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2. Structural proteins of baculovirus occlusion bodies and virions

Infectious baculoviruses can be generated from the transfection of naked viral DNA into susceptible insect cells indicating that the transcription of early genes required for initiating the infection is accomplished via the host cell RNA polymerase. Therefore, viral structural proteins, although necessary for protecting the viral genome and initiating infection, very likely are not required for functions such as transcription or DNA replication. Below is an overview of the proteins that have been associated with major baculovirus virion structures. These include occlusion bodies, budded virus (BV) and occlusion derived virus (ODV) envelopes, and proteins that are components of nucleocapsids. Many of the latter category are shared between BV and ODV (1).

Occlusion body evolution

In temperate climates and in many tropical areas where there are wet and dry seasons, insect populations are transitory and expand dramatically during warm, moist periods, and then collapse with the onset of colder temperatures or drought when food sources are reduced or eliminated. In addition, even during seasons optimal for insect growth and reproduction, their populations are normally limited by predators, pathogens, normal temperature cycles, and food sources. However, under certain circumstances, insect populations can expand dramatically when a combination of conditions greatly increases their food supply, facilitates high levels of reproduction, or eliminates predators and pathogens. These cycles of population expansion are well documented for forest insects and may be separated by long periods of time. For example, the Douglas fir tussock moth, a pest of forests in western North America, has cycles of 7 to 10 years, and other insect epizootics are separated by 5 to 40 years (reviewed in (2)). Viruses often play a major role in causing the collapse of these large insect populations.

A major consequence of the cyclic nature of insect populations is that their pathogens are left without hosts either seasonally, or for much longer periods of time. Viruses, in general, have developed several methods to insure their survival until their hosts reappear. If their hosts are seasonal, such as those of insect pathogens, viruses could be present in the eggs or pupae of over wintering insects, or they might persist in alternate hosts, or some might remain stable outside their hosts. Although there is some evidence for baculoviral persistence within host insects (see Chapter 3), stability outside their hosts mediated by their presence within occlusion bodies appears to be a common feature of all baculoviruses and some nudiviruses. By immobilizing the virus within the crystalline protein lattice of the occlusion body, an environment is provided that allows virions to remain viable indefinitely as long as they are protected from extremes of heat and from UV light. In addition, the stability provided by occlusion may be of such a selective advantage that it has apparently been incorporated into the life cycle of three different types of insect viruses; in Reoviridae (cypoviruses, double-stranded RNA viruses with segmented genomes), the Poxviridae (entomopox viruses-dsDNA viruses with cytoplasmic replication), and in

the Baculoviridae/Nudiviridae. No primary amino acid sequence relatedness is evident between these different categories of occlusion body proteins and the primary sequence of the occlusion body protein of a dipteran baculovirus appears to be unrelated to other baculovirus polyhedrins (3), and the polyhedrins from some cypoviruses also appear to be only distantly related to each other (4). Similarly, the occlusion body protein of a nudivirus of the pink shrimp, *Penaeus monodon,* did not appear to be related to baculovirus polyhedrin (5), even though sequence data from other predicted proteins indicated the virus was related to the baculovirus lineage (6). The structure of both cypovirus and baculovirus polyhedrins have been determined, and they appear to be unrelated and, therefore, may be examples of convergent evolution (7, 8), although one report suggests that they may be distantly related (4).

Polyhedra are thought to stabilize baculovirus virions, and in one example it was estimated that only about 0.16% of OpMNPV occlusion bodies remained infectious one year after an epizootic. However, considering that over 3 x 10^{15} occlusion bodies were estimated to have been produced per hectare during the outbreak, this would still be a substantial number of infectious viruses (reviewed in (2)).

Occlusion body proteins

In addition to virions, occlusion bodies are composed of the matrix that occludes the virions and an outer membrane-like structure on the surface. Several other proteins are also associated with polyhedra (Table 1). The major protein forming occlusion bodies is polyhedrin/granulin.

Table 1 Proteins Associated with Baculovirus Occlusion Bodies

AcMNPV orf # and name	Distribution in the Baculoviridae	Effect of Deletion[2]
Ac8 Polyhedrin	All[1]	Viable
Ac131 Polyhedron envelope/Calyx	All except CuniNPV	Viable
Enhancin	A few NPVs and GVs	Viable
Ac137 p10	Group I/II; some GVs	Viable
Alkaline proteases	Non baculovirus contaminants	

[1] CuniNPV polyhedrin is unrelated to that of other baculoviruses.

[2] For details see Chapter 12

Polyhedrin/Granulin. Polyhedrin and granulin are closely related and are the major structural components of occlusion bodies (polyhedrin in NPVs and granulin in GVs). They initially were challenging to characterize because the occlusion bodies are naturally contaminated with a protease (see below) that caused degradation of the protein upon dissolution of polyhedra. However, once it was found that the proteases could be heat inactivated, polyhedrins from several viruses were characterized and were found to contain about 250 aa (30 kDa) and are one of the most conserved baculovirus proteins. They form a crystalline cubic lattice that is interrupted by and surrounds embedded virions (Figure 1). Orthologs of polyhedrin/granulin are found in all baculovirus genomes, except for that of the dipteran virus (CuniNPV). Surprisingly, CuniNPV has an occlusion body protein that appears to be unrelated in primary amino acid sequence to the polyhedrin of other baculoviruses and is about three times as large (3, 9). The polyhedrin of an NPV pathogenic for pink shrimp also appears to be unrelated to that from other baculoviruses in its primary sequence (5). However, it was reported that it did react with antiserum to AcMNPV and TnGV polyhedrin even though at 50 kDa it appears to be twice as large as the insect baculovirus polyhedrins (10).

Despite forming a natural crystal in nature, determining the structure of polyhedrin by crystallography was unsuccessful because it was not possible to recrystallize it from solution. However, advances in crystallography, plus the observation that a single amino acid change (G25D in AcMNPV polyhedrin) resulted in larger than

normal crystals (11) allowed the determination of the structure of polyhedrins from two different Alphabaculoviruses (AcMNPV and *Wiseana* sp NPV) using occlusion bodies produced by viral infection (8) (12). A structure for a granulin from CpGV has also been determined (13). The 30 kDa polyhedrin subunits form trimers that are then arranged into dodecamers (four trimers) via disulfide bonds. This structure interlocks with another dodecamer to form the cubic-shaped unit cell of the crystal (Figure 2). Hydrophobic and salt bridge interactions between the cubes likely form the linkages at the crystal interfaces that are disrupted by the alkaline pH of the insect midgut (see below). Occlusion of virion bundles might displace up to 20,000 polyhedrin subunits and could destabilize the crystals. This possibly led to the evolution of the envelope structure that is thought to stabilize these structures (12). The presence of disulfide bonds may have prevented the attempts to crystallize purified polyhedrin.

The calyx/polyhedron envelope (PE). The calyx/polyhedron envelope is an electron-dense structure that forms a smooth, seamless surface that surrounds polyhedra. The function of the calyx/PE appears to be to seal the surface of polyhedra and to enhance their stability. In the laboratory when polyhedra are subjected to alkaline treatment, the crystalline lattice is dissolved, but the polyhedron envelope remains as a bag-like structure in which many virions are trapped (Figure 3). Although the calyx/polyhedron envelope was originally found to be composed of carbohydrate (14), a phosphorylated protein component was subsequently identified (15). This protein appears to be an integral component of the calyx/PE (16). Homologs of the PE protein (Ac131) are found in the genomes of all lepidopteran NPVs. It is likely that when polyhedra are ingested by susceptible insects, they are dismantled by a combination of the alkaline pH of the insect midgut and proteinases that are present in the midgut and associated with polyhedra. This combination would likely contribute to the disruption of the polyhedron and polyhedron envelope to facilitate virion release. The PE protein is associated with p10 fibrillar structures (Figure 4), and p10 appears to be required for the proper assembly of the polyhedron envelope (17-20). Polyhedra from viruses with either the p10 or PE gene deleted have a similar appearance; they have a rough pitted surface and the PE appears to be fragmented or absent (Figure 5). In addition, in some viral genomes, genes are present that appear to be fusions of both PE and p10 protein domains, further suggesting a fundamental relationship between these two proteins (see below). A partial structure for the PEP of CpGV has been determined (21).

Polyhedron-associated proteins

Ac68 may be involved in polyhedron morphogenesis. Homologs of Ac68 appear to be present in all baculoviruses. When Ac68 was deleted, no major effects were detected other than a longer lethal time in larvae (22). Similar results were obtained when the ortholog in BmNPV (Bm56) was deleted from a bacmid. However, the polyhedra produced by the mutant BmNPV bacmid were abnormal and lacked virions, suggesting that Bm56 may be involved in polyhedron morphogenesis (23).

P10 (Ac137). Although p10 does not appear to be a major occlusion body protein, it colocalizes with the PE protein and appears to be required for the proper formation of the polyhedron envelope. When p10 is phosphorylated, it becomes associated with microtubules (24) (25). This could be related to the structures it forms, including microtubule-associated filaments and tube-like structures that surround the nuclei of infected cells (26, 27). Deletion of P10 results in polyhedra that resemble those produced by mutants lacking the calyx/polyhedron envelope protein; they are fragile, have a rough surface showing cavities where virions have apparently become dislodged, and often show an incomplete calyx/polyhedron envelope (16, 28, 29) (see Figure 5). Serial block-face scanning electron microscopy (SBFSEM) has been used for the 3-D characterization of p10 structures in AcMNPV infected Tn-368 cells (Figure 6) (30). It forms large vermiform structures that surround nuclei and also structures within nuclei that are associated with 'electron dense spacers' which appear to contain Ac131, the polyhedron envelope/calyx associated protein (19). In cultured cells p10 facilitates occlusion body release by nuclear lysis (31) (30) and an increase in infectious virus (28). Homologs of p10 are found in the genomes of all Group I/II NPVs and most GVs, in some instances in multiple copies, e.g., PlxyGV has 3 copies

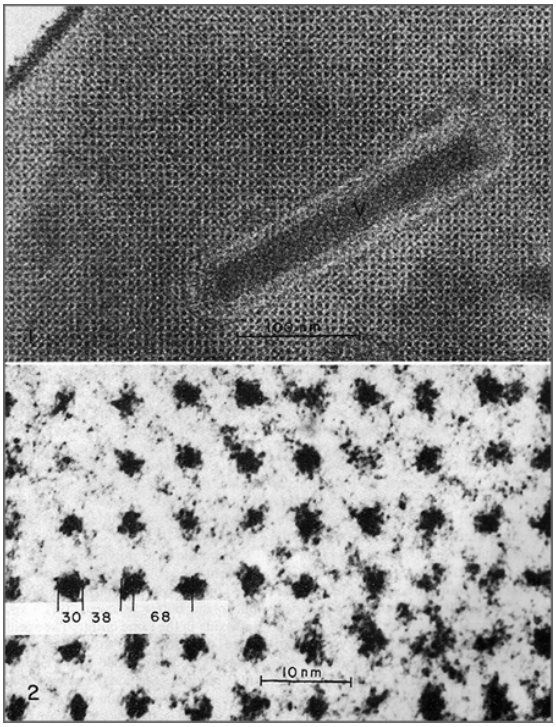

Figure 1. Sections of portions of polyhedra showing crystalline structure. Top panel is from an NPV of *Pseudohazis eglanterina* (western sheep moth). Bottom panel is a higher magnification from an NPV of *Nepytia freemani* (false hemlock looper). Measurements are shown in Angstroms. From K. Hughes (238). Reproduced with permission of Elsevier Limited via Copyright Clearance Center.

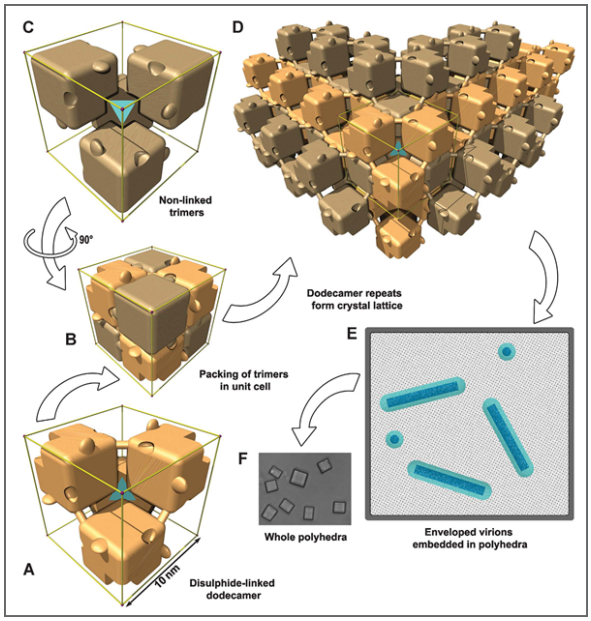

Figure 2. The assembly of polyhedrin into polyhedra. Polyhedrin trimers are depicted as simplified cubic blocks. To clarify interpretation, the edges of the unit cell are shown in gold and a cyan tetrahedron symbolizes the cell center. Within a unit cell, disulphide-linked trimers with one polarity are colored light beech (A) and those with the opposite polarity are colored light brown (C). (B) All eight trimers in the unit cell. The disulphide bond connecting adjoining trimers is shown as a dowel. (D) The crystal lattice is built up from repeats of the dodecameric unit. (E) Sketch of a cross-section through a polyhedron. The lattice spacing of the unit cells is illustrated as a dot pattern into which are embedded nucleocapsids (dark blue) surrounded by an envelope (cyan). (F) Light microscopy image of G25D mutant AcMNPV polyhedra. From Ji et al (12). Reproduced with permission via Copyright Clearance Center.

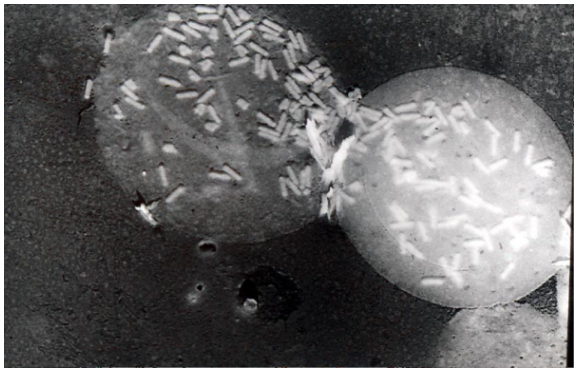

Figure 3. Two adjacent dissolved polyhedra showing rod-shaped virions trapped by the collapsed polyhedron envelope. Photo by K. Hughes.

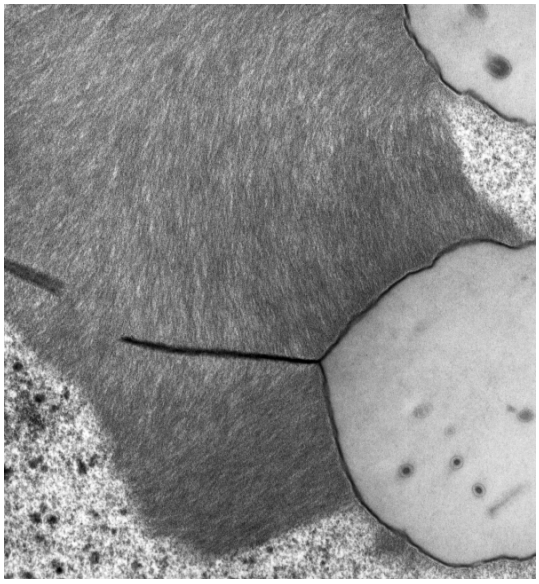

Figure 4. Fibrous p10-containing material aligned with the calyx/polyhedron envelope. Photo courtesy of G. Williams. From (239), with permission.

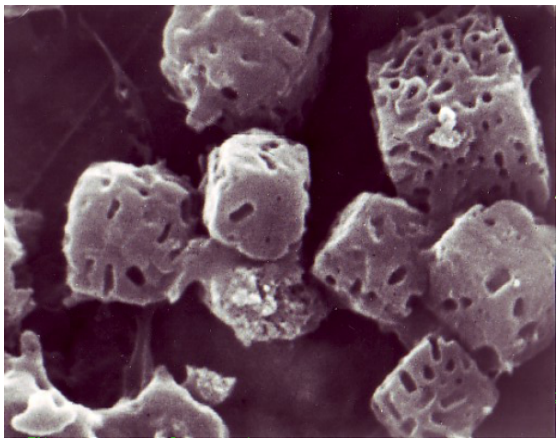

Figure5. Polyhedra from OpMNPV with the polyhedron envelope protein and p10 genes deleted. From (16), with permission.

(32). Their phylogeny is complicated somewhat by the fact that some of the GV genes appear to consist of a combination of p10 and polyhedron envelope protein domains (33, 34). A p10 homolog has also been characterized in an entomopox virus (35).

Viral enhancing factors: enhancin. Enhancins are a class of metalloproteinases (36) that are encoded by a few lepidopteran NPVs (e.g., Ld-, Cf-, and MacoNPV) and GVs (e.g., Ag-, As-, Tn-, XcGVs), but not AcMNPV. In one study of TnGV, enhancin was estimated to comprise up to 5% of the mass of occlusion bodies (37). In another virus, LdMNPV, it was found to be associated with ODV envelopes (38). Enhancin genes are often present in multiple copies, e.g., the XecnGV genome has four copies (39). In LdMNPV which encodes two enhancins, deletion of either results in a 2- to 3-fold reduction in potency, whereas deletion of both caused a 12-fold reduction (40). Enhancin is thought to facilitate baculovirus infection by digesting the peritrophic matrix (PM) (see Figure 2, Chapter 3). The PM forms a barrier in insect guts that prevents the ready access of pathogens to the epithelial cells. The PM is rich in chitin and intestinal mucin protein, and enhancins appear to target the degradation of the mucin, thereby facilitating access of virions to the underlying cells (41). Enhancins show sequence homology with high levels of significance (e.g., 3e-29) to predicted proteins of a number of pathogenic bacteria, e.g., *Clostridium botulinum*, and a variety of *Bacillus* (e.g., *B. anthracis*) and *Yersinia* (e.g., *Y. pestis*) species. To investigate their function, enhancin homologs from *B. cereus*, *Y. pseudotuberculosis*, or TnGV were cloned into a construct of AcMNPV that yielded occluded viruses. Although the LD50 of these constructs was found to be about half of wt, only the construct expressing the TnGV enhancin caused a reduction in survival time. In addition, the bacterial enhancins failed to degrade insect intestinal mucin. It was suggested that the bacterial enhancins may have evolved an activity distinct from their viral homologs (42).

Proteinases. The initial research on occlusion body structure was hindered by the presence of proteinases that degraded the proteins under investigation. These preparations were derived from insect carcasses in various states of disintegration and decay. In hindsight, it is not surprising that these preparations showed proteinase activity. The finding that the proteinases could be heat inactivated (43), led to the ability to purify and eventually sequence a number of polyhedrin proteins before the advent of DNA sequencing technology (44, 45). Subsequently, it was found that the proteinases associated with occlusion bodies had properties similar to enzymes associated with the insect gut (46) and that polyhedra produced in cell culture lacked associated proteinases (47-49). Although many baculoviruses encode a cathepsin-like proteinase, in AcMNPV (Ac127) it was most active under acidic (pH 5) conditions (50). Therefore, the proteinases associated with occlusion bodies are likely a combination of enzymes derived from bacteria, the insect gut, and the virus.

Baculovirus virions: The envelope proteins

For AcMNPV and other relatively well-characterized lepidopteran NPVs, there are two types of virions produced during the virus infection; in insects the infection is initiated by occlusion-derived virus (ODV) that are released into the insect midgut upon dissolution of the occlusion bodies. ODV initiate infection in the midgut epithelium, and the virus propagated in these cells are budded virus (BV) that exit the cells in the direction of the basement membrane and spread the infection throughout the insect. Late in the infection, virions become occluded within the nuclei of infected cells and are released into the environment upon the death and disintegration of the insect. The major difference between BV and ODV is the origin of their envelopes. BVs derive their envelopes as they bud through the host cell plasma membrane that has been modified by viral proteins. In contrast, ODV obtain their envelope in the nucleus and it may be derived from nuclear membranes that are modified with a number of viral proteins. Whereas viral contributions to the BV envelope may be limited to one or two proteins, ODV envelopes are very complex. They appear to contain a number of virally encoded proteins, and in some instances, it is difficult to separate them from capsid proteins.

Cell entry: Baculovirus envelope fusion proteins

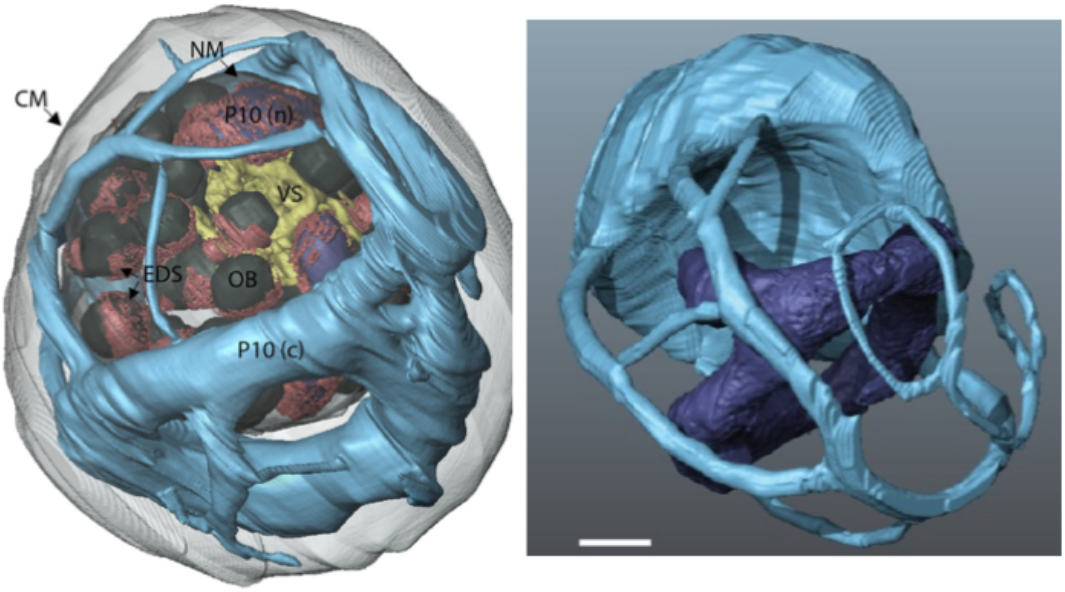

Figure 6. Whole cell 3D reconstruction of P10 structures in AcMNPV infected Tn-368 cells (30) using serial block-face scanning electron microscopy Left image: 72 hpi with the following structures labeled: virogenic stroma (VS); P10 (nuclear, n; cytoplasmic, c); electron dense spacers (EDS); and occlusion bodies (OB) as well as the cytoplasmic (CM) and nuclear membranes (NM). Right image: Nuclear (dark blue) and cytoplasmic P10 (light blue) structures at 96 hpi. Images are from (30) via Creative Commons.

Many viruses are surrounded by a lipid envelope and enter cells either by fusion with the cell surface membrane or an endosomal membrane after phagocytosis by a cell. The merging of the membranes is activated by fusion proteins. There are three classes of viral envelope fusion proteins, I, II, and III (reviewed in (51) and baculoviruses employ categories I and III. Class I fusion proteins are activated by cleavage of a trimeric single-chain precursor protein. Cleavage is usually activated by exposure to low pH. Examples are the influenza hemagglutinin (HA), the HIV gp41, and the baculovirus F fusion proteins. Class II fusion proteins are found in small icosahedral viruses such as flaviviruses and alphaviruses and are present in a 1:1 complex with a chaperone protein that inhibits fusion. The inhibition is removed by cleavage of the chaperone. Class III fusion proteins are activated by low pH, but are not cleaved, and are found in rhabdoviruses (e.g. vesicular stomatitis virus - VSV-G), herpes viruses (gB), and some baculoviruses and orthomyxovirues (gp64).

Envelope proteins of budded virions

AcMNPV the most well studied baculovirus may enter cells by two different processes; direct fusion, and by receptor mediated endocytosis (52). Probably the most well characterized baculovirus structural protein is the envelope fusion protein of Group I baculoviruses, GP64 (Class III), because a relatively simple assay for its fusion activity was developed early in its investigation (53). Because of early studies elucidating the importance of this protein for AcMNPV BV infectivity (54, 55), it was unexpected when genome sequence analyses of additional baculoviruses revealed that many of them lacked homologs of the *gp64* gene (56, 57). Furthermore, it was determined that these viruses use a different fusion protein called F (Class I) and that homologs of F are retained in *gp64*-containing viruses (Figure 7). These F homologs in viruses encoding GP64 are inactive as fusion proteins, suggesting that *gp64* was obtained by a baculovirus and displaced the fusion function of the F protein, but the F gene was retained. These proteins are described below.

GP64 (Ac128) is present in all Group I NPVs, a single GV, and certain lineages of orthomyxoviruses. GP64 is a fatty acid acylated glycoprotein (58) and a low pH activated envelope fusion protein (59-61) that is one of the three most abundant proteins, along with vp39 and p6.9, found associated with AcMNPV budded virions (62).

The presence of the *gp64* gene is one of the major distinguishing features of the Group I alphabaculoviruses. Deletion of AcMNPV *gp64* results in viruses that replicate in a single cell, but cannot bud out and infect surrounding cells (55, 63). It was originally thought that all Group I viruses use GP64 for the entry of BV into cells, whereas all other baculoviruses lack a *gp64* homolog and appear to use the F (Ac23 homolog) protein as their envelope fusion protein except for hymenopteran NPVs, which lack both genes (Figure 7). However, it was subsequently found that *Diatraea saccharalis* (the sugarcane borer) granulovirus, DisaGV, encodes both F and gp64 fusion protein orthologs (64). This was the first example outside of the Group I nucleopolyhedroviruses where a baculovirus possesses gp64 in addition to the F protein. It is thought that gp64 was incorporated into the Group I viruses in a recombination event (Figure 8) that may have involved up to 11 other additional genes specific to Group I NPVs (Chapter 1, Table 3). After incorporation, it is thought that gp64 displaced the fusion function of the F protein which has been retained but is not an essential gene (65). An AcMNPV bacmid deleted for gp64 and pseudotyped with DisaGV gp64 was infectious although at a lower level than wt AcMNPV. In cultured sf9 cells, it infected about 60% of the cells, vs wt which infected close to 100%. Also, although DisaGV gp64 was fusogenic for Sf9 cells at low pH, it was less efficient than AcMNPV gp64 (66). DisaGV gp64 shows 67-74% identity to many NPV gp64 sequences in genbank. Phylogenetic analysis of DisaGV gp64 suggests that its incorporation into the GV genome is not a recent event (Figure 9) although it does clearly belong to the baculovirus gp64 lineage. It has not been determined if gp64 is the main fusion protein of DisaGV or if its F protein is active and is also involved in fusion.

GP64 and the Orthomyxoviridae. In addition to the Group I NPVs, orthologs of *gp64* are also found in several genera of the Orthomyxoviridae. Currently, there are 7 genera of Orthomyxoviridae, four that appear to be specific to vertebrates (birds and mammals)(Influenza virus A, B, C, and D), and encode a hemagglutinin (Class I) that facilitates viral attachment to sialic acid residues and fusion. Genera A and B also encode a neuraminidase that digests the receptor and prevents the binding of newly replicated virions to cells, whereas genera C and D encode a combination hemagglutinin-esterase (HE) that has both functions. The Isavirus genus of Orthomyxoviridae (infectious salmon anemia virus -ISAV), a pathogen of Atlantic salmon, appears to encode the attachment and fusion domains on two different molecules. Although evolutionarily distant, structural evidence suggests that the ISAV F protein is a Class I fusion protein related to those from other orthomyxoviruses (67, 68). In contrast, the Quaranjaviruses and Thogotovirus genera lack genes similar to HA and neuraminidase and instead encode an ortholog of baculovirus gp64 (Class III) that is involved in both attachment and fusion. These viruses are associated with ticks and birds, but some have been reported to infect humans. In addition, an unclassified Orthomyxovirus, Sinu virus, associated with mosquitoes also encodes gp64. Primary sequence and structural information indicate that the baculovirus and orthmyxovirus gp64 molecules have a common origin (69). Phylogenetic analysis of gp64 indicates that the Sinu and baculovirus gp64s have a common lineage distinct from the other orthomyxovirus gp64 suggesting that the baculovirus gp64 may have originated from a virus in the Sinu virus lineage (Figure 9). The post-fusion structure of AcMNPV GP64 indicates that six of seven disulfide bonds are conserved between thogotovirus and baculovirus GP64 and one forms an intermolecular bond involved in trimer formation (70). The data indicate that the fusion peptide and receptor binding sites co-localize to a hydrophobic patch located in two loops (L1 and L2) at the tip of the trimer (Figure 10). Further evidence for the involvement of L1 and L2 was subsequently provided in studies that employed alanine scanning mutagenesis (71) (72). A third loop (L3) attached to L2 was not involved in binding or fusion. It was suggested that transient forms of GP64 embed hydrophobic side chains into cell membranes triggering endocytosis independent of specific receptor molecules. This lack of specific receptors (see below) and the affinity of GP64 for cell membranes may explain its ability to facilitate the entry of AcMNPV into a wide variety of different cell types. In addition, it was suggested that the lack of a high affinity receptor is consistent with AcMNPV systemic infections. Since the infection has already been initiated by midgut infection using a mechanism independent of GP64, GP64 has evolved to spread the infection to as many cell types as possible via systemic infection. A comparison of the major structural features of GP64 and F proteins is shown in Figure 10.

Evolution of Group I (GP64 containing) baculoviruses. As indicated in Chapter 1, Group I baculoviruses appear to have been a lineage that had, in addition to gp64, 11 genes not present in other lepidopteran NPVs. Since many groups of NPVs have sets of genes not present in other lineages, this would not have been extraordinary as such genes might be lost and gained over time. However, the incorporation of *gp64* appears to have changed the biology of the lineage enough so that they evolved into a distinct group of viruses (73). In addition, this evolutionary event was recently recapitulated with the demonstration that *gp64* could pseudotype and partially rescue a Group I virus deleted for its F protein (74) (75). However, in those experiments, the recombinant virus lacked both the F protein and the other genes unique to the Group I lineage. The latter includes genes encoding the transactivator IE-2 (Ac151) and the global transactivator ortholog among others that could have facilitated the evolution of this lineage after it incorporated *gp64* (73).

Fusion protein-F, (Ac23). Although F (Ac23) is not an active envelope fusion protein in AcMNPV, in Group II NPVs, GVs and the dipteran virus (CuniNPV), orthologs of F are likely used as the fusion protein, because all these viruses lack homologs of *gp64*. An exception appears to be the hymenopteran NPVs that lack homologs of both the F and GP64 proteins (Figure 7) (see discussion in Chapter 3). Several reports have described investigations in cultured cells on AcMNPV in which *gp64* was substituted with the *F* gene from other baculoviruses. In one study, a construct with Ac23 was not completely rescued by Se8 (77), whereas in another, the presence of Ac23 appeared to be required for the elevation of infectivity to near wt levels (78). It was suggested that these contrasting results might be due to the different strength of the promoters used to express Se8 in the two constructs (78). F proteins of Group II NPVs function as low-pH envelope fusion proteins (79) (80) and can also rescue AcMNPV lacking *gp64* (77) (81). The F protein of granuloviruses has been implicated as a fusion protein due to their lack of a *gp64* ortholog. Although the gene encoding one such protein (PlxyGV orf26) failed to mediate fusion when incorporated into the genome of an AcMNPV mutant bacmid lacking *gp64* (77), another from *Agrotis segetum* GV (Agse orf108) was able to rescue AcMNPV lacking *gp64* (82). Other GV F proteins can also substitute for gp64 (83). F proteins appear to be members of a large and diverse family of viral envelope fusion proteins called Class I (see above), reviewed in (70). They are present as homotrimers and are synthesized as a precursor that is cleaved by a furin-like proteinase into two subunits, and near the amino terminus of C-terminal peptide is a hydrophobic fusion peptide (Figure 10). Class I fusion proteins include many other viral fusion proteins such as influenza HA (e.g., see (84, 85)). In addition, orthologs of the baculovirus F gene are also found as the env gene of insect retroviruses (Figure 8) (reviewed in (65) and are also present in some insect genomes (86, 87) (see Chapter 11). With the recent determination of the structure of a variety of viral envelope fusion proteins, when baculovirus F and insect retrovirus env proteins are analyzed using the Hhpred structure prediction program (88), they all have predicted structures similar to the fusion proteins of paramyxoviruses. Some probabilities are quite high; the PxGV or26, 95%; Kanga env 97%; LD130, 92%. Even the nonfunctional Ac23 from the virus that uses gp64 as its fusion protein, scored about 90%. Paramyxoviruses are negative strand RNA viruses and they only seem to be found in vertebrates, although there is one report from a parasitic insect of birds. Although that data might have been due to the insect feeding on an infected host (89). In addition, recent evidence suggests that the F and GP64 categories of fusion proteins may be distantly related (70).

Although inactive as a fusion protein in Group I viruses, the F protein homolog in OpMNPV (Op21) is glycosylated. Similarly, in Helicoverpa armigera NPV (HearNPV) where F is the active fusion protein, it is N-glycosylated at 5 of 6 sites (76). In OpMNPV it was associated with the envelope of BV and with the membranes of OpMNPV infected cells (90). In AcMNPV, the F homolog (Ac23) is also associated with BV membranes and its deletion from the genome results in infectious virus with titers similar to wt in cultured cells, but the time to kill larvae is somewhat extended (91). In addition, antibodies against some selected regions of GP64 appeared to inhibit binding of BV to Sf9 cells of Ac23 deleted virus to a greater extent than wt virus. This was interpreted to suggest that Ac23 may increase the binding of BV to Sf9 receptor molecules (92). It could also indicate that Ac23 is closely associated with GP64 in the virion envelope and when it is absent, the antibodies have greater access to GP64. Proteomic studies found Ac23 to be associated with AcMNPV ODV as was the homolog in CuniNPV (93,

94). This suggests that it is transported to the nuclei of infected cells. The significance of this is not clear. Whereas AcMNPV is able to enter a variety of vertebrate cells, when the gene encoding its envelope fusion protein, gp64, was replaced by F genes from 5 different NPVs, although some replicated well in insect cells, none of these recombinants was capable of entering vertebrate cells (81) suggesting that they have a much greater cell specificity than GP64.

Identification of virion proteins using mass spectrometry and bacmid knockouts: Is a protein really an essential component of a virion's structure?

A major advance in the enumeration of proteins associated with virion components has involved the use of mass spectrometry. Fractionating BV and ODV into envelope and nucleocapsid fractions and analyzing their content using mass spectrometry in conjunction with data from DNA sequencing, has provided a wealth of information of many proteins associated with these structures. This information can include both host proteins and also modifications of proteins. These studies also identify proteins that would not be predicted to be part of the virion structure (93-95) including a variety of proteins involved in DNA replication and transcription. It is unknown whether the presence of these proteins is adventitious or if they are bona fide structural proteins and play a role in accelerating the initiation of the infection cycle. The presence of proteins, such as DNA polymerase, likely reflects an intimate relationship between DNA replication and packaging, and nucleocapsid assembly and envelopment. In addition, lipids can be 'sticky,' and proteins in close proximity during virion assembly could adhere to the envelope and co-purify with ODV. Evidence from proteomic analysis of BV support this theory as they appear to lack most proteins associated with DNA replication and transcription that have been reported from ODV, suggesting that they were stripped off the nucleocapsids as they moved through the cell to the cytoplasmic membrane (62) (96). Furthermore, the facultative association of proteins with BV was demonstrated when it was found that BV can trap baculovirus expressed chloramphenicol acetyl transferase (97). Likewise, some proteins that are present in polyhedra and associated with occluded virions may have a lesser affinity for the envelope and may be lost during the ODV purification process. Proteomic analysis combined with the use of bacmid knockout constructs provides additional information regarding whether the protein is required for virion structure. The bacmid data can provide its own set of complications such as when an observed structural defect may be due to a secondary effect on some other structure. An example is the DNA binding protein DBP (Ac25). It is not associated with virions, but when deleted, virions appear to be structurally defective. Since DBP associates with the virogenic stroma, and when deleted, this structure is absent, it has been suggested that the role of DBP in virion structure is caused by the contributions of DBP to the structure of the virogenic stroma. Without a properly formed virogenic stroma, the assembly of virions is aberrant (98). Consequently, the best data is derived from a combination of proteomic, structural investigations using knock out bacmids, and immunological data that can provide definitive information on the location of a protein. Fortunately, the latter information can be often conveniently derived from the use of epitope tagged repair viruses produced using the bacmid technology. In the following overview, I have concentrated on the proteins for which there is information in addition to that provided by mass spectrometry. Information on AcMNPV proteins, that are not covered below can be found in (1) and Chapter 12.

Host proteins associated with BV envelopes. Evidence for host proteins associated with virions is described at the end of this chapter.

Cell receptors and virus entry for budded virions

Evidence indicates that GP64 is the receptor binding protein of AcMNPV (99). It is also well documented that GP64 can mediate the entry of AcMNPV into a wide variety of vertebrate cell lines (e.g., (100)). However, the identification of the cell receptor for budded virions has remained elusive. In one study, it was suggested that cell surface phospholipids might be involved in the AcMNPV BV (GP64 mediated) entry into vertebrate cells since

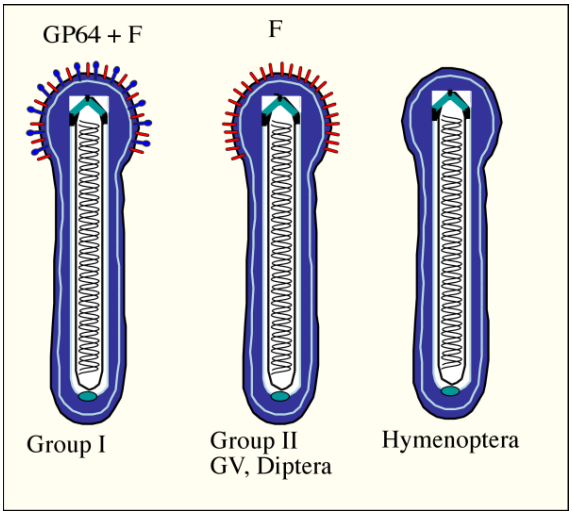

Figure 7. Distribution of envelope fusion proteins. Group I have homologs of both GP64 and F, but F is not a fusion protein. Group II, GVs and dipteran viruses have homologs of F, whereas the hymenopteran viruses have homologs of neither GP64 nor F. As noted in the text, there is one instance of a GV encoding *gp64*.

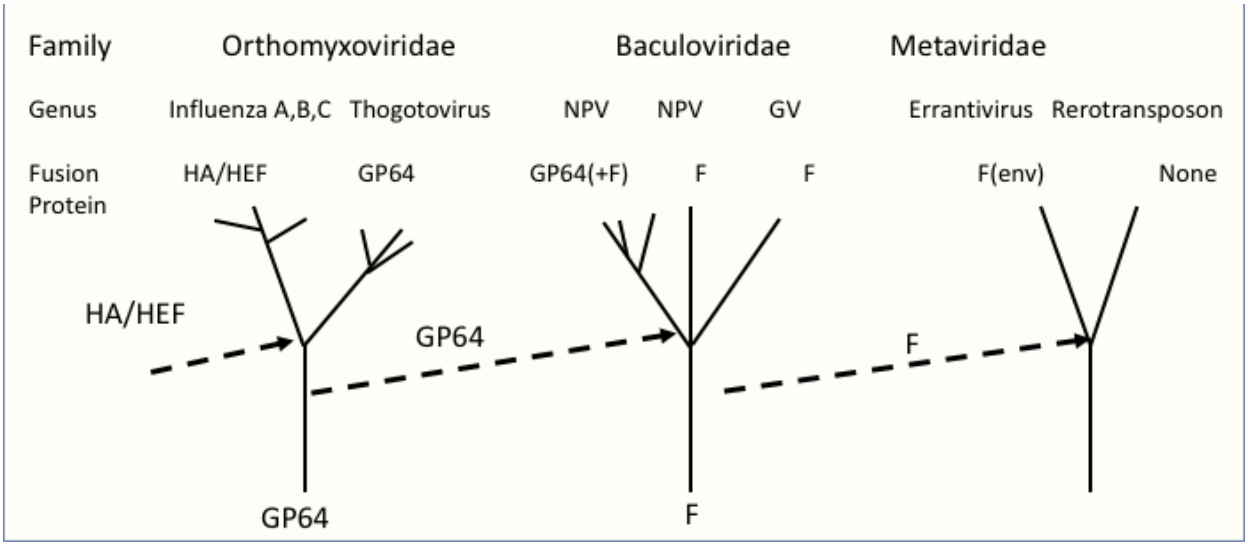

Figure 8. A model for the transfer and incorporation of envelope fusion proteins (EFP) between viral families. Orthomyxoviridae: The original orthomyxoviruses likely had gp64 as their EFP because they originated as pathogens of invertebrates at a time predating the evolution of vertebrates. At some point after vertebrates evolved, one lineage incorporated the HA/HEF EFP and lost GP64. Baculoviridae: A main lineage of baculoviruses had F as its EFP and one lineage incorporated GP64 which displaced the fusion function of F but F was retained. The Metaviridae: An insect retrotransposon lacking an EFP incorporated F from a baculovirus leading to the evolution of the errantivirus lineage.

treating cells with phospholipase C reduced reporter gene expression in cells (101). In addition, acidic phospholipids in giant unilamellar vesicles are required for fusion with AcMNPV BV envelopes (102). A role for membrane spanning heparin sulfate proteoglycans (syndecans) in virus binding has also been proposed for entry into mammalian cells (103). Investigations also have suggested that macropinocytosis, dynamin- and clathrin-dependent endocytosis, and cholesterol in the plasma membrane, all may be involved in the entry of AcMNPV into mammalian cells (103). In mammalian cells, AcMNPV entry is facilitated by low pH. In addition, when the basicity of the basic loop in GP64 was increased, the ability of the virus to enter mammalian cells was elevated. However, this mutant failed to spread between Sf9 cells. Viruses grown in insect cells from different species showed differing efficiency of mammalian cell entry suggesting that some host factors incorporated into

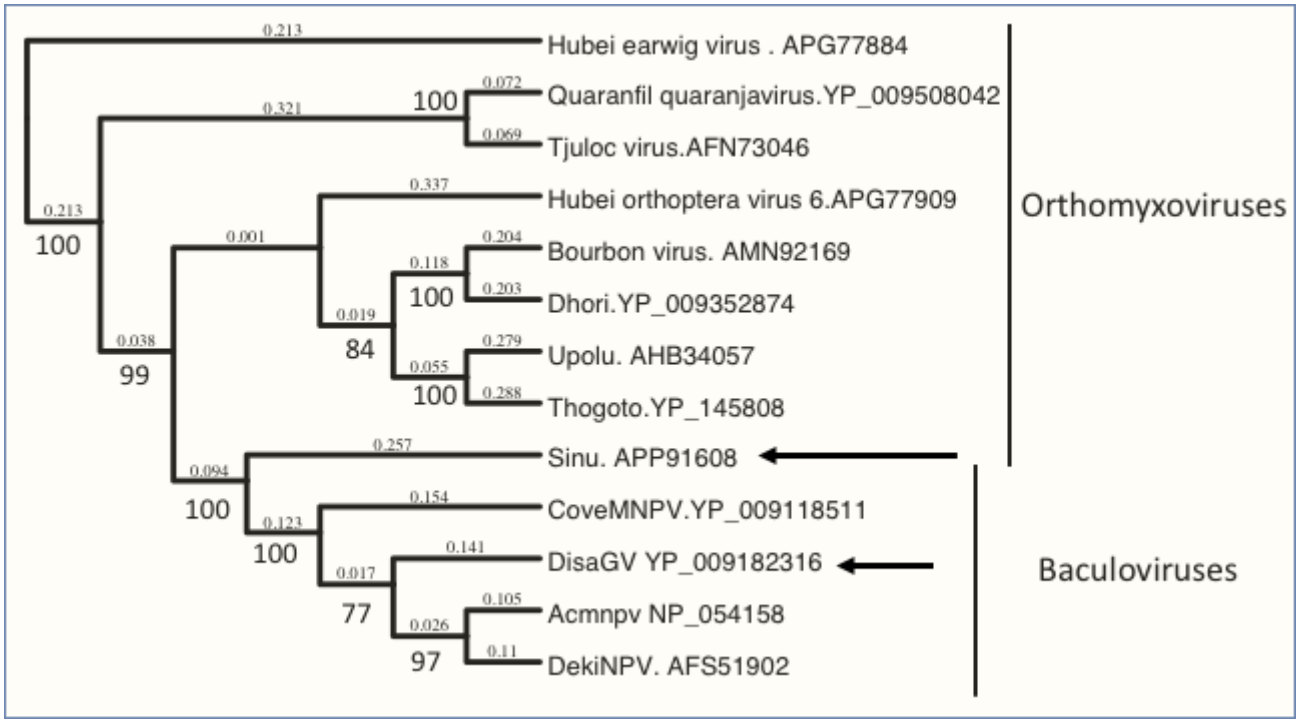

Figure 9. Phylogeny of the GP64 viral envelope fusion protein in the Baculoviridae and Orthomyxoviridae. This is a combination tree: Neighbor Joining Best Tree with Neighbor Joining Bootstrap (1000 replicates). The numbers in bold indicate Bootstrap values ~~(1000 repeats)~~ of over 70%. The GenBank numbers are shown to the right of each viral lineage. The upper arrow indicates the gp64 of the Sinu virus, an orthomyxovirus-like virus. Based on this analysis the Sinu virus gp64 shares a common lineage with baculovirus gp64 with high levels of confidence. The lower arrow indicates the GP64 present in a GV. Baculovirus abbreviations: Cove=Condylorrhiza vestigialis; Disa= Diatraea saccharalis; Deki= Dendrolimus kikuchii.

the virus might facilitate cell entry (104). As described above, the lack of specific receptors and the affinity of GP64 for cell membranes may explain its ability to facilitate the entry of AcMNPV into a wide variety of different cell types and this might facilitate systemic infections where many different cell types are encountered (104). Other reports indicate that BV of a virus that used an F fusion protein did not have the ability to enter the array of vertebrate cells as AcMNPV. Consequently, it was suggested that the F fusion proteins might use a different receptor from GP64 (105) (106). In contrast, another report described the ability of UV-inactivated HearSNPV expressing either F and GP64 was able to compete for receptors with an F expressing wt virus suggesting that they have similar modes of entry (74). For further information see (1).

Envelope proteins of occlusion-derived virus that are also BV associated

The source and content of the envelope is the major distinguishing feature between BV and ODV. In contrast to BV, where a few virus-encoded proteins have been identified as envelope associated, the ODV envelope is much more complex (Figure 11). There may be five or more such proteins categorized as envelope proteins along with a set of about 9-12 proteins called per os infectivity factors (PIF) (107) (108) (109) that are likely envelope components. Some of these proteins contain an N-terminal hydrophobic sequence in combination with several adjacent positively charged amino acids. These have been predicted to be motifs that target these proteins to intranuclear microvesicles that are the likely precursors from which the envelopes of occluded virus are derived (110). The following proteins (see also Table 2) have been characterized and are likely to be components of ODV envelopes (see also Figure 11).

BV/ODV-E26, (Ac16). Homologs of Ac16 are found in lepidopteran Group I NPVs. Evidence for the association of Ac16 with BV and ODV envelopes has been reported for AcMNPV (111) (93) (62). However, in BmNPV, the

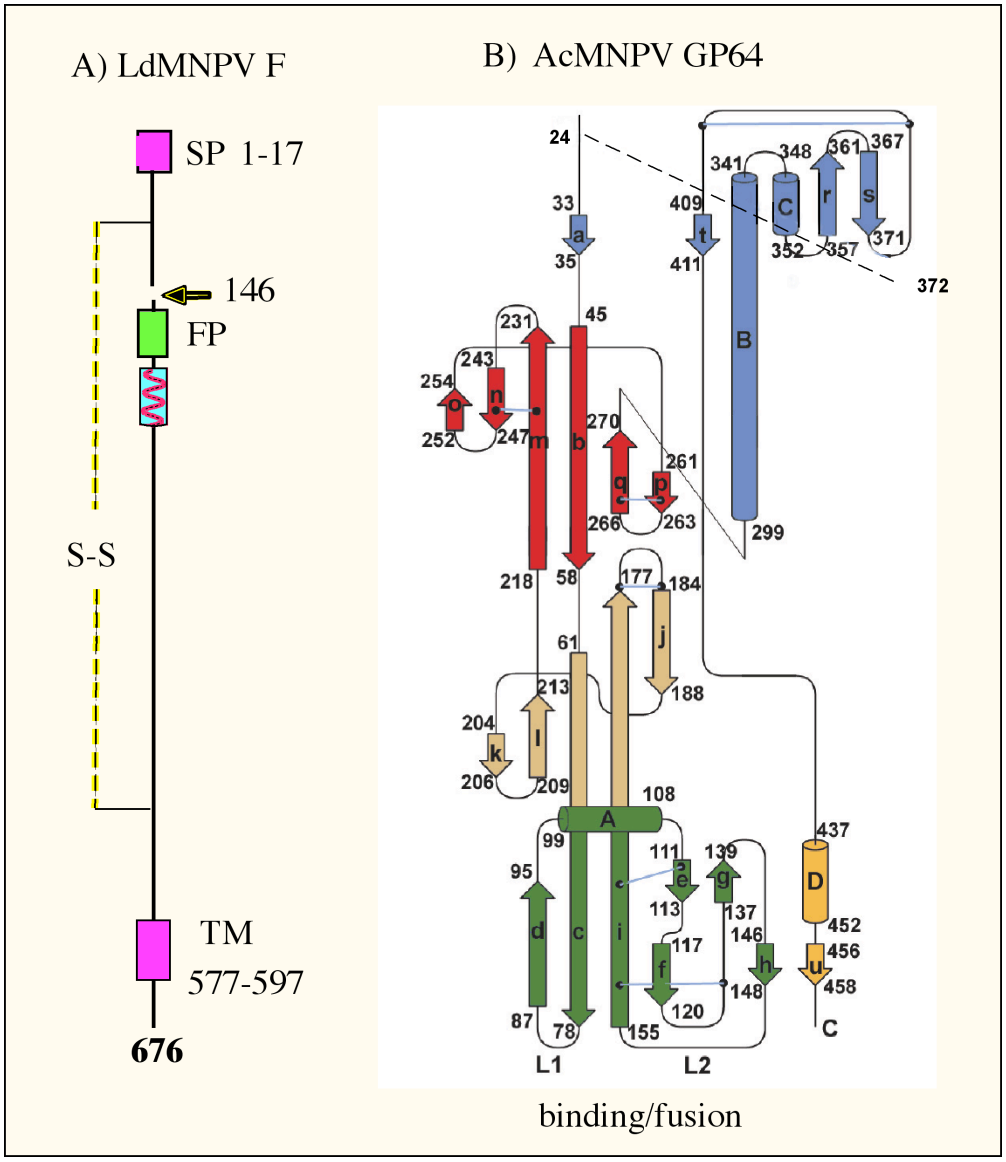

Figure 10. Structure of the baculovirus F (fusion) protein (Ld130) from LdMNPV and AcMNPV GP64. A) Ld130 F protein. Shown is a predicted signal peptide (SP), fusion peptide (FP) and transmembrane domain (TM) including the amino acid coordinates. The cleavage site is indicated by the arrow. A predicted coiled coil domain is also indicated. The disulfide bond is predicted from (240). B) GP64: Shown are 7 disulfide bonds, all of which are intramolecular except for the one from aa 24 which is connected to aa 372 in an adjacent molecule. This and the coiled-coil (299-341) region are involved in trimer formation. All the disulfide bonds are conserved except 178-184 which is not present in thogotovirus GP64. The receptor binding/fusion peptide region is shown at the base of the diagram. GP64 is from Kadlec et al (70). Copyright 2008 by Nature Publishing Group. Reproduced with permission of Nature Publishing Group via Copyright Clearance Center.

homolog of AcMNPV Ac16 (Bm8) was not identified as a virion structural protein (112). It was suggested that these conflicting results were due to the different sources of the antibodies used to detect the protein (110). Ac16 interacts with FP25 (Ac61), forms a complex with cellular actin (111), and is palmitoylated (113). A mutant in which AcMNPV orf16 (called DA26) was insertionally inactivated was viable and showed no difference from wt in infections of *T. ni* or *S. frugiperda* cells or larvae (114). In another report, a virus deleted for Ac16 infected cells showed a delay in BV production (115). It has also been shown to interact with both IE-1 and IE-0 and may be involved in the regulation of these gene products (116).

ODV-E25, (Ac94). Ac94 is a core gene present in all baculovirus genomes (117). The protein encoded by this gene was originally identified in OpMNPV, and immunogold staining with a specific antibody against Ac94 was

localized to ODV envelopes (118). It has also been shown to be associated with BV and ODV of AcMNPV and HearNPV (93, 95) (62) (119) and is highly expressed in midgut cells (120). The hydrophobic N-terminal 24 aa of AcMNPV ODV-E25 appears to be a nuclear targeting signal (121). Ac94 associates with NSF and may be involved in the nuclear entry and egress of BV (122). Deletion of Ac94 resulted in a 100 fold reduction in infectious BV and ODV were not evident and virions were not occluded in polyhedra (123). When ODV-E25 is expressed as an early gene under the IE-1 promoter, it accumulates on the cytoplasmic side of the nuclear membrane rather than within nuclei, and budded virus production is severely reduced. This suggests that it might play a role in the shift from BV to ODV virions. An open reading frame of ODV-E25 encodes a microRNA that down regulates ODV-E25 expression. It was suggested that this might result in a reduction in infectious virus production and be involved in the shift to occluded virus production (124) (125). In addition, expression of Ac94 from the very late polyhedrin or p10 promoter reduced and delayed occlusion body formation suggesting that it may play a role in virion occlusion (126) (127).

ODV-EC43, (Ac109). Ac109 is a core gene present in all baculovirus genomes. Evidence suggests that it is ODV-associated in AcMNPV (93) and *Helicoverpa armigera* NPV (Ha94=ODV-EC43) (95, 128) and also is BV-associated in AcMNPV (62) (129). Four studies have examined deletions of Ac109 and demonstrate that it is an essential gene and when deleted, DNA replication is not affected. One study reported that deletion of Ac109 resulted in a block in nucleocapsid and polyhedron formation (130). However, the other reports described different results. One indicated that polyhedra and virions were produced by Ac109 deletions, but the virus was not infectious (129). Another study found similar results, but also showed that the nucleocapsids had defects in envelopment and the polyhedra lacked virions (131). A fourth report also described similar findings, but indicated that the BV produced by a Ac109 knockout could enter the cytoplasm, but not nuclei, and also noted that the occlusion bodies lacked virions (132).

ODV-E18, (Ac143). Ac143 is a core gene present in the genomes of all baculoviruses. An antibody generated against an Ac143-GST fusion reacted with a protein of 18 kDa in the ODV envelope fraction and Ac143 was named ODV-E18 (133). Ac143 and its HearNPV homolog were found in surveys of ODV-associated proteins by proteomic analysis (93, 95). Deletion of Ac143 results in single cell infections that produce polyhedra and therefore appears to be essential for BV production (134). In addition to its presence in ODV envelopes, it is also BV associated (62). Hhpred (135) predicts the presence of a transmembrane helix and similarity to a protein translocation complex of bacteria.

Ac144 (ODV-EC27) See below.

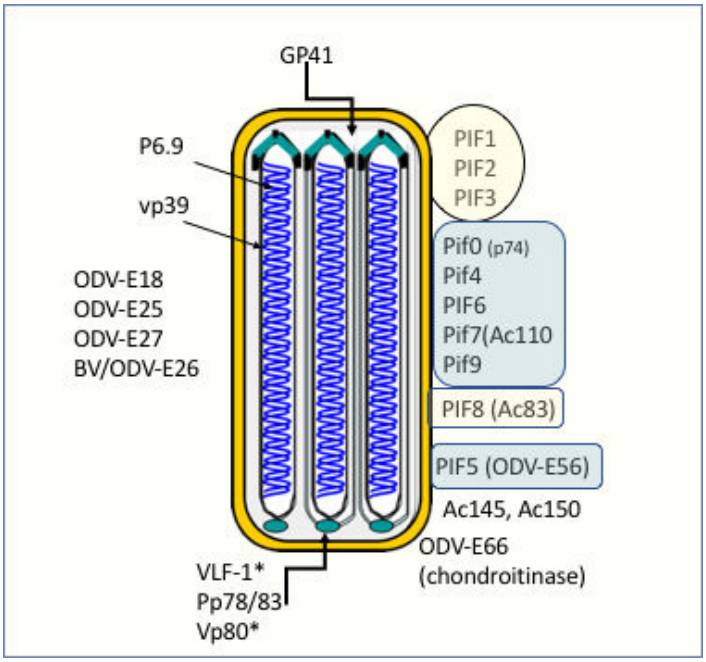

Figure 11. Selected structural proteins of ODV. Shown are envelope associated proteins (ODV-E), the PIF proteins (also envelope associated), the tegument protein, gp41, the DNA binding protein, p6.9, and two basal end-associated proteins, pp78/83 and VLF-1. For convenience, VLF-1 and Vp80 is shown here located at the basal end, however, they could be located at the apical end (227) (167). PIFs 1-3 are the core PIF complex. Without them, the other PIFs will not assemble. PIFs 0, 4, 6, 7, and 9 comprise a larger complex dependent on the pif1-3 complex, while pif8(ac83) binds to that larger complex (108, 109, 136). PIF5 is not part of the complex. There are a variety of other capsid proteins, but they appear to have a more generalized distribution. For details see the text.

Table 2. Occlusion Derived Virus Envelope Proteins and Per os infectivity factors

AcMNPV orf # and name	Distribution in the Baculoviridae	Effect of Deletion
ODV envelope proteins		
Ac16, BV/ODV-E26	Lep. I	Viable (114)
Ac94, ODV-E25	Lep. I, II, GV	Not viable (144)
Ac109, ODV-EC43	All	Not viable (130) (129)
Ac143 ODV-E18	Lep. I, II	Not viable (134)
Per os infectivity factors		
Ac22, pif-2	All	Viable (not by per os) (138)
Ac46, ODV-E66	Lep. I, II, GV	Viable (144) (150)
Ac68, pif-6	All	Viable (not by per os) (242)
Ac83	All	See (145)
Ac96, pif-4	All	Viable (not by per os) (243)
Ac110, pif-7	All	Viable (not by per os) (244)
Ac115, pif-3	All	Viable (not by per os) (138)
Ac119, pif-1	All	Viable (not by per os) (138)
Ac138, pif-0 p74	All	Viable (not by per os) (245)
Ac145	All but CuniNPV	Viable (reduced per os) (155)
Ac148, ODV-E56 pif-5	All	Viable (not by per os) (246)

Table 2. continued from previous page.

AcMNPV orf # and name	Distribution in the Baculoviridae	Effect of Deletion
Ac150	Lep. I (a few)	Viable (155)

Per os infectivity factors (PIF); ODV envelope associated proteins required for midgut infection

Ac22 (*pif*2), Ac68 (*pif*6), Ac83(*pif*8), Ac96 (*pif*4), Ac108(*pif*9), Ac110 (pif7), Ac115 (*pif*3), Ac119 (*pif*1), Ac138 (p74-*pif*0), Ac148 (*pif*5). Per os infectivity factors were originally identified because they were required for infection of insects, but dispensable for infection of cultured cells (107) (108, 109, 136). There are at least 10 *pif* genes encoded by AcMNPV (Figure 11). Orthologs of PIF genes (except PIF9) are present in all baculovirus genomes and most are also found in genomes of nudiviruses (137) and some are found in other virus pathogenic for invertebrates (see Chapter 1). Three additional possible PIF genes have also been noted (Ac46 (ODV-E66), Ac145, and Ac150) (see below). AcMNPV mutants lacking *pif 1*, *2*, or *3* are not orally infectious for *T. ni* or *S. exigua* larvae based on feeding of 10,000 PIB of the deleted virus. They are also not orally infectious for *H. virescens*, except for the PIF2 mutant that shows limited infectivity. In contrast, injection of 1 pfu of the three deletion mutants into third instar larvae of these three species caused over 80% mortality.

PIF function; binding and entry to midgut cells. Most of the PIF proteins contain a hydrophobic region that is thought to be involved integration into the ODV envelope. Nine of the PIF proteins form a complex that is involved in binding and entry of ODV into midgut cells. It includes three that form a stable core complex (PIFs 1-3) and five that associate with this complex (PIFs -0(p74), -4, -6, -7, and -9) to form a larger complex. In addition, Pif8(Ac83) can bind to the larger 8-subunit complex (reviewed in (108) (109, 136) (Figure 11). PIF1, PIF2, and p74 mediate specific binding of ODV to midgut cells, suggesting that they are directly involved in virus cell interaction as an initial step in infection (138) (138, 139). Although PIF-3 appears to be a component of the PIF complex, evidence suggests that it is not involved in specific binding and its function is not clear (140). PIF-5 is apparently not part of the PIF complex (141). A 35-kDa binding partner for AcMNPV P74 was detected in extracts of brush border membrane vesicles from host larvae (*S. exigua*), but not from a non-host (*H. armigera* larvae) (142). The identity of this host protein has not been determined.

Ac83 (VP95)(PIF8); essential for nucleocapsid assembly and is a PIF protein. Ac83 (PIF8) encodes a virion associated protein called VP91 that was originally characterized in OpMNPV (143). It is a core baculovirus gene and is also found in nudiviruses (137) and possibly in several insect genomes, e.g., *Anopheles gambiae* (E = 5e-04). It is an essential gene because a deletion mutant in BmNPV (Bm69) did not produce BV (144). It was found to associate with the PIF complex (141) and although the complete Pif8 gene is required for viability, when a predicted region containing three zinc fingers was deleted, the virus was not infectious when fed to larvae, but could replicate when injected into the haemocoel confirming that it is a PIF protein (145). Originally the zinc finger region was predicted to contain a chitin binding domain, but evidence indicated that AC83 does not bind to chitin (145). The zinc finger region appears to be involved in the assembly and localization of the PIF complex to ODV envelopes and the binding or entry of ODV into midgut cells (146). It was also determined that the ac83 gene contains a cis-acting nucleotide sequence essential for nucleocapsid assembly and is called the nucleocapsid assembly-essential element (NAE) (147). Therefore, the Ac83 gene appears to encode at least two functions; the zinc fingers are involved in the PIF function, whereas the NAE is a nucleotide sequence involved in nucleocapsid assembly. For more information, see Chapter 5.

Ac46 (ODV-E66), a chondroitinase and a PIF protein. Ac46 is a component of ODV envelopes (148) is the only known viral chondroitinase (149). It interacts with several members of the PIF complex (reviewed in (108)). Homologs of Ac46 are found in the genomes of all Group I NPVs, GVs, and most Group II NPVs, but not in hymenopteran or dipteran viruses. Two copies of the gene are present in some genomes (e.g., SeMNPV). In a

study of AcMNPV, an ODV-E66 deletion was observed to kill *Plutella xylostella* larvae as efficiently as wt when injected into larvae, however when infected per os, the LD50 was1000 fold greater for the mutant than wt virus. Therefore it was suggested that ODV-E66 is a per os infectivity factor (150) and could be designated PIF10. A truncated form of Ac46 lacking the N-terminal 66 amino acids was found to be secreted into the medium by infected cells and had chondroitinase activity (149). Chondroitinases have been shown to regulate cytokine and growth factors and can influence a variety of processes including development, inflammation, and organ morphogenesis. Chondroitin sulfate is present in the peritrophic matrix (PM) of *B. mori* and it can be digested by Ac46. This has led to the suggestion that Ac46 enhances the primary infection by digesting the chondroitin sulfate in the PM (151). This activity could be the reason Ac46 is a per os infectivity factor. Chondroitin glycosaminoglycans have been found to localize to the apical midgut microvilli of *Anopheles gambiae* (152) suggesting that if Lepidoptera are similar, this enzyme might also be involved in recognition or entry into midgut cells. Ac46 was previously shown to have hyaluronan lyase activity suggesting that it might be involved in penetrating the extracellular matrix which is composed of hyaluronan (153). However, its activity as a hyaluronan lyase was minimal in the chondroitinase study (149). When the N-terminal 23 amino acids of ODV-E66 are fused to a reporter gene, it is targeted to the nucleus (121). This is consistent it being a protein involved in ODV envelope which develops in association with the nucleus. The crystal structure has been determined (154).

Ac 145 and 150; more possible PIF proteins. These two genes encode small proteins (~9 and 11 kDa, respectively) that are related to one another (23% amino acid sequence identity) and localize to ODV envelopes (155). Close relatives of Ac 145 are found in all baculoviruses except the dipteran NPV. In contrast to Ac145, close relatives of Ac150 are only found in a few NPVs closely related to AcMNPV. Ac145 and 150 are predicted to encode a domain thought to bind to chitin (156). In one study (155), deletion of AcMPNV Ac145 led to a six-fold drop in infectivity in *T. ni,* but not *H. virescens* larvae. An effect of deletion of AcMNPV Ac150 was not detected. Deletion of both genes causes a major (39 fold) reduction of infectivity for *H. virescens*. Injection of BV of the double mutant intrahemocoelically produced the same level of infection as injected wt BV, suggesting that these genes play a role in oral infection. Ac145 and 150 were found to be associated with both BV and ODV (155). In another study (157), occluded virions deleted for Ac150 were found to be significantly less virulent when administered per os than the wt virus in *Heliothis virescens*, *Spodoptera exigua* and *Trichoplusia ni* larvae. Evidence suggested that the mutant had a reduction in its ability to establish primary infections in midgut cells.

Nucleocapsid Structure

Baculovirus nucleocapsids have a defined rod-shaped capsid this is capped by distinct apical and basal structures (Figure 12). Although many proteins appear to be capsid associated, a few appear to be located to end structures. Three of these VP80, VLF-1 and pp78/83 are shown in Figure 11. Although shown located to the basal region, this has not been definitively shown for VP80 or VLF-1.

Essential BV and ODV Nucleocapsid associated proteins encoded by all baculoviruses

As described above many proteins have been associated with BV and ODV nucleocapsids (reviewed in (1) however their role, if any, is unknown. Rather than an exhaustive list of these proteins, in this section I will focus on proteins that evidence suggests are essential components of both BV and ODV nucleocapsids (Table 3). The following are core baculovirus proteins that appear to be nucleocapsid associated.

VP1054, (Ac54). VP1054 was named for the size of its orf and encodes a protein required for nucleocapsid assembly. A ts mutant failed to produce nucleocapsids at the non-permissive temperature, indicating that it is an essential gene. It is found in both BV and ODV (158) and it interacts with 38K (Ac98) (22). Ac98 is likely to be a phosphatase. VP1054 also associates with BV/ODV-C42 and VP80, but not VP39 suggesting that it is critical for

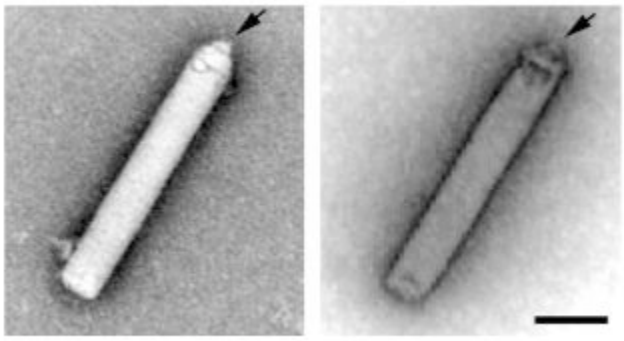

Figure 12. Morphology of AcMNPV capsids stained with uranyl acetate. The figure shows the morphology of the two ends, one with a conical shape (arrow). Scale bar, 50 nm .From Au and Pante (241). Reproduced with permission of Elsevier Limited via Copyright Clearance Center.

nucleocapsid assembly (159). When the vp1054 gene was deleted from a bacmid, the bacmid was not infectious and nucleocapsids appeared to be replaced with tube-like structures. It was suggested that vp1054 is related to a cellular protein called PURα that binds to purine-rich sequences and may be involved in DNA packaging and it was shown to bind to single stranded DNA or RNA sequences that contained runs of GGN. Therefore, it was suggested that it might interact with the orf1629/p/78/83 (ac9) sequence which encodes a series of prolines and therefore is rich in GGN codons (160).

Ac66. Orthologs of Ac66 are present in all baculovirus genomes. An AcMNPV bacmid deleted for Ac66 was severely compromised and BV titers derived from transfected cells were reduced by over 99% compared to wt. In addition, at low titers the mutant BV appeared to infect single cells and was unable to spread to other cells. Although the nucleocapsids appeared to be normal and had an electron-dense core suggesting that they contained DNA, they appeared to be trapped in the virogenic stroma, indicating that Ac66 was required for the efficient egress of virions from nuclei. The deletion did not affect the levels of DNA replication or polyhedrin transcription, but the production of occlusion bodies was eliminated (161). This suggests that Ac66 is required both for egress of virions from nuclei and also may be involved in the nucleation of polyhedra. Ac66 in BV but not ODV is ubiquitinated by the viral ubiquitin and appears to interact with Ac141 (predicted to be an E3 ubiquitin ligase). It was suggested that this might be a signal for BV egress in contrast to ODV that remain in nuclei (162, 163). For more information see Chapter 12.

VLF-1, (Ac77). The very late factor, VLF-1, is a member of the lambda integrase (Int) family of proteins and was originally identified because it influences the hyperexpression of very late genes (164) possibly by binding to their regulatory regions (165). VLF-1 appears to be a structural protein present in both BV and ODV (166) and is clearly required for the production of nucleocapsids. Bacmid deletion mutants produce tube-like structures that stained with vp39 antiserum suggesting that the lack of VLF1 prevents normal capsid assembly. Bacmids with point mutations in the conserved tyrosine form normal appearing capsids, but are also not infectious. VLF-1 localized to the end regions of nucleocapsids further suggesting that it is a structural protein (167). VLF-1 was also associated with ODV virions as determined by proteomic analysis in AcMNPV and CuniNPV, but not HearNPV (93-95). For additional information, see Chapter 5 and Chapter 12.

GP41, tegument protein, (Ac80). GP41 is modified with O-linked N-acetylglucosamine, and is located between the virion envelope and capsid in a structure called the tegument (168, 169). Homologs are present in all baculovirus genomes. Based on the characterization of a ts mutant, it is an essential gene required for the egress of nucleocapsids from the nucleus (170). Under the restrictive temperature, this mutant produces no BV and

infection is limited to single cells. Proteomic studies identified GP41 in both BV and ODV of HearSNPV (119). In addition, it was found that oligomerization of gp41 was required for BV production (171).

VP39, (Ac89). VP39 is thought to be the major capsid protein. It is one of the three most abundant proteins found by proteomic analysis of BV (62). Homologs of vp39 are present in all baculovirus and nudivirus genomes. It has been observed that VP39 interacts with a conserved domain of kinesin 1 and it has been suggested that this interaction is involved in the transport of nucleocapsids destined to become BV to the cell membrane after their assembly in nuclei (172). Based on mutagenic analysis it appears to be required for proper DNA packaging and nucleocapsid assembly (173).

Ac92, (P33), a sulfhydryl oxidase. Ac92 is an essential core gene that encodes a sulfhydryl oxidase, and is likely involved in the production of disulfide bonds in viral proteins (174) (175). It forms a stable complex with the tumor suppressor gene p53 and appears to enhance its apoptotic function (176) (177). P33 was found to be associated with ODV virions by proteomic analysis in several baculoviruses (93-95) (119) and in both AcMNPV BV and ODV in western blot analyses (175) (178). It is unclear whether Ac92 is required for virion structure or is associated with virions because it facilitates disulfide bond formation during virion assembly. Further information on Ac92 is available in Chapters 5, 7, and 12.

Ac98 (38K). Ac98 is encoded by all baculoviruses and is associated with both BV and ODV nucleocapsids. By yeast two-hybrid assays it interacted with VP1054, VP39, VP80, and itself (22). When deleted, tube-like structures devoid of DNA but that stain with vp39 antibody are produced (179). It is related to a set of enzymes including CTD phosphatases and evidence indicates that it dephosphorylates the p6.9 DNA binding protein which allows it to be packaged with the viral DNA (180).

P6.9, (Ac100). P6.9 is a DNA binding protein and one of the three most abundant proteins found in proteomic analysis of BV (62). It is a small (55 aa) arginine/serine/threonine rich protein (181). Homologs appear to be encoded by all baculovirus genomes, but may be difficult to identify in computer analyses because of their small size and repetitive amino acid content. P6.9 was originally identified as a DNA binding protein in a GV (182) and the homolog was subsequently identified from AcMNPV (181). The high concentration of arg and ser/thr residues is similar to protamines that are also small molecules of 44-65 amino acids (183, 184) present in sperm nuclei of many higher eukaryotes and are involved in the production of highly condensed DNA. Arginine has a high affinity for the phosphate backbone of DNA, and the polyarginine tracts in protamines neutralize the phosphodiester backbone, whereas the ser and thr residues interact with other protamine molecules, thereby yielding a neutral, highly compact DNA-protein complex that is biochemically inert. P6.9 localizes to the nuclear matrix during infection (185). Once viral DNA has been delivered to the nucleus, p6.9 is phosphorylated (see below) resulting in both DNA and p6.9 being negatively charged. It is thought that this causes the removal of p6.9 from the viral DNA thereby allowing access to transcription factors (186). AcMNPV has two candidate kinases that could be involved in this process. Protein kinase 1, PK1 (Ac10), is encoded by lepidopteran baculoviruses (GVs and NPVs), whereas PK2 (Ac123) is encoded by only a few baculoviruses closely related to AcMNPV. Neither protein was found to be associated with ODV by mass spectrometry (93-95). However, a kinase was found to be associated with BV and ODV, although its source was not determined (187). P6.9 was found to co-localize with viral DNA and to fractionate with RNA polymerase II at 24 hpi (141). After synthesis, p6.9 is hyperphosphorylated, at least in part by pk-1 and this is associated with high levels of expression of very late genes (188) (189). This occurs immediately upon synthesis and p6.9 is dephosphorylated by the 38k protein (180) before being complexed with DNA (189). Using an AcMNPV bacmid deleted for p6.9, nucleocapsids were not produced although tube-like structures similar to those associated with the deletion of VLF-1 and Ac98 (see above) were observed. The mutant appeared to synthesize normal amounts of DNA, but did not produce infectious virus (190). BmNPV micro RNA-3 (mir-3) appears to regulate, at least in part, the expression of BmNPV p6.9 (191).

BV/ODV-C42, (Ac101). Ac101 is a core gene and encodes a capsid-associated protein found in both BV and ODV (93). In addition, it was reported to interact in a yeast two-hybrid assay and by native gel electrophoresis (192) with the actin nucleation factor pp78/83 (Ac9) (described below) and is required for its transport into nuclei (193). It stabilizes P78/83 by inhibiting its degradation. C42 also interacts with Ac102 and this suppresses ubiquitination of C42 further regulating the stability of P78/83 (194-196). Deletion of Ac101 affected nucleocapsid formation but, although lethal, did not appear to affect DNA synthesis (197).

Ac141 (exon0). Ac141 is conserved in all lepidopteran baculoviruses and is associated with both BV and ODV nucleocapsids (198) (62) and interacts with BV/ODV-C42 (Ac101) and FP25 (Ac61) (199). It appears to be required for the efficient transport of nucleocapsids from nuclei through the cytoplasm (198, 200). It appears to both co-localize with and co-purify with β-tubulin. In addition, inhibitors of microtubules reduced BV production by over 85%. Therefore it has been suggested that the interaction of Ac141 with microtubules might be important in the egress of BV (201). It has also been shown to interact with a conserved domain of kinesin 1, a motor protein involved in transporting cargo along microtubules to the periphery of the cell further supporting a role for microtubules in transport of virions to the cell surface (172, 202). Hhpred (135) predicts with over 90% probability that the C-terminal ~90 amino acids has structural similarity to E3 protein ubiquitin ligase along with several other proteins. Deletion of ac141 and vubi results in single cell infection and BV were not produced. The ubiquitination of Ac141 was essential for optimal production of BV. BV but not ODV nucleocapsids were ubiquitinated by vUbi. The target was Ac66 and it was shown to co-localize with vUbi and Ac141 at the nuclear periphery. It was suggested that the ubiquitination of capsid proteins may be a signal for BV egress from nuclei (162).

Ac142, (p49). Ac142 is encoded by a core gene and is associated with both BV and ODV virions. Deletion of Ac142 appeared to affect nucleocapsid formation but, although lethal, did not appear to affect DNA synthesis (197). Similar results were reported for the homolog in BmNPV (Bm118) (203). Another study describing a different bacmid construct that deleted less of the Ac142 gene showed similar results except that nucleocapsids, although fully formed, were not enveloped in nuclei and were not occluded (204). It is unclear whether the difference in the two studies was due to the removal of a 3' processing signal for the upstream Ac141 gene in the former investigation, or to the presence of a significant portion of the Ac142 orf in the latter study. Proteomic analysis also suggests that Ac142 is ODV-associated in three different viruses (93-95). In addition, it was reported that Ac142 interacts with components of the ESCRT-III complex and may be involved in the release of nucleocapsids at the nuclear membrane (205).

Ac144. Ac144 is a core gene and was originally named ODV-EC27 and it was suggested that it is a cyclin (206). However, another investigation using an HA-tagged Ac144 recombinant virus found that Ac144 was expressed as an ~ 33.5 kDa protein which conforms to the predicted MW (197). In addition, it was found to be BV associated (62, 197). Proteomic analysis suggests that Ac144 is ODV-associated in three different viruses (93-95). A variety of investigations have been conducted on Ac144. Initially, it was confirmed that its transcript initiates at a late promoter element (133). It was reported to interact in a yeast two-hybrid assay with Ac101 described above (also named C42) and with both Ac101 and p78/83 (Ac9) in native gel electrophoresis assays (192). It was also found that deletion of Ac144 resulted in amorphous electron dense structures that stained with vp39-capsid antibodies, but no nucleocapsids were evident. Although lethal, deletion of Ac144 did not appear to affect DNA synthesis (197).

Table 3 Selected Proteins Associated with Baculovirus Nucleocapsids

Name and AcMNPV orf #	Distribution in the Baculoviridae	Effect of Deletion or mutation
Ac100, P6.9 DNA binding	All	Not viable (190)
Ac89, VP39 capsid	All	Not viable (144)
Ac80 GP41 tegument	All	Not viable (170)

Table 3 continued from previous page.

Name and AcMNPV orf #	Distribution in the Baculoviridae	Effect of Deletion or mutation
Ac98 38K	All	Not viable (179)
Ac142	All	Not viable (197, 204)
Ac144	All	Not viable (197)
Ac66	All	Severely compromised (161)
Ac92 (P33)	All	Not viable (175, 178)
Ac54 (VP1054)	All	Not viable (158)
Ac77 VLF-1	All	Not viable (247)
Ac104, VP80	Lep. I and II NPV	Not viable (225)
Ac9, PP78/83	Lep. I and II NPV	Not viable(211)
Ac129, P24	Lep. I, II, GV	Viable (235)

Other structural proteins

The following structural proteins are found in some, but not all baculovirus genomes. This could indicate either that they are not present or have evolved to such an extent that their relatedness can no longer be identified in the genomes in which they are not found.

PP78/83, (Ac9). PP78/83 is a phosphorylated protein that is located at one end of nucleocapsids (207, 208). It is a Wiskott-Aldrich syndrome protein (WASP)-like protein. Such proteins are involved in nuclear actin assembly, and it has been demonstrated that pp78/83 serves this function during AcMNPV infection (209) (210) (see Chapter 3). Homologs of pp78/83 are found in all lepidopteran NPV genomes. It is an essential gene, and because it is located adjacent to the polyhedrin gene, it was originally manipulated via complementation to elevate the frequency of obtaining recombinant baculoviruses at the polyhedrin locus (211).

GP37 (Ac64). Orthologs of gp37 have been found in the genomes of all alpha- and betabaculoviruses (212). In AcMNPV it is expressed as a late gene (213) and is nonessential for replication in cell culture or *T. ni* larvae (214). The BmNPV homolog (Bm52) was also found to be non essential in BmN cells (144). The gp37 homolog in SpltNPV has been reported to contain chitin binding domains and is capable of binding to chitin (215). The gp37 of CpGV also binds chitin and was able to enhance per os infections (216). Insect proteins, such as the coagulation protein hemolectin, also have chitin-binding domains (217). Whether gp37 somehow inhibits or redirects such pathways remains to be determined. GP37 was reported to be polyhedron associated in AcMNPV and to be N-glycosylated (218). It was also found to be BV associated (62). In OpMNPV infected *L. dispar* cells, GP37 was found to be an N-glycosylated protein located in cytoplasmic occlusions late in infection (219). In entomopox viruses the gp37 ortholog forms crystallized spindle-like structures. These structures have been suggested to digest the peritrophic matrix (220). Structural analysis of these spindles indicated that they contained a globular domain that is related to lytic polysaccharide monooxygenases of chitinovorous bacteria. It is thought that upon ingestion by the host, the spindles are dissolved and the monooxygenase domain is exposed and can then digest the chitin-rich peritrophic matrix (221) thereby facilitating the passage of virions through the peritrophic matrix and allowing them access to midgut cells. It has been reported that GP37 can degrade the peritrophic matrix and also facilitates the binding of ODV to midgut cells (222). The *Spodoptera litura* NPV genome was found to contain a gene that is a fusion of ubiquitin and gp37 (for discussion in Chapter 12, see *Ac35, ubiquitin*) and the protein was associated with the envelopes of BV and ODV (223). For more information see Chapter 12.

Vp80 (Ac104). Orthologs of Ac104 are found in all Group I and II lepidopteran NPV genomes, but not in those of GVs or hymenopteran or dipteran NPVs. It is capsid associated in both OpMNPV (224) and AcMNPV (93, 225, 226) and interacts with 38K (Ac98) (22). Deletion of Vp80 showed that it is an essential gene and resulted in nucleocapsids that were unable to move from the virogenic stroma (227). It appears to localize in nuclei near actin scaffolds that may connect the virogenic stroma to the nuclear envelope. In addition, it co-immunoprecipitates with actin. It also appears to localize to one end of nucleocapsids and contains sequences similar to paramyosin motifs that may be involved in the transport of virions to the periphery of nuclei (227). It forms dimers, contains a C-terminal region that was predicted to contain a basic helix-loop-helix domain, and binds to DNA (228).

Ac109 (ME53). Homologs of *ac139* are present in the genomes of all the lepidopteran NPVs and GVs, but have not been reported in hymenopteran or dipteran baculovirus genomes. It is BV and ODV associated (229). One study indicated that AcMNPV deleted for this gene is not viable and fails to replicate its DNA and does not produce nucleocapsids. However, cells transfected with DNA from the mutant showed early stages of cpe, including nuclear enlargement and the formation of granular material in the nucleus (230). This suggests that the mutant is blocked in an early gene function. This is consistent with its original characterization as a major early gene (231). However, another study showed that deletion of Ac139 did not alter DNA replication, but results in a 1000-fold reduction in BV titer. In addition, it was found that it appears to be required both early and late in infection (229). ME53 fused to GFP localized mostly to the cytoplasm early and to nuclei late in infection. However, foci of ME53 were also noted at the cell periphery late in infection and co-localized with gp64 and VP39-capsid and was capsid associated in BV. It was suggested that it may provide a connection between the nucleocapsid and the viral envelope (232).

P24-capsid, (Ac129). Ac129 (P24) was found to be associated with both BV and ODV of AcMNPV and OpMNPV by Western blot and electron microscopic analyses (233). It is likely to be nonessential as interruption of this gene with a transposable element in a strain of AcMNPV has been reported (234, 235) and it can also be deleted from BmNPV with no detectable effects (144). Homologs of Ac129 are present in all Group I /II and GV genomes. The Ac129 homolog was not reported to be associated with ODV of HearNPV (95). It was also reported to be BV associated (62).

Additional virion associated proteins. Many more proteins have been found to be associated with AcMNPV virions. Information on these and summarized in (1) and Chapter 12.

Host proteins and protein modification

Host proteins. Analysis of BV from AcMNPV using mass spectrometry identified 48 virally encoded proteins and 11 host proteins (62). In addition, a variety of host proteins were also associated with preparations of HearSNPV ODV and BV, with 21 and 101 host proteins identified, respectively. Many of these proteins could be simply trapped as the virions are assembled or are present as normal components of the membranes from which their viral envelopes were derived. However, some components could be essential for virion function. One such protein, cyclophilin A was associated with both HearSNPV BV and ODV. Cyclophilin A catalyzes the isomerization of peptide bonds from *trans* to *cis* at proline residues, a process that can be important in protein folding. In HIV-1, but not other primate immunodeficiency viruses, cyclophilin A associates with a proline rich region of the GAG polyprotein and is required for the production of infectious virions (236). The 11 host proteins that were identified in AcMNPV BV (62) included cytoplasmic actin and actin depolymerizing factor, perhaps reflecting the role actin plays in BV transport (210). In addition, it was observed that baculoviruses grown in different insect cells show differing efficiencies in mammalian cell entry suggesting that some host factor may facilitate cell entry (104).

Post translational modification of structural proteins. Proteomic analysis using several techniques was applied to both BV and ODV of the HearSNPV (119). They reported N-glycosylated proteins in only BV, but not in

ODV. These included F, FGF, V-CATH, ChiA, P26 (=Ac136), and ODV-E18 (=Ac143). One of these, ODV-E18, is present in both BV and ODV. They also observed differing phosphorylation profiles between the two phenotypes with 38 sites in ODV associated proteins, and 4 in BV. Serine was the predominant phosphorylation site (72.1%) followed by Thr (22.3%) and Tyr (4.6%). The phosphorylated proteins (phosphoproteome) of B. mori cells infected with BmNPV has been examined. Many phosphorylated host and viral proteins were identified, some of which were hyperphosphorylated including p6.9 (237).

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