Identification and expression of two baculovirus gp37 genes

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The gp37 genes of the Mamestra brassicae and Lymantria dispar multicsapd nucleopolyhedroviruses (MbMNPV and LdMNPV) have been identified and characterized. Both genes were similar to other baculovirus gp37 genes and to entomopoxvirus fusolin genes. Phylogenetic analysis showed that baculovirus gp37 genes and entomopoxvirus fusolin genes form two distinct and well-separated clades. There was no evidence of recent gene transfer between the two groups. The gp37 genes also showed a distant similarity to bacterial cellulose- and chitin-binding protein genes, but the significance of this is unclear. MbMNPV and LdMNPV gp37 were both transcribed from consensus baculovirus late transcription start sites. MbMNPV gp37 was additionally transcribed from a putative early transcription start site. Tunicamycin treatment of MbMNPV-infected cells confirmed that MbMNPV GP37 is N-glycosylated. Confocal immunofluorescence microscopy revealed that the protein is located exclusively in the cytoplasm, probably in the endoplasmic reticulum.

Introduction

The baculoviruses and entomopoxviruses (EPVs) represent distinct families of large DNA viruses that share the property of infecting insect hosts. They both form two distinct types of inclusion body within infected cells. The larger of these, termed polyhedra or polyhedral inclusion bodies (PIBs) for baculoviruses and spheroids for EPVs, consist of virus particles embedded in a proteinaceous matrix (Miller et al., 1999). In both groups of virus, these bodies serve as a major vehicle for horizontal transmission, apparently allowing the virus to persist in the environment prior to infection of a new host. The proteins that make up the matrix of these occlusion bodies (OBs), polyhedrin and spheroidin, respectively, show no obvious similarity to each other at the amino acid sequence level. This is a remarkable example of unrelated groups of viruses independently evolving the same strategy for efficient propagation in insect hosts.

The other type of inclusion body formed by these viruses, termed spindle bodies, are bi-pyramidal in shape and do not contain virus particles. These are found in the cytoplasm and are smaller than PIBs or spheroids. Indeed, there are instances among the EPVs where the spindle bodies are incorporated into the spheroids (Goodwin et al., 1991). In contrast to OBs, the proteins that form the matrix of the spindle body, termed GP37 in baculoviruses and fusolin in EPVs, are clearly related, sharing 30–40% amino acid sequence identity (Dall et al., 1993; Gauthier et al., 1993; Gross et al., 1993; Hayakawa et al., 1996; Liu & Carstens, 1996; Mitsuhashi et al., 1997; Vialard et al., 1990). Both display a high cysteine content, presumably involved in crystal formation, and many of these proteins are glycoproteins.

Given that gp37/fusolin genes are found in baculoviruses and EPVs but not in the chordopoxviruses (poxviruses related to EPVs that do not infect insects), it seems likely that they are involved in efficient virus infection of an insect host (Sriskantha et al., 1997). There is accumulating evidence that this is the case for fusolin proteins. Hayakawa et al. (1996) purified a factor present in Bombyx mori (Ps) EPV OB preparations that could enhance Ps nucleopolyhedrovirus (NPV) infection of this insect. Cloning and sequence analysis of this factor revealed it to be a fusolin protein. More recently, Mitsuhashi et al. (1998) have reported that Anomala cuprea EPV (AncuEPV) spindle bodies can enhance Bombyx mori (Bm) NPV infection of B. mori up to 104-fold. It is not yet known whether baculovirus GP37 proteins can also enhance virus infectivity.
There appears to be some variation in the subcellular localization of baculovirus GP37 proteins. The first to be characterized, the Autographa californica multicapsid NPV (AcMNPV) GP37, then erroneously termed spheroidin-like protein, was reported to be present within viral PIBs in the nucleus (Vialard et al., 1990). In contrast, Gross et al. (1993) found that Orgyia pseudotsugata (Op) MNPV GP37 is localized within spindle-body-like structures in the cytoplasm. These authors re-examined the localization of AcMNPV GP37 and confirmed that it can be detected associated with PIBs in the nucleus. However, they reported that it can also be detected in spindle body structures in the cytoplasm of AcMNPV-infected cells. AcMNPV GP37 is currently the only member of the GP37/fusin family of proteins known to occur in the nucleus.

In this study we describe two further baculovirus GP37 proteins, from the viruses Mamestra brassicae (Mb) MNPV and Lymnaea dispar (Ld) MNPV. A phylogenetic analysis of known GP37 and fusin proteins is presented. The expression of both genes has been characterized at the transcriptional level. We have raised an antiserum to MbMNPV GP37 and used it to characterize the subcellular localization of the protein. These studies show that MbMNPV is localized predominantly in the cytoplasm.

**Methods**

**Cells, insects and viruses.** Trichoplusia ni TN368 cells (Hink, 1970) and Lymnaea dispar IPLB-Ld652Y cells (Goodwin et al., 1978) were maintained in TC100 medium (Gibco) supplemented with 10% foetal calf serum and 0.26% tryptose broth. IPLB-Ld652Y cells were provided by D. Lynn (USDA-ARS, Beltsville, MD, USA). T. ni eggs were provided by J. Cory (IVEM, Oxford, UK) from a laboratory colony. The larvae were reared at 25 °C on an artificial diet (Hunter et al., 1984). The LdMNPV isolate A21-MPV (Slavicek et al., 1996) was used. This was provided by J. Slavicek (US Forest Service, Delaware, OH, USA) and propagated on IPLB-Ld652Y cells. The Oxford strain of MbMNPV (Possee & Kelly, 1988) (provided by J. Cory) was used. This was propagated by infecting third or fourth instar T. ni larvae with 10^5 OBs per insect and allowing the infection to proceed until death. OBs were purified from infected cadavers as described previously (O’Reilly et al., 1992). Budded virus stocks were prepared by bleeding infected insects 4–5 days post-infection (p.i.). The infected haemolymph was diluted 4-fold in complete TC100. A small amount of phenylthiourea was added to inhibit melanization. Virus was amplified by infection of 2.5 × 10^5 TN368 cells in a 35 mm dish with 0.5 ml of the diluted haemolymph and incubation at room temperature for 5 days. The pass-1 stock generated in this way was further amplified by an additional one or two rounds of infection of TN368 cells. Budded virus stocks were titrated by plaque assay as described previously (O’Reilly et al., 1992), except that the assays were incubated at room temperature.

**Nucleotide sequence analysis.** A 1.9 kbp Xhol–HindIII fragment of the MbMNPV genome, encompassing the region from the left end of the Xhol H fragment to the left end of HindIII J (Possee & Kelly, 1988), was subcloned into pBluescript (Stratagene) and sequenced completely. Similarly, a 2.1 kbp EcoRI–SphI clone of LdMNPV DNA, from 43.3 to 44.6 mu (Thiem et al., 1996), was subcloned into pBluescript and sequenced completely. Clones for sequence analysis were generated by a combination of subcloning restriction fragments and by the generation of nested deletions from both ends of the inserts using the protocol of Henikoff (1984). Custom-designed primers were also used to facilitate sequencing of specific regions. Sequence determination was by dyeoxy chain termination (Sanger et al., 1977) with T7 DNA polymerase or a Circumvent sequencing kit (New England Biolabs). Sequence assembly and analysis were performed using the Genetics Computer Group package (Devereux et al., 1984). BLAST, TBLASTN, FASTA and TFASTA algorithms were used for searches of the GenBank, EMBL and SWISS-PROT databases.

**Phylogenetic analysis.** Conceptual translations of LdMNPV and MbMNPV gp37 genes were compared with a variety of homologous proteins obtained from BLAST searches of the EMBL/GenBank/DDJB non-redundant sequence database. Multiple sequences were aligned by using CLUSTAL W (Thompson et al., 1994) with the following parameters: pairwise alignments; slow, gap opening 10, gap extension 0.1, matrix BLOSUM, 30; multiple alignments; gap opening 10, gap extension 0.05. Alignments were refined by eye to exclude regions that could not be aligned unambiguously. A total of 207 amino acids were used in the final alignment. Phylogenetic analyses were performed by using both maximum parsimony and neighbour-joining methods and utilized the computer package PAUP4d-04 (written by D. L. Swofford) and the ‘protpars’ matrix. Single maximum parsimony trees were obtained after 25 random-addition replicates. Maximum parsimony and neighbour-joining bootstrap values were obtained from 100 and 500 replicates, respectively. Topological constraint trees were constructed by using the same search strategy as that used to find the minimum (unconstrained) trees.

**Primer extension analysis.** T. ni larvae were infected with MbMNPV OBs as described above. At 4 days p.i. they were harvested and ground to a fine powder in liquid nitrogen. Total RNA was extracted from the powder by guanidinium isothiocyanate lysis and centrifugation through cesium chloride (O’Reilly et al., 1992). For LdMNPV, IPLB-Ld652Y cells were infected with LdMNPV at an m.o.i. of 8 p.f.u. per cell. After 1 h adsorption, the inoculum was removed and cells were re-fed with complete medium. The infected cells were harvested at 48 h p.i. Time zero was defined as the time when the inoculum was removed and incubation at 27 °C was initiated. Mock-infected cells were treated in the same way, except that incomplete medium was used as inoculum. Total cellular RNA was isolated by a single-step method (Chomczynski & Sacchi, 1987). Primer extension analyses, with 50 or 20 μg (MbMNPV or LdMNPV) total RNA, were carried out essentially as described by Sambrook et al. (1989). The primers were an 18-mer (MbMNPV) or a 20-mer (LdMNPV) annealing downstream of the gp37 translational start site. The same primers were used to prime DNA sequencing reactions to provide markers for the primer extension products.

**Generation of anti-GP37 antiserum.** The complete MbMNPV gp37 gene was amplified by PCR and subcloned into the vector pET-28b (Novagen) in-frame with the N-terminal polyhistidine tag present in this plasmid. The resulting plasmid was designated pETGP37. The insert was sequenced completely to confirm authenticity. pETGP37 was transformed into E. coli BL21 cells and fusion protein expression was induced by incubation in the presence of 1 mM IPTG when the OD600 reached ~ 0.6. One hour later, the cells were harvested and lysed by sonication in ice-cold 5 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9. The lysate was centrifuged at 10,000 g for 15 min and the pellet (containing the fusion protein) was dissolved in the above buffer containing 8 M urea. The fusion protein remained insoluble even after urea treatment. The urea-insoluble material was separated by SDS–PAGE (Laemmli, 1970), the GP37 band was excised and the gel was fragmented by multiple passages through a narrow-gauge syringe. This purified protein was used as the immunogen to raise a GP37-specific antiserum.
approximately 100 μg purified protein was emulsified in Freund's complete adjuvant and injected intramuscularly into a Halfop rabbit (Froxfield Farms). Three further intramuscular booster injections were given followed by three injections directly into the lymph node in the lower leg. Boosts were given at 3-week intervals using purified protein in incomplete adjuvant. The serum was collected as described by Harlow & Lane (1988) and tested for its response to GP37 by immunoblotting.

### Immunoblotting

TN368 cells (5 x 10^5 per 60 mm dish) were infected with MbMNPV at an m.o.i. of approximately 0.1 and incubated at room temperature. One day later, a subset of the cells was incubated in the presence of 5 μg/ml tunicamycin. All samples were collected 60 h later and lysed in SDS–PAGE loading buffer. Uninfected cells were analysed in parallel as a control. Proteins were separated by SDS–PAGE and blotted onto nitrocellulose membranes. Duplicate blots were prepared and processed for immunoblotting as described by Harlow & Lane (1988). One blot was incubated in the presence of pre-immune serum and the other in the presence of the anti-GP37 immune serum. Both sera were diluted 1:100. Bound antibodies were revealed by incubation with a 1:3000 dilution of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Promega) and detection of alkaline phosphatase activity as described by the manufacturer.

### Immunofluorescence analysis

TN368 cells were seeded onto glass coverslips (5 x 10^4 cells per 35 mm dish) and incubated overnight at 27 °C. They were infected with MbMNPV at an m.o.i. of approximately 0.1 and incubated at room temperature for 5 days. Uninfected cells were analysed in parallel as controls. The cells were fixed by immersion in acetone–methanol (7:3) for 15 min at −20 °C and processed for immunofluorescence as described by O'Reilly et al. (1992).

The primary antibody was either pre-immune serum or anti-GP37 immune serum, both diluted 1:100. The secondary antibody was FITC-conjugated goat anti-rabbit antiseraum (Sigma), also diluted 1:100. The samples were visualised by using a Nikon Optiphot microscope with an MRC-600 laser scanning confocal imaging system.

### Results

**MbMNPV and LdMNPV gp37 genes**

In the course of sequencing the genomes of MbMNPV and LdMNPV for other reasons, we have identified and sequenced gp37 gene homologues in both viruses. In MbMNPV, the gp37 gene is located at approximately 20 μm and is orientated clockwise on the circular genome map. The complete sequence of LdMNPV has subsequently been determined (Kuzio et al., 1999). The gp37 gene extends from nt 63583 to nt 64392 and is orientated anticlockwise on the circular map. Both genes display significant similarity at the amino acid sequence level to other baculovirus gp37 genes and to entomopoxvirus fusolin genes (Fig. 1A).

Overall, an N-terminal region spanning approximately 250 amino acids is well conserved but the C-terminal portions are highly divergent. LdMNPV and MbMNPV GP37 are the shortest proteins of the group (267 and 262 amino acids, respectively) and both lack most of this divergent C-terminal section. Within the N-terminal part, five conserved regions identified by Viallard et al. (1990) are also conserved in the new sequences presented here. In addition, there are significant conserved regions N-terminal of the motifs they highlighted. The tripeptide motif His–Gly–Tyr is absolutely conserved near the N terminus of all proteins. This is the mature N terminus of several of the fusolins and the amino acids N-terminal of it are considered to constitute a signal sequence (Dall et al., 1993; Hayakawa et al., 1996; Mitusahi et al., 1997; Yuen et al., 1990). LdMNPV GP37 includes two potential signals for N-linked glycosylation, at amino acids 180–182 and 195–197. The position of the latter signal is conserved in many of the other GP37 and fusolin proteins. MbMNPV GP37 has a single potential N-glycosylation site, at amino acids 178–180, corresponding to the first LdMNPV site. Six conserved cysteine residues identified by Liu & Carstens (1996) are present in both new sequences.

Phylogenetic analysis of GP37/fusolin proteins

Sequence alignments of GP37 and fusolin proteins showed that the proteins are clearly related to each other. To explore the evolutionary relationships between these proteins further, we constructed phylogenetic trees based on existing GP37 and fusolin amino acid sequences. GP37 and fusolin proteins clustered into two well-separated groups (Fig. 2). This division was strongly supported by bootstrap analysis. The fusolin sequences were further subdivided into two clades, comprised of sequences from viruses infecting lepidopteran hosts [Choristoneura biennis (Cb) EPV, Heliothis armigera (Ha) EPV and PsEPV] and from those infecting coleopteran hosts [Melolontha melolontha (Mm) EPV and AncuEPV], AcMNPV, BmNPV, Choristoneura fumiferana defective NPV (CfdefNPV) and OpMNPV GP37 proteins grouped together in a well-supported clade, separate from the other GP37s. We further tested the robustness of the monophyly of the GP37 and fusolin groups by constructing topological constraint trees in which the GP37 group was forced to become polyphyletic (data not shown). We then compared the resultant tree with the minimum tree by using the Kishino–Hasegawa and Templeton test options in PAUP*4d-64 (Kishino & Hasegawa, 1989; Templeton, 1983). All tests indicated that the topology in which the GP37 and fusolin groups were monophyletic was significantly better (P = 0.0044, Kishino–Hasegawa test; P = 0.0093, Templeton test; 591 as opposed to 607 steps).

Transcription start sites of MbMNPV and LdMNPV gp37 genes

Where their expression has been examined, baculovirus gp37 genes are expressed as late genes. We examined the transcription of the MbMNPV and LdMNPV gp37 genes.
Fig. 1. For legend see facing page.
Motifs overlaps with the TATA box. These data suggest that flanking it both represent TAAG motifs. The distal TAAG likely to represent an early start site. The initiation sites nucleotides downstream of a potential TATA box motif and is identified, 12, 98 and 130 nucleotides upstream of the ATG, for expressed as a late gene. In contrast, three start sites were upstream of the ATG, indicating that LdMNPV consensus baculovirus late start site (TAAG) 7–11 nucleotides were identified by primer extension analysis (Fig. 3). For LdMNPV-infected IPLB-Ld652Y cells and gp37 (Fig. 3B). The central site is located 30 gp37 is transcribed from both early and late transcription was initiated within a consensus baculovirus late start site (TAAG) 7–11 nucleotides upstream of the ATG, indicating that LdMNPV gp37 is expressed as a late gene. In contrast, three start sites were identified, 12, 98 and 130 nucleotides upstream of the ATG, for MbMNPV gp37 (Fig. 3B). The central site is located 30 nucleotides downstream of a potential TATA box motif and is likely to represent an early start site. The initiation sites flanking it both represent TAAG motifs. The distal TAAG motif overlaps with the TATA box. These data suggest that MbMNPV gp37 is transcribed from both early and late promoters.

Total RNA was isolated from MbMNPV-infected T. ni larvae or LdMNPV-infected IPLB-Ld652Y cells and gp37 start sites were identified by primer extension analysis (Fig. 3). For LdMNPV (Fig. 3A), transcription was initiated within a consensus baculovirus late start site (TAAG) 7–11 nucleotides upstream of the ATG, indicating that LdMNPV gp37 is expressed as a late gene. In contrast, three start sites were identified, 12, 98 and 130 nucleotides upstream of the ATG, for MbMNPV gp37 (Fig. 3B). The central site is located 30 nucleotides downstream of a potential TATA box motif and is likely to represent an early start site. The initiation sites flanking it both represent TAAG motifs. The distal TAAG motif overlaps with the TATA box. These data suggest that MbMNPV gp37 is transcribed from both early and late promoters.

**Glycosylation and subcellular localization of MbMNPV GP37**

There are intriguing differences in the published subcellular localization of other baculovirus GP37 proteins. AcMNPV GP37 is reported to be found both in the nucleus and the cytoplasm (Vialard et al., 1990); whereas OpMNPV GP37 is found only in the cytoplasm (Gross et al., 1993). In this context, we chose to examine the subcellular localization of MbMNPV GP37. To facilitate this, a polyclonal antiserum was raised against MbMNPV GP37. This was then used to study its expression and glycosylation as well as its localization within infected cells. The complete gp37 gene was expressed in a bacterial expression vector as a fusion with an N-terminal polyhistidine tag. The fusion protein was gel-purified and used as immunogen for the generation of the antiserum. Immunoblot analysis of MbMNPV-infected TN368 cells with this antiserum allowed the detection of a single protein of approximately 30 kDa (Fig. 4). No immunoreactive proteins were detected in lysates of uninfected TN368 cells. Similarly, no cross-reacting proteins were revealed when a similar blot was probed with pre-immune serum (data not shown). To examine whether MbMNPV GP37 is glycosylated, infected TN368 cells were incubated in the presence of tunicamycin, an inhibitor of N-glycosylation, prior to immunoblot analysis. Tunicamycin treatment led to the appearance of a second form of GP37 of approximately 27 kDa (Fig. 4). These data indicate that MbMNPV GP37 is at least partially glycosylated.

The subcellular localization of MbMNPV GP37 was examined by immunofluorescence analysis. Uninfected TN368 cells exhibited low levels of background fluorescence (Fig. 5 B).
Fig. 3. Primer extension analysis of LdMNPV and MbMNPV gp37 transcription. Total RNA was extracted from (A) uninfected (U) or LdMNPV-infected (I) IPLB-Ld652Y cells 48 h p.i. or (B) uninfected (U) or MbMNPV-infected (I) T. ni larvae 4 days p.i. and gp37 transcription start sites were mapped by primer extension with primers that annealed downstream of the gp37 start codons. The sequence data generated with the same primers are also presented. The primer extension products are indicated with arrows. The sequences at the bottom show the positions of the start sites (marked with asterisks) relative to the gp37 coding sequences. The A of the ATG is residue +1. Consensus baculovirus late transcription start sites (TAAG motif) are underlined and a putative TATA box element is indicated by a line above the sequence.

Fig. 4. Immunoblot analysis of MbMNPV GP37 expression. TN368 cells were infected with MbMNPV and 1 day p.i. were treated with tunicamycin (IT). The cells were lysed 60 h later and analysed by immunoblotting with the anti-GP37 antiserum. Uninfected cells (U) and infected cells not treated with tunicamycin (I) were analysed in parallel. The positions of glycosylated and non-glycosylated GP37 are shown. The positions of the molecular mass markers (in kDa) are indicated.

Use of the pre-immune serum as primary antibody resulted in no detectable fluorescence in either infected or uninfected cells (Fig. 5C, D). In contrast, after staining of infected TN368 cells with the anti-GP37 antiserum, GP37 was clearly visible in the cytoplasm of infected cells (Fig. 5A). It was typically distributed in a double semicircle or ring. There was no detectable fluorescence in the nucleus.

Discussion

The Baculoviridae and Poxviridae comprise fundamentally distinct families of large DNA viruses differing in morphology, genome structure and replication mechanisms. However, within the family Poxviridae, the EPVs share with baculoviruses the property of infecting insect hosts. In several cases, a baculovirus and an EPV that infect the same host species are known. Sriskantha et al. (1997) have proposed that these groups of viruses should share strategies for optimal infection of insect hosts and consequently should share genes not found in non-insect infecting viruses. The study of these genes would provide insights into central features of the interaction of an insect virus with its host. The gp37 and fusolin genes represent one such family of genes.

In this paper, we have characterized the gp37 genes of the baculoviruses MbMNPV and LdMNPV. Transcription analysis indicated that both genes initiate from consensus baculovirus
late start sites, suggesting that, like other baculovirus gp37 genes, they are transcribed in the late phase of infection. However, MbMNPV gp37 transcription also appeared to initiate from an early start site, 30 nucleotides downstream of a TATA box motif (Fig. 3B). MbMNPV does not grow well in cell culture. Because of this it was not possible to obtain the high-titre stocks necessary for synchronous infection of cells in culture. Consequently, it is difficult to draw firm conclusions about the temporal pattern of MbMNPV gp37 expression. However, the data indicate that it is likely to be expressed both early and late in the infection cycle. The significance of early expression of this gene is not clear.

The subcellular localization data revealed that MbMNPV GP37 is predominantly cytoplasmic (Fig. 5). In this respect, it resembles OpMNPV rather than AcMNPV GP37. Consistent with this, we were unable to detect any GP37 associated with purified MbMNPV PIBs by immunoblotting (data not shown). The fact that MbMNPV GP37 is glycosylated (Fig. 4) indicates that it is transported into the endoplasmic reticulum (ER) of infected cells. This is supported by the amino acid sequence alignment data, which strongly suggest that all GP37 and fusolin proteins possess a signal sequence (Fig. 1A). Spindle body formation is likely to take place within the ER, where the reducing environment would facilitate the formation of disulphide bonds between the cysteine residues in GP37/fusolin proteins. Mature spindle bodies have a bilamellar envelope derived from the ER membrane. In this respect, the fact that AcMNPV GP37 can be detected in the nucleus of infected cells is notable. Since it is glycosylated and can form cytoplasmic spindle bodies, it is presumably transported to the ER after synthesis. It is not apparent how an ER-localized protein can be subsequently directed to the nucleus.

The phylogenetic analyses of GP37 and fusolin proteins revealed a deep division between the baculovirus and EPV sequences. Within the baculovirus clade, the GP37 phylogeny is consistent with previously published baculovirus phylogenies based on other proteins (Clarke et al., 1996; Kang et al., 1998; Zanotto et al., 1992). Notably, the group I NPVs, AcMNPV, BmNPV, OpMNPV and CiMNPV, clearly clustered together and were distinct from the other NPVs examined. Within the EPVs, the viruses infecting lepidoptera, ChEPV, HaEPV and PsEPV, formed a separate group from the coleopteran viruses AncuEPV and MmEPV. This division was well supported by bootstrap analysis and suggests there has been little or no recent gene exchange between the two groups of viruses.

There are at least three possibilities that would account for the gp37/fusolin gene family occurring in both baculoviruses and EPVs. Firstly, a putative common ancestor of both groups of virus may have possessed a gp37/fusolin gene. However, it is known that not all EPVs possess a fusolin gene. In particular, the complete genome sequence of Melanoplus sanguinipes EPV has recently been determined (Afonso et al., 1999) and shown to lack a fusolin gene. This hypothesis would therefore require that the vertebrate poxviruses and fusolin-negative EPVs have lost the gene after diverging from the baculoviruses. Secondly, both baculoviruses and EPVs may have acquired a gp37/fusolin gene independently from the host genome. Thirdly, a gp37/fusolin gene may have been transferred from one virus group to the other, for example by transposon-mediated horizontal transmission or by direct recombination. The division of the baculovirus and EPV sequences into separate clades is strongly supported both by bootstrap analysis (Fig. 2) and by topological constraint analysis. These data indicate that there has been no recent horizontal transfer of gp37/fusolin genes between the two groups of viruses. In other respects, however, the evidence does not support firm conclusions on the origin of this gene family in baculoviruses and EPVs.

The formation of spindle bodies by GP37/fusolin proteins is consistent with a role in enhancing virus infection of an insect, perhaps acting at the stage of the initial infection within the insect gut. It seems plausible that these bodies might be relatively stable in the environment, analogous to virus OBs or the Bacillus thuringiensis δ-endotoxin crystals, and serve as a vehicle to deliver active protein to the gut of an insect host. The spindle bodies are released from larval cadavers along with the OBs and are therefore likely to be ingested when an insect ingests OBs. This situation is assured in those cases where the

Fig. 5. Subcellular localization of MbMNPV GP37. MbMNPV-infected (4 days p.i.) (A, C) or uninfected (B, D) TN368 cells were fixed on glass coverslips and incubated with anti-GP37 antiserum (A, B) or pre-immune serum (C, D). Bound antibody was detected by staining with FITC-conjugated goat antiserum against rabbit antibodies and visualized by confocal microscopy.
spindle bodies are embedded within the OBs. The presence of AcMNPV GP37 in PIBs may represent a similar strategy. Baculoviruses are already known to encode a protein that enhances virus infection at the stage of the initial formation of the midgut (Derkson & Granados, 1988; Gallo et al., 1991). This protein, known as enhancin, or viral enhancing factor, is a protease that acts by disrupting the peritrophic membrane lining the lumen of the insect midgut (Wang & Granados, 1997). The peritrophic membrane is thought to represent a barrier to virus infection of the midgut epithelial cells. Enhancins are generally associated with virus OBs. There is no detectable amino acid similarity between enhancins and GP37/fusolin proteins. Nonetheless, it may be that GP37/fusolin also acts to disrupt the peritrophic membrane. The sequence alignments we have presented show that there is some similarity between GP37/fusolins, a chitinase from Pseudalderemonas and a CBP from the actinomycete Streptomyces halstedii. CBP is known to be divided into two domains by a threonine/proline-rich linker (Fig. 1B) (Garda et al., 1997). The C-terminal domain has been identified as the cellulose-binding domain. This portion is missing from the GP37/fusolin proteins identified to date, suggesting that they are unlikely to be cellulose-binding proteins per se. The function of the N-terminal domain is less clear. However, it displays weak similarity to a Streptomyces olivaceoviridis chitin-binding protein (Schnellmann et al., 1994; data not shown). The peritrophic membrane is composed of both chitin and protein. Thus, these data may indicate that GP37/fusolin proteins facilitate virus infection by targeting the chitin component of the peritrophic membrane in some way.

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