the plasmid p_{XXL}POL, which is employed in the development of stable packaging insect cell lines [11], that resulted in an abnormal phenotype of polyhedra in Sf9 cells. In order to confirm that the morphological changes in polyhedra were a consequence of the mutation of polyhedrin protein, a recombinant baculovirus named *Ac*POL_{E44G} was constructed by introducing an A-to-G substitution at position 130 of the polyhedrin nucleotide sequence (GenBank: K01149.1). The coding sequence for polyhedrin bearing the point mutation was amplified from plasmid p_{XXL}POL using sense oligonucleotide POL-ATG (5′TA AATATGCCGGATTATTCA3′) and antisense oligonucleotide POL-STOP (5′TTTTAATACGCCGGACCA GT3′). A PCR product of the expected size was cloned into pFastBac-1 plasmid (Invitrogen), and a recombinant *Ac*MNPV bacmid was obtained using the Bac-to-Bac system (Invitrogen). *Ac*POL_{E44G} baculovirus was produced by transfection of Sf9 cells with Cellfectin Reagent (Invitrogen) following the supplier's instructions, and the inoculum was titered by endpoint dilution [12]. Figure 1A shows the cytopathic effect observed in Sf9 cells infected with *Ac*POL_{E44G} or wild-type (wt) *Ac*MNPV baculovirus. The abnormal phenotype of occlusion bodies became evident after 3 days post-infection. Cells infected with *Ac*POL_{E44G} baculovirus showed a mean value of 1.9 ± 1.1 large cubic polyhedra per cell, as compared to 12.2 ± 3.4 normal polyhedra observed in cells infected with wt AcMNPV. The means were significantly different ($P < 0.05$) by Student′s t-test.

The first approach to characterizing the mutant virus was to compare the ability of these two viruses to replicate in Sf9 cells. The growth curves obtained were very similar (data not shown), suggesting that the point mutation in the polyhedrin gene of *Ac*POL_{E44G} did not affect virus replication. To study the kinetics of polyhedrin production for both viruses, cells were infected with *Ac*POL_{E44G} or wt *Ac*MNPV at a multiplicity of infection (moi) of 10 plaque-forming units (pfu) per cell and harvested at 6, 18, 24, 30, 42, 48, 54 and 66 hours postinfection (hpi). The samples were standardized, and equal quantities analyzed by SDS-PAGE. Western blot was performed using a polyclonal anti-polyhedrin antibody (GenTex) conjugated to alkaline phosphatase, and protein bands were made visible using NBT-BCIP (Promega). Kinetics of polyhedrin production for both viruses was evaluated by western blot using a polyclonal anti-polyhedrin antibody (GenTex) conjugated to alkaline phosphatase and NBT-BCIP substrate (Promega). Figure 1B shows that, although the quantity of mutant polyhedrin produced was approximately 4 times

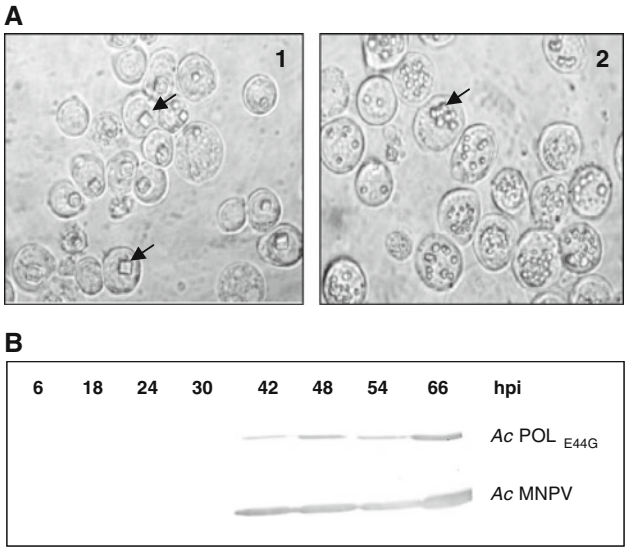

Fig. 1 **A**) Microscopic visualization of Sf9 cells infected with *Ac*POL_{E44G} baculovirus. Cytopathic effect of *Ac*POL_{E44G} at 4 days postinfection [1] in contrast to wt *Ac*MNPV [2]. Arrows show polyhedra inside the cells. Magnification 100X. **B**) Analysis of polyhedrin kinetics. A western blot was performed using a polyclonal anti-polyhedrin antibody to detect polyhedron expression in cells infected with *Ac*POL_{E44G} or wt *Ac*MNPV from 6 to 66 hours postinfection (hpi)

lower than that of wt (as estimated by densitometry, not shown), the progression of polyhedrin synthesis was similar for both mutant and wt baculoviruses, indicating that there was no apparent alteration in temporal polyhedrin gene expression.

In order to determine whether the large polyhedra produced by *Ac*POL_{E44G} were able to occlude baculoviruses, Sf9 cells were infected at a moi of 1 pfu per cell. As a control, cells were infected with wt *Ac*MNPV. The infection was left to proceed for six days, and polyhedra were purified by extraction with 0.5% SDS and 0.5 M NaCl [12]. Western blot assays were performed using approximately 1×10^8 wt or mutant polyhedra, which had been sedimented and re-suspended in disruption buffer (120 mM Tris-HCl, pH 6.8, 4% SDS, 0.02% bromophenol blue, 1.4 M 2-mercaptoethanol, 20% glycerol). Samples were heated at 65°C for 10 min to eliminate baculoviruses on the polyhedron surface. After centrifugation at 9000x*g*, the first supernatant (S1) was kept, and the pellets were washed for a second time. The second supernatant (S2) was kept, and the final pellet (P) was treated with 0.1 N Na_2CO_3. Equal volumes of supernatants and pellet were loaded onto a gel and electrophoresed under denaturing conditions. The presence of occluded virions was inferred by detection of the nucleocapsid major protein VP39 by western blot using a monoclonal antibody (1:2,000) [13] and a secondary alkaline phosphatase-conjugated anti-mouse antibody (1:15,000) (SIGMA). Figure 2A shows the detection of a

band corresponding to VP39 in the P fraction of mutant and wt polyhedra as well as in the S1 fraction of *Ac*POL$_{E44G}$ polyhedra. This result suggests the presence of additional baculoviruses adsorbed on the surface of the cubical polyhedra.

To analyze the proportion of structurally aberrant polyhedra with respect to normally shaped ones and to confirm the presence of virions within mutant polyhedra, cells infected with *Ac*POL$_{E44G}$ baculovirus were analyzed by transmission electron microscopy (TEM). Briefly, infected cells were fixed with 2.5% glutaraldehyde, post-fixed in 2% osmium tetroxide and packaged in agar. Blocks were then carved and sliced with an ultramicrotome to obtain section pieces of about 60-90, nm which were collected on copper grids and contrasted with heavy salts (coloration of uranyl-Reynold's). Samples were visualized in a TEM JEOL 1200EX-II microscope. An extensive scan of the field revealed that the proportion of abnormally-shaped polyhedra with respect to normally-shaped ones was of 54.5%; only 47.3% of the abnormal polyhedra were found to have baculoviruses inside. Figures 2B 1 and 2 show two mutant cubic polyhedra, one of them occluding virions, and the other not. The dimensions of the abnormal crystals ranged from 4 to 15 μm, with a mean ± standard deviation (SD) value of 5.6 ± 1.8 μm in comparison to wt polyhedra, which were typically 1-2 μm, with a mean value of 1.7 and a SD of 0.2. We noticed the presence of large quantities of pre-occluded baculoviruses outside the OBs, which was probably related to the detection of VP39 in the S1 fraction (Fig. 2A). This observation suggests that the mutation responsible for altering the phenotype of OBs also generates a deficiency in viral occlusion by an unknown mechanism.

In order to determine the infectivity of mutant polyhedra, groups of twelve randomly selected third instar *Rachiplusia nu* larvae were orally infected with increasing doses of *Ac*POL$_{E44G}$ or wt *Ac*MNPV polyhedra (up to 1,600) on artificial food cubes. Another group was mock-infected as a negative control. The number of deaths was recorded every day until the control larvae pupated. The experiment was performed three times.

Lethal times$_{50}$ (LT$_{50}$) were significantly different for wt *Ac*MNPV and *Ac*POL$_{E44G}$ at the three doses of polyhedra assessed (8.21 vs 11.37, 7.11 vs 10.59 and 6.94 vs 9.15 days postinfection, for 100, 400 and 1,600 polyhedra, respectively, $p < 0.05$, ANOVA with Tukey's multiple comparisons post-test) and were consistent with a diminished LD$_{50}$ in *Ac*POL$_{E44G}$ with respect to wt (mean 56.5, SD 18 vs mean 5.7, SD 2.9, Probit analysis by BioStat 4.1).

This diminished infectivity is in accordance with electron micrographs, which demonstrated that only half of the polyhedra produced by *Ac*POL$_{E44G}$ occluded baculoviruses. Probably, the lower mortality observed with the abnormal polyhedra is a result of their occlusion deficiency.

When the effect of the infection process became apparent in larvae, haemolymph samples were extracted and observed under a light microscope. Polyhedra found in the haemolymph from larvae infected with mutant polyhedra were morphologically abnormal and characteristic of *Ac*POL$_{E44G}$ infection, whereas haemolymph from larvae infected with wt polyhedra contained normal-sized occlusion bodies (data not shown).

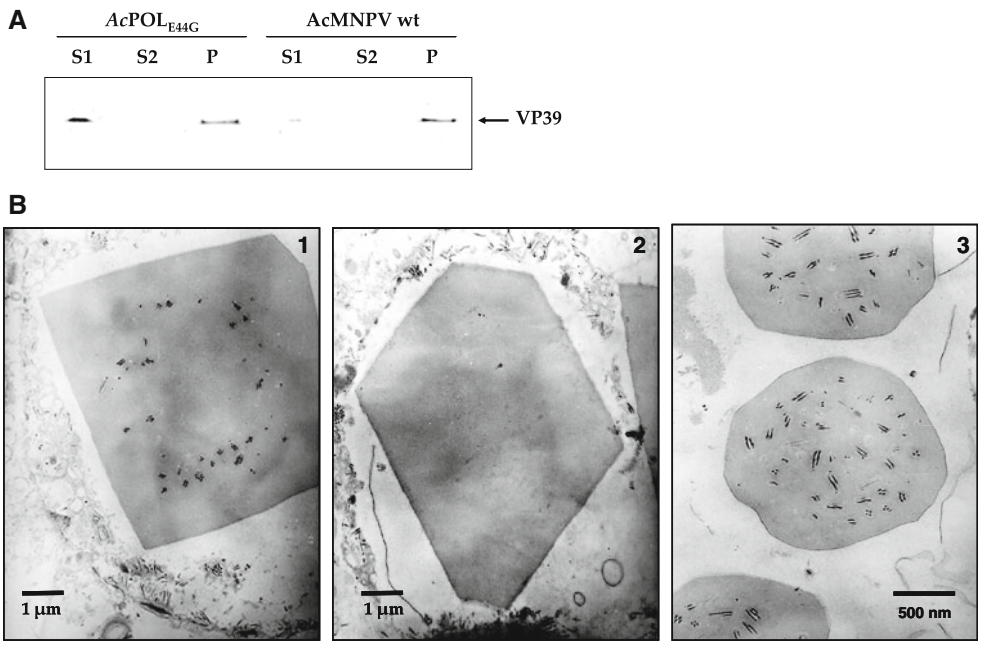

Fig. 2 **A**) Immunoblot assay to determine baculovirus occlusion in mutant polyhedra. VP39 protein detection assays were performed on samples from inside and outside polyhedra. Polyhedra were extracted with SDS, washed twice and finally treated with Na_2CO_3 to dissolve polyhedrin and release virions (S1 and S2: supernatants of washing steps 1 and 2, respectively; P: pellet; see text). **B**) Electron micrographs of cells infected with *Ac*POL$_{E44G}$ or wt *Ac*MNPV 1) Mutant polyhedron with virions inside. 2) Mutant polyhedron with no virions inside. 3) Wild-type polyhedra occluding virions

Finally, secondary structures and 3-D models of the wt and mutant polyhedrins were predicted using the Scratch and I-Tasser servers, respectively. The substitution of a negatively charged glutamic acid by a small neutral glycine residue occurred in a region of disordered residues that remained unresolved in the tertiary structure of the protein obtained by crystallography (PDB ID:2WUY) [10]. Although the predicted secondary structure of mutant polyhedrin exhibited an interruption of an alpha helix (Fig. 3A), the alignment of predicted tertiary structures for both proteins did not show relevant changes (Fig. 3B), and the model obtained for the quaternary structure of the mutant polyhedrin trimer did not reveal any apparent differences when compared to the structure of the wt polyhedrin trimer determined by X-ray crystallography (not shown).

Our results are in accordance with previous reports that naturally occurring amino acid substitutions in polyhedrin, most of them single point mutations, produce variable phenotypic changes, such as the formation of large cubic polyhedra that occlude no or few virions [8, 14, 15]. The mutation E44G, which corresponds to the class ii classification according to Ji et al. [10], generates large cubic polyhedra that inefficiently occlude baculovirus, and it alters infectivity as well.

As the mechanisms and the genetic basis of the occlusion process are still unknown, the cause of the decrease in occlusion within certain mutant polyhedra remains to be determined. Polyhedra of similar morphology to the wt polyhedra and without occluded virions are not rare in nature [16]. The absence of selection in the culture system, in contrast to what happens naturally, may explain the appearance of this phenotype. Nevertheless, ODV integrity is not a prerequisite for polyhedron morphogenesis. A mutant of *Ac*MNPV that generates morphologically normal polyhedra that do not contain virions inside has been described elsewhere [17]. These authors found that deletion of the ac142 gene leads to a defect in ODV envelopment, and as a result, no occlusion is detected. Thus, in the case of the polyhedrin point mutants, this deficiency may be due to a conformational change in polyhedrin assembly that prevents normal packaging of ODVs. Also, the region between residues 32 and 48 is less conserved among members of the different baculovirus species. This variability suggests that this region plays an important role in the specificity of virus packaging, and baculoviruses would

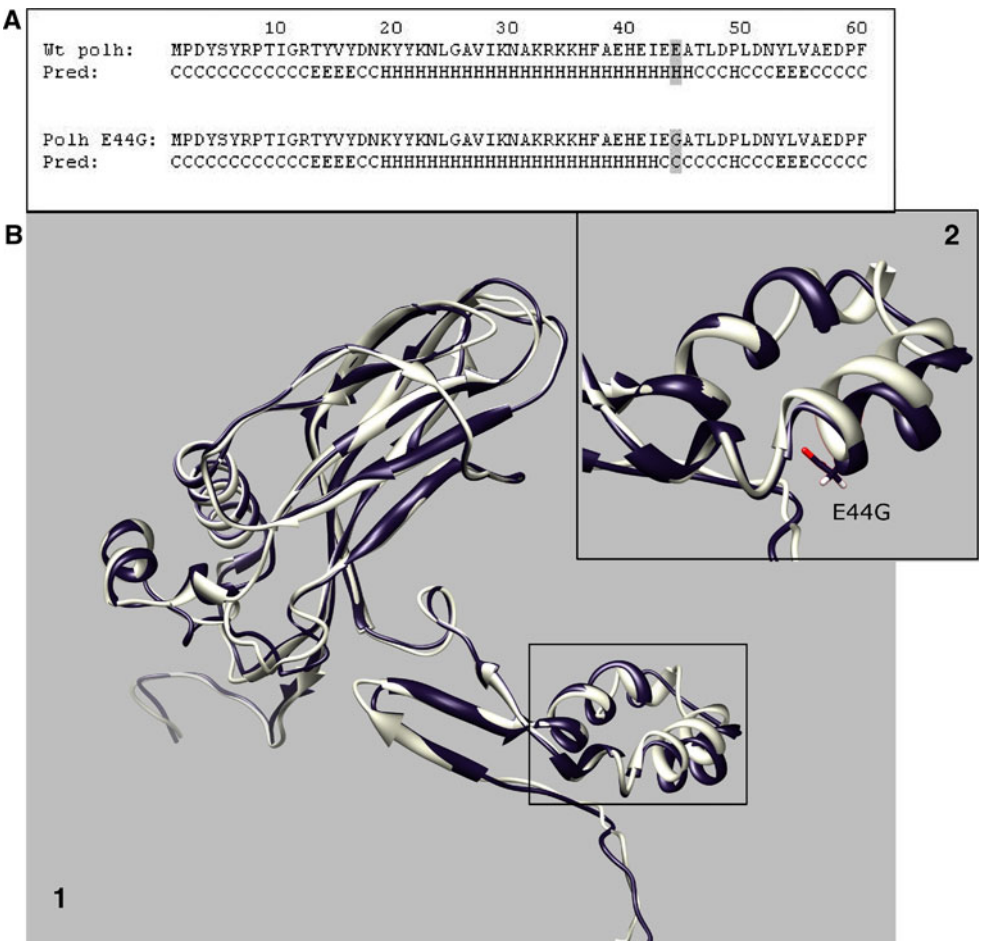

Fig. 3 **A**) Prediction of secondary structures of E44G mutant and wt polyhedrin proteins (polh) using the Scratch server. The position of the amino acid substitution is highlighted in grey. H: helix; E: strand; C: coil. Pred: prediction. **B**) 3-D model alignment of polyhedrin structures using the I-Tasser server. E44G polyhedrin is represented in a dark color, and wt polyhedrin in a light color [1]. The region of disordered residues that was not resolved by crystallography for *Ac*MNPV polyhedrin (PDB 2WUY) is boxed. [2] Closer view of the highlighted region, where the side chain of glutamic acid at position 44 of wt sequence is shown in a ball-and-stick representation

require certain flexibility in areas that interact with the virion envelope proteins [10]. It is not known in detail if this asymmetry is needed to occlude the irregular packages of nucleocapsids or if it is not relevant, since polyhedrin may interact nonspecifically with baculovirus proteins. However, this hypothesis is supported by the observation that mutations close to this region or within it interfere with viral occlusion. In the case of the mutant described in this work, although it forms part of a variable region, residue 44 is highly conserved among members of different baculovirus species [2], and we observed that its substitution affects polyhedron morphology and baculoviral occlusion, probably by altering the assembly of polyhedrin building blocks and giving viruses less physical space to adhere to the crystal structure.

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