Analysis of a naturally-occurring deletion mutant of *Spodoptera frugiperda* multiple nucleopolyhedrovirus reveals *sf58* as a new *per os* infectivity factor of lepidopteran-infecting baculoviruses

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ABSTRACT

The Nicaraguan population of *Spodoptera frugiperda* multiple nucleopolyhedrovirus, SfMNPV-NIC, is structured as a mixture of nine genotypes (A–I). Occlusion bodies (OBs) of SfMNPV-C, -D and -G pure genotypes are incapable of oral transmission; a phenotype which in SfMNPV-C and -D is due to the absence of *pif1* and *pif2* genes. The complete sequence of the SfMNPV-G genome was determined to identify possible factors involved in this phenotype. Deletions of 4860 bp (22,366–27,225) and 60 bp (119,759–119,818) were observed in SfMNPV-G genome compared with that of the predominant complete genotype SfMNPV-B (132,954 bp). However no genes homologous to previously described *per os* infectivity factors were located within the deleted sequences. Significant differences were detected in the nucleotide sequence in *sf58* gene (unknown function) that produced changes in the amino acid sequence and the predicted secondary structure of the corresponding protein. This gene is conserved only in lepidopteran baculoviruses (alpha- and betabaculoviruses). To determine the role of *sf58* in peroral infectivity a deletion mutant was constructed using bacmid technology. OBs of the deletion mutant (Sf58null) were not orally infectious for *S. frugiperda* larvae, whereas Sf58null rescue virus OBs recovered oral infectivity. Sf58null DNA and occlusion derived virions (ODVs) were as infective as SfMNPV bacmid DNA and ODVs in intrahemocelically infected larvae or cell culture, indicating that defects in ODV or OB morphogenesis were not involved in the loss of peroral infectivity. Addition of optical brightener or the presence of the orally infectious SfMNPV-B OBs in mixtures with SfMNPV-G OBs did not recover Sf58null OB infectivity. According to these results *sf58* is a new *per os* infectivity factor present only in lepidopteran baculoviruses.

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1. Introduction

Nucleopolyhedroviruses (NPVs) are double-stranded, circular DNA viruses belonging to the family Baculoviridae that infect insects, particularly the larval stages of Lepidoptera. To complete the infection cycle, NPVs require two types of virions that are genetically identical, but morphologically distinct (Volkman, 2007). Occlusion-derived virions (ODVs) initiate the primary infection in epithelial cells of the host midgut, following ingestion of contaminated foliage. Infected cells give rise to budded virions (BVs) that transmit the infection from cell to cell within the host. Later in the infection cycle, virions containing single (SNPV) or multiple (MNPV) nucleocapsids per envelope are occluded in proteinaceous occlusion bodies (OBs) that permit virus survival in the environment. Multiple genotypes or multiple copies of the same genotype can therefore be occluded within each OB, and even within the same virion in the case of MNPVs (Bull et al., 2001; Clavijo et al., 2010; Hodgson et al., 2004). After host death, OBs are released into the environment permitting transmission to susceptible hosts (Williams and Faulkner, 1997). Coinfection of host cells by multiple genotypes results in a high prevalence of mixed genotype disease (Bull et al., 2003). This genotypic heterogeneity appears to be a common characteristic of alphabaculovirus populations (Hodgson et al., 2004; Muñoz et al., 1999; Simón et al., 2004).

Nine distinct genotypes (SfMNPV-A to -I) have been isolated from the Nicaraguan strain of *Spodoptera frugiperda* multiple nucleopolyhedrovirus, SfMNPV-NIC, that can be clearly identified by their restriction endonuclease profiles (Simón et al., 2004). The SfMNPV-B genotype with the largest genome, 132,954 bp (accession number HM595733; Simón et al., 2011), is the predominant

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genotype in the wild-type population. All the other genotypes present one or more deletions compared to SfMNPV-B (Simón et al., 2005). OBs produced in pure SfMNPV-C, -D and -G infections cannot be transmitted orally (Simón et al., 2004) and survive by complementation with other genotypes in mixed infections (López-Ferber et al., 2003; Simón et al., 2005). For SfMNPV-C and -D, the deletion includes two genes *pif1* and *pif2* that are essential for cell binding and penetration during the primary infection process (Kikhno et al., 2002; Pijlman et al., 2003). Progeny OBs obtained after cotransfection of SfMNPV-C and -D DNAs with a plasmid that encompassed both *pif* genes recovered oral infectivity. However OBs of SfMNPV-G did not recover infectivity using a similar approach, indicating that these genes were not responsible for the loss of oral infectivity in this genotype (Simón et al., 2004). In addition SfMNPV-C and -D genotypes encompassed the deletion present in SfMNPV-G, suggesting that another not previously described gene is likely responsible for the non-infectious phenotype.

The *per os* infectivity factors (PIFs) comprise the proteins required only during the primary infection of host midgut cells. ODVs, which bind to and fuse with the microvilli of midgut cells (Okhawa et al., 2005; Peng et al., 2010), are structurally complex entities and the number of ODV-associated PIF factors that have been identified continues to increase. However, not all PIFs are involved in binding and fusion (Okhawa et al., 2005). To date, they include P74 (PIF0) (Faulkner et al., 1997), PIF1 (Kikhno et al., 2002), PIF2 (Fang et al., 2006; Pijlman et al., 2003), PIF3 (Okhawa et al., 2005), PIF4 (Fang et al., 2009) and PIF5 (ODV-E56) (Harrison et al., 2010; Xiang et al., 2011).

Our aim in this study was to determine the factor(s) involved in the absence of oral infectivity of SfMNPV-G genotype OBs. For this, the complete sequence of the SfMNPV-G genome was determined and compared with that of SfMNPV-B, in order to identify differences at the genomic level that could account for this phenotype. A *sf58* deletion mutant was constructed by bacmid recombination and experiments were performed to determine its role in the oral infectivity of SfMNPV OBs.

2. Materials and methods

2.1. Insects and virus

Larvae from a laboratory colony of *S. frugiperda* were maintained on wheatgerm-based semi-synthetic diet (Greene et al., 1976) at 26 ± 2 °C, 16 h:8 h L:D, 70–80% RH. The defective genotype SfMNPV-G, isolated by plaque assay purification from the Nicaraguan SfMNPV wild-type population, was amplified by injection of fourth instar *S. frugiperda* larvae with ODVs released from OBs by alkaline lysis (Simón et al., 2004). OBs of the complete genotype SfMNPV-B were amplified by oral infection and used as reference. Infected larvae were homogenized in sterile distilled water and OBs were filtered through muslin, washed with 0.1% sodium dodecyl sulfate (SDS) and 0.1 M NaCl, pelleted by centrifugation, and resuspended in distilled water. OB suspensions were quantified in triplicate using an improved Neubauer counting chamber and stored at 4 °C until used. SfMNPV-G DNA was purified using CsCl (King and Possee, 1992) and used for sequencing (LifeSequencing S.L. and Sistemas Genómicos S.L., Paterna, Valencia, Spain).

2.2. DNA sequencing and sequence analysis

A genomic library of SfMNPV-G DNA was constructed into a sequencing vector. A total of 2304 sequencing reactions were performed that corresponded to 1152 clones. The depth of sequence coverage across the genome was 4–8-fold. Sequencing reactions were set up using the ABI Prism Big Dye Terminator Cycle Sequencing Reaction kit on a 9600 PE thermocycler. The reaction products were loaded in an automated DNA sequencer ABI PRISM. The genomic sequence of SfMNPV-G (GenBank accession number JF899325) was compared with that of SfMNPV-B published previously (accession number HM595733; Simón et al., 2011) using the Clone Manager program (Scientific & Educational Software, 1994–2007). Sequence alignments and gene-parity plots were performed using NCBI BLAST alignment tools (Altschul et al., 1990; Pearson, 1990) to examine genome organization and order of homologous ORFs. Protein sequence analyzes were performed by using tools available in Geneious Pro software, version 5.3.6 (Drummond et al., 2010). Sequence conservation of predicted protein sequences was analyzed using the multiple sequence alignment MAFFT tool (v6.814b). Conserved domains and putative signal peptide sequences were predicted using InterProScan and signal cleavage sites using the Emboss (Rice et al., 2000) sigcleave module. The transmembrane prediction tool (TMHMM) was used to identify transmembrane regions of the protein sequences. Secondary structure of proteins was predicted using the Emboss garnier module, whereas the Emboss antigenic tool was used to determine putative antigenic regions. Selection pressure analysis was performed using two maximum likelihood methods; Paml4 (Yang, 2007) and the HyPhypackage (Kosakovsky Pond et al., 2005), to calculate non-synonymous/synonymous (d_N/d_S) nucleotide substitution ratios based on codon alignment and phylogenetic tree. To determine whether the amino acids were conserved (buried or exposed) in the alignment the Consurf server (Ashkenazy et al., 2010) was employed. Representative DNA and protein sequences used for comparison were obtained from the GenBank database. Baculovirus sequences used in the comparative analysis were GenBank (accession number); SfMNPV-G (JF899325), SfMNPV-B (HM595733), *Spodoptera litura* NPV (AF325155), *Spodoptera exigua* MNPV (AF169823), *Agrotis segetum* NPV (DQ123841), *Agrotis ipsilon* NPV (EU839994), *Helicoverpa armigera* NPV NNg1 (AP010907), *Mamestra configurata* NPV A (U59461), *M. configurata* NPV B (AY126275) and *Chrysodeixis chalcites* NPV (AY864330).

2.3. Temporal expression of sf58 genes in SfMNPV-B and -G genotypes

To determine the temporal expression of the *sf58* gene RT-PCR was performed using the Access RT-PCR System kit (Promega). Total RNA was extracted from larvae that had been orally infected using SfMNPV-B OBs or larvae intrahemocelically-infected with SfMNPV-G ODVs at 0, 2, 4, 6, 12, 24, 48, 72, 96, 120 and 144 hpi with Trizol reagent (Gibco) as described in the manufacturers' protocol. RNA samples were treated with DNase prior to RT-PCR to ensure absence of contaminant DNA, and a PCR was performed to confirm this. First strand cDNA synthesis was performed using AMV reverse transcriptase and the internal oligonucleotide Sf58.2 (5′-TTACGTAGGTGCTGGAGGAG-3′; nt 54,983 → 55,002 referring to SfMNPV-B genotype). The cDNA mixtures were then amplified by PCR in the presence of Sf58.1 (5′-GTCCTCGGTGCTGAATCAGG-3′; nt 55,270 ← 55,289) and Sf58.2 primers and amplification products were visualized in 1% agarose gels. For comparison, RT-PCR was performed on the SfMNPV-B infected larvae with two primers specific for the very late transcribed *polyhedrin* gene (*Sfpolh*), Sfpolh.1 (5′-CCCGACACCATGAAGCTGGT-3′; nt 241 → 260) and Sfpolh.2 (5′-TTAGTACGCGGGTCCGTTGTA-3′; nt 721 ← 741).

2.4. Generation of sf58 deletion virus

A PCR and bacmid-based recombination system was used to delete the *sf58* gene from a complete SfMNPV bacmid (Sfbac) that included the complete genome of the SfMNPV-B genotype as previously described (Simón et al., 2008a). Briefly, bacteria containing

Sfbac were made electrocompetent and transformed with the Red/ET plasmid pSC101-BAD-gbaA (Gene Bridges GmbH). These cells were also made electrocompetent and induced with arabinose (0.1–0.2% w/v) to express the recombination proteins (gbaA). The Tn5-neo template, a PCR-template for generating a Tn5-neomycin (kanamycin) cassette, was amplified sequentially using two primer sets; Sf58del.1 (5′-CATGGTAACCAATTTAAATTTACGTTGGACAGC AAGCGAACCGGAATTGC-3′; nt 54,958 → 54,982) and Sf58del.2 (5′-CACCTAGCCAGCACAGGACG ACGACTCAGAA GAACTCGTCAA GAAGGCG-3′; nt 55,328 ← 55,352), and Sf58del.3 (5′-TGATCGATTC ACTCTTTTTGACGTTCATGGTAACCAATTTAAATTTA CGT-3′; nt 54,933 → 54,982) and Sf58del.4 (5′-CAGATACAATTACTTTTT TTAAGAGCACCTAGCCAGCACAGGACGACGAC-3′; nt 55,328 ← 55,377) to add 50 nucleotide terminal sequences corresponding to either the 3′ or 5′ untranslated regions of *sf58*. The resulting product was used to transform the electrocompetent cells containing Sfbac, and pSC101-BAD-gbaA that produced gbaA. Recombinants were selected as resistant colonies on medium containing chloramphenicol and kanamycin. To confirm deletion of the *sf58* gene, restriction endonuclease analysis of bacmid DNA and PCR amplifications were performed. The corresponding Sf58null rescue virus was also constructed by replacing the *sf58* gene in the Sf58null bacmid. For that, a PCR product containing the *sf58* coding region was used for the reintroduction of the *sf58* gene into the Sf58null bacmid. The *sf58* coding region was PCR amplified using primers outside the coding region, Sf58reg.1 (5′-GTCATGATCATG CATGTTCG-3′; nt 53,983 → 54,002) and Sf58reg.2 (5′-TACGCA ATCTGATCGTGTAC-3′; nt 56,307 ← 56,326), and the SfMNPV-B DNA as template. Fourth instars of *S. frugiperda* were co-infected with 5 µl from a DNA suspension containing 50 µl of Sf58null bacmid (100 ng/µl), 50 µl of the PCR product that covered the *sf58* region (500 ng/µl) and 50 µl of lipofectin reagent. Inoculated larvae were individually transferred to 25-compartment dishes and provided with diet. Larvae were reared at 25 °C and virus mortality was recorded every day. The OBs were purified from virus-killed insects and viral DNA extraction was performed as described below. A PCR was performed using viral DNA from OBs and primers outside the coding region (Sf58reg.1 and Sf58reg.2) to determine whether or not recombination had occurred. Viral DNAs from the complementation assay were transfected into DH5α electrocompetent cells. Colonies were grown on medium containing chloramphenicol and the DNA from thirty colonies was purified by alkaline lysis. Restriction endonuclease analysis and a PCR were performed to confirm the transposition of the *sf58* gene into the Sf58null and corresponding rescue bacmid. Colonies that carried the restored sf58 gene were selected for PCR amplification and the sequences upstream and downstream from the sf58 gene were determined using the Sf58reg.1 and Sf58reg.2 primers. In this way the authenticity of the sf58null rescue virus was confirmed to avoid selecting Sf58null rescue viruses with modifications that could influence the transcription of adjacent ORFs.

To produce OBs from the Sfbac, Sf58null and Sf58null rescue bacmids, *S. frugiperda* fourth instars were injected with a DNA suspension including bacmid DNAs and lipofectin transfection reagent (Invitrogen) in a 2:1 proportion. A 200 µl volume of each bacmid DNA containing 100 ng/µl was mixed with 100 µl of lipofectin. After 10 min, 5 µl of this suspension was injected into each larva (333 ng/larva) and inoculated larvae were transferred to diet. Larvae were reared at 25 °C and virus mortality was recorded every day. OBs from virus-killed larvae were extracted and filtered through cheesecloth. These were washed twice with 0.1% SDS and once with 0.1 M NaCl and finally resuspended in bidestilled water. The OB suspensions were quantified using a Neubauer hemocytometer and stored at 4 °C.

2.5. Sf58null OB infectivity

The oral potency of Sfbac and Sf58null OBs were determined by *per os* bioassay in *S. frugiperda* second instars using the droplet feeding technique (Hughes and Wood, 1987). For Sfbac and Sf58null viruses, fivefold dilutions between 1.2×10^6 and 1.9×10^3 OBs/ml were used. Each dose range was previously determined to kill between 95% and 5% of Sfbac-treated larvae (Simón et al., 2008a). Bioassays with 25 larvae per OB concentration and 25 larvae as controls were performed four times. Larvae were reared at 25 °C, and virus mortality was recorded every 12 h until the insects had either died or pupated. Virus induced mortality was subjected to probit analysis using the PoloPlus program (LeOra Software, 1987). The Sf58null rescue virus OBs were fed to second instar larvae at a single concentration of 1.2×10^6 OBs/ml to confirm that oral infectivity had been restored.

Sf58null ODV infectivity was also determined in Sf9 cells and *S. frugiperda* larvae. The mean virion titers of Sfbac and Sf58null OBs were estimated by end point dilution of ODVs as previously described (Lynn, 1992; Simón et al., 2008a). For this a 500 µl of 10^9 OBs/ml were mixed with an equal volume of 0.1 M Na_2CO_3. A 30 µl volume of 5.4 M HCl was added to decrease the pH. This suspension was filtered through a 0.45 µm filter and diluted in TC100 medium (1:10, 1:50. 1:250, 1:1250 and 1:6250). A 10 µl volume of each dilution was used to infect 10^4 Sf9 cells in 96 well plates. Twenty-four independent infections were performed for each dilution. Plates were prepared in triplicate, sealed with masking tape, incubated at 28 °C and examined for virus infection after 5 days. The results were analyzed by the Spearman–Kärber method (Lynn, 1992) to provide 50% tissue culture infectious dose ($TCID_{50}$) estimates. $TCID_{50}$ values were subsequently converted to infectious units per 5×10^8 OBs for presentation in the figures. To determine ODV infectivity *in vivo*, a 500 µl volume containing 5×10^8 OBs was mixed with an equal volume of 0.1 M Na_2CO_3 and 5 µl of this mixture was individually injected intrahemocoelically into *S. frugiperda* fourth instars. Bioassays with 25 larvae per virus were performed three times. Larvae were reared at 25 °C, and virus mortality was recorded every 12 h until the insects had either died or pupated. The results were subjected to *t*-test using SPSS.

2.6. Effect of an optical brightener on Sf58null OB infectivity

Enhancins are components of the ODVs of some baculoviruses and are known to disrupt the peritrophic membrane, thereby facilitating ODV access to the midgut cells (Slavicek and Popham, 2005). Tinopal UNPA-GX (F-3543, Sigma) is a stilbene-derived optical brightener known to degrade the peritrophic membrane, favoring the passage of ODVs (Wang and Granados, 2000). We reasoned that if SF58 had a mode of action that targeted the porosity of the host peritrophic membrane then Tinopal UNPA-GX should favor the passage of Sf58null ODVs and restore OB infectivity. To determine whether Tinopal UNPA-GX affected the potency of Sf58null OBs two sets of experiments were performed, with groups of 25 s instar and 25 four instar larvae. It is known that the degree of OB potentiation by optical brighteners is instar-dependent, being higher as the instar increases (Martínez et al., 2003). Second instar *S. frugiperpa* were inoculated orally by the droplet feeding technique (Hughes and Wood, 1987) with 1.2×10^6 and 5×10^4 OBs/ml of Sfbac or Sf58null OBs in the presence or absence of 1% (wt./vol.) Tinopal UNPA-GX. For Sfbac OBs, these concentrations were predicted to result in mortalities of 90% and 50%, respectively, in the absence of Tinopal UNPA-GX; mortality levels that were sufficiently high and low to quantify the potential enhancement effect of the optical brightener. Fourth instars were inoculated with 4×10^7 and 4×10^4 OBs/ml in the presence or absence of 1% Tinopal UNPA-GX, concentrations that were expected to result in

mortalities of 90% and 20%, respectively. Groups of 25 s and four in-star larvae were used as mock-infected controls with and without 1% Tinopal UNPA-GX. The experiment was performed three times for each instar.

2.7. Complementation bioassays

Complementation assays were performed in order to determine whether SfMNPV-G genotype OBs recovered oral infectivity in the presence of genotype B OBs. Purified OB suspensions were quantified by counting in triplicate. OBs of each genotype were then mixed in a 1:1 proportion and used directly in insect bioassays. The potency of the mixture was determined by *per os* bioassay in *S. frugiperda* second and fourth instars following the droplet feeding technique (Hughes and Wood, 1987) and compared with OBs of pure genotypes SfMNPV-B and -G. The effect of the presence of 1% Tinopal UNPA-GX in mixed OB inocula was also evaluated. To determine OB potency second instars were inoculated with five-fold dilutions between 1.2×10^6 and 1.9×10^3 OBs/ml, whereas to determine OB potency in the presence of Tinopal UNPA-GX between 5.0×10^4 and 3.1×10^3 OBs/ml were mixed with 1% Tinopal UNPA-GX and used in droplet feeding bioassays. For fourth instar larvae OB concentrations ranged between 4.0×10^7 and 9.8×10^3 OBs/ml in the absence of Tinopal UNPA-GX and between 4.0×10^4 and 4.9×10^2 OBs/ml in mixtures with 1% Tinopal UNPA-GX. Each range of concentrations was previously determined to kill between 95% and 5% of the insects inoculated with wild-type SfMNPV-NIC OBs (Martínez et al., 2003). Bioassays with 25 larvae per OB concentration and 25 larvae as mock-infected controls with and without Tinopal UNPA-GX were performed four times. Larvae were reared at 25 °C and virus mortality was recorded every 12 h until the insects had either died or pupated. Virus induced mortality was subjected to probit analysis using the PoloPlus program (LeOra Software, 1987).

3. Results

3.1. SfMNPV-G genome sequence analysis

The genome of the defective genotype SfMNPV-G is 128,034 bp, which is 4920 bp smaller than the genome of the reference genotype SfMNPV-B (Simón et al., 2011). This size difference between the two genotypes was accounted for almost entirely by a deletion of 4860 bp located between nucleotides (nt) 22,366 and 27,225 (referring to SfMNPV-B). This deletion affected the 3′-end of the *sf23* ORF (unknown function; nt 21,915 → 22,556), the complete ORFs *sf24* (*gp37*; nt 22,660 → 23,460), *sf25* (*ptp-2*; nt 23,457 ← 23,960), *sf26* (*egt*; nt 24,056 → 25,633), *sf27* (unknown function; nt 25,820 → 26,344), *sf28* (unknown function; nt 26,351 → 26,998) and the 3′-end of the *sf29* gene (unknown function; nt 27,018 ← 29,654), reducing its length by 207 nucleotides. *Sf29* has been shown to determine the number of virions occluded in OBs. This deletion resulted in a reduction in the number of ODVs within OBs which was reflected in reduced pathogenicity, but did not eliminate oral infectivity, as seen in SfMNPV-G OBs (Simón et al., 2008a). However, none of these genes have been shown to be necessary for oral infectivity. In addition, other genotypes present in the SfMNPV-NIC isolate with an orally infectious OB phenotype presented larger deletions that included these genes among others (Simón et al., 2004, 2005). A second deletion of 60 bp was detected between nt 119,759–119,818. This deletion affected *sf113* (nt 119,321 → 120,220), the ortholog of the *Autographa californica* MNPV (AcMNPV) ORF136, described as *p26* (Rodems and Friesen, 1993; Simón et al., 2008b).

Other minor differences were found in the nucleotide sequence; a number of these modifications produced significant changes in the deduced amino acid sequences that could result in significant alterations in the functionality of the protein. The first of these was detected in *sf38* (*alk-exo*; nt 36,680 → 37,396). The nt 37,118 of the SfMNPV-B genome changed from a cytosine to a thymine in the SfMNPV-G genome sequence, which resulted in an amino acid change from a His (CAT codon) to a Tyr (TAT codon). These amino acids are relatively similar, suggesting little or no effect on the functionality of the protein. Similar changes were also observed in *sf115* (*odv-e66b*; nt 110,768 ← 108,639), an ortholog of *ac46*, described as *odv-e66* (Ayres et al., 1994). nt 110,686 of the SfMNPV-B sequence changed from guanine to adenine in the SfMNPV-G genome, which resulted in a putative amino acid change from Ser (AGC codon) to Asn (AAC codon). In contrast, nucleotide sequence differences in the *sf58* gene (unknown function; between nt 54,983 ← 55,327) resulted in changes in five amino acids located close to the deduced amino terminal region. The sequence located between nucleotides 55,241 and 55,258 included 14 different nucleotides of the *sf58* polyhedrin antisense gene. The 5′-CAATTGGTGTCGCGCAAT-3′ sequence of SfMNPV-B genome was present as a 5′-ATCAAAGAATCCACGCAA-3′ sequence in SfMNPV-G. However, the Ile-Lys-Glu-Thr-Gln amino acid sequence in SfMNPV-G (24-IKESTQ-29) differed from the equivalent Gln-Leu-Val-Arg-Asn amino acid motif in SfMNPV-B (24-QLVSRN-29). Analysis of the translated sf58 gene revealed that the QLVSRN sequence of the SfMNPV-B genotype is conserved with 100% identity with other alphabaculoviruses such as SfMNPV-3AP2, SpltNPV, SeMNPV, AgseNPV or AgipNPV (Fig. 1) (Harrison et al., 2008; Simón et al., 2011). The *sf58* ORF is 345 nt long and encodes a protein of 114 amino acids with a predicted molecular weight of 12.88 kDa. Sequence analysis revealed the presence of a late promoter motif (TAAG) 32 nt upstream the start codon. The deduced amino acid sequence of SF58 of SfMNPV-G retains the 11 kDa conserved domain of these uncharacterized baculovirus proteins (Lapointe et al., 2004; Zhang et al., 2005). Homologs of this protein are present in alpha- and betabaculovirus genomes which infect Lepidoptera, but are not present in delta- or gammabaculoviruses which infect Diptera and Hymenoptera, respectively. Analysis of the secondary structure of the protein revealed that these changes dramatically affect the predicted secondary structure of SfMNPV-G SF58 (Fig. 2) from alpha helix (24-IKE-26)/coil (27-S-27)/alpha helix (28-TQ-29) to a beta strand (24-QLV-26)/coil (27-S-27)/turn (28-RN-29) in the putative protein produced by SfMNPV-B genotype. The secondary structure pattern (beta strand/coil/turn) is also maintained in other orally infectious alpha- or betabaculoviruses, whereas the altered pattern (alpha helix/coil/alpha helix) an exclusive feature of the SfMNPV-G genotype. In addition, InterProScan tool (SignalP module) predicted a putative signal peptide (SP) of 58 amino acids in the SF58 proteins in both SfMNPV-B and -G genotypes (Fig. 2), and similar signal sequences were also recognized in the homologous proteins from other orally infectious baculoviruses (data not shown). This long, but not unusual putative signal peptide is similar in length to the signal peptide found in the Lassa virus GP-C glycoprotein (Eichler et al., 2003). Based on these results, the altered sequence of the SF58 protein of SfMNPV-G genotype may likely affect the functionality of the predicted signal peptide.

ConSurf analysis indicated that the valine (26-V-26) residue present in SfMNPV-B is predicted as structural (highly conserved and buried). This residue changed to a glutamic acid (26-E-26) in the SfMNPV-G genotype (Fig. 2), which is predicted to be functional (highly conserved and exposed). This implied alterations in the hydrophobicity and the structure of this region, which could lead to major functional changes in the SF58 protein of the SfMNPV-G genotype. Moreover, the predicted antigenic region
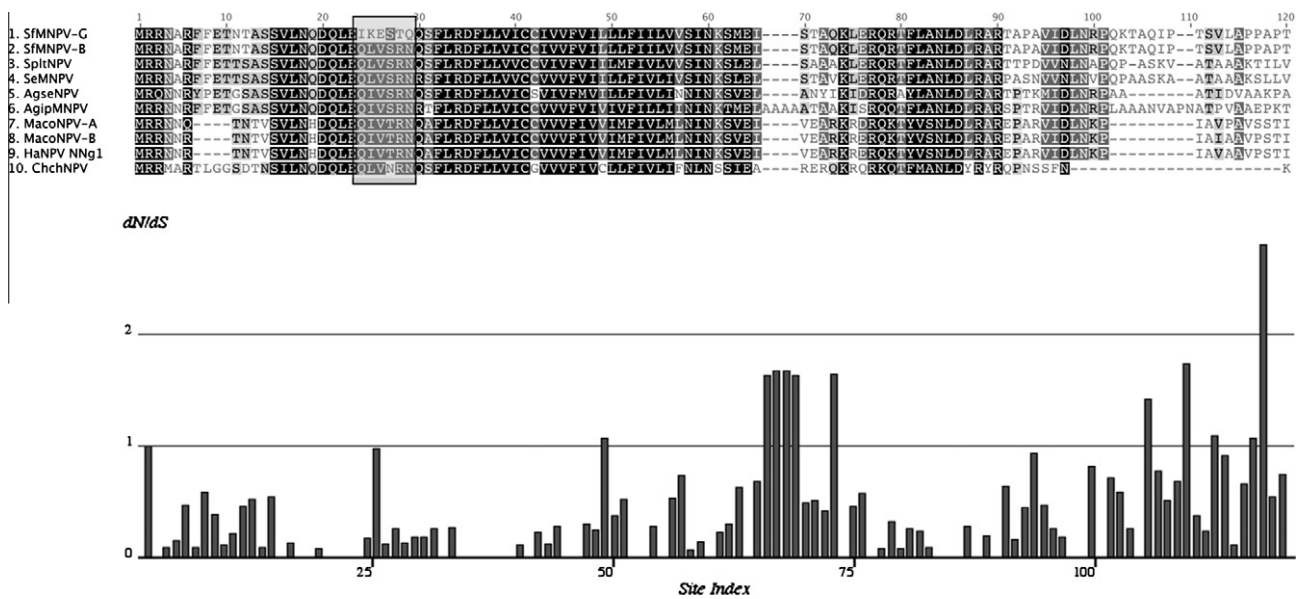
Fig. 1. Multiple sequence alignment of amino acid sequences of SF58 proteins of selected baculoviruses. Gray box indicates the conserved region of SF58 proteins, except in SfMNPV-G. Similarity between amino acids in the same position is indicated by shading (black 100% similar, dark gray 80–100% similar, light gray 60–80% similar and white less than 60% similar). Bar chart represents significant negative selective forces acting on the protein ($P < 0.05$), positively selected sites ($d_N/d_S > 1$) were not significant ($P > 0.05$). Baculovirus sequences used in the comparative analysis were GenBank (accession number); SfMNPV-G (JF899325), SfMNPV-B (HM595733), *S. litura* NPV (AF325155), *S. exigua* MNPV (AF169823), *Agrotis segetum* NPV (DQ123841), *A. ipsilon* MNPV (EU839994), *Helicovepra armigera* NPV NNg1 (AP010907), *Mamestra configurata* NPV A (U59461), *M. configurata* NPV B (AY126275) and *Chrysodeixis chalcites* NPV (AY864330).
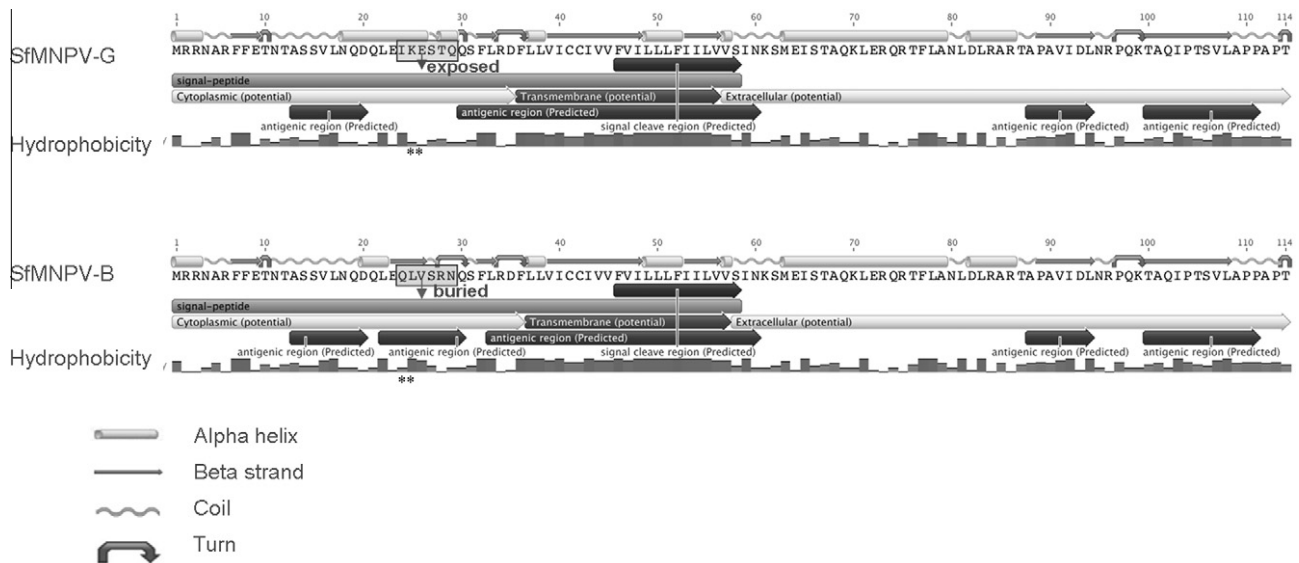
Fig. 2. Amino acid translation of the *sf58* gene from (A) SfMNPV-G and (B) SfMNPV-B genotypes. Boxes indicate major changes into the amino acid sequence and secondary structure of the proteins. Asterisks indicate changes into the hydrophobicity of the amino acids. The predicted exposed status of each site, buried or exposed, is indicated.

(22-LEQLVSRNQ-30) present in the SF58 protein of SfMNPV-B genotype and in the homologs of other alpha- and betabaculoviruses is absent in SF58 of SfMNPV-G genotype (Fig. 2). Analysis of the amino acid sequence with TMHMM indicated that the SF58 proteins of both SfMNPV-B and -G genotypes possess prominent transmembrane domains at the N terminus between amino acids 36 and 58 (Fig. 2). Despite the significant changes in the amino acid terminal region of the SF58 protein of SfMNPV-G, the transmembrane domain, the internal and external regions and the predicted cleavage site are conserved and identical to those of the SF58 protein of SfMNPV-B and -3AP2 genotypes.

Analysis of the number of non-synonymous (d_N) and synonymous (d_S) substitutions per site was performed to detect

evolutionary forces acting on each amino acid residue of the SF58 protein of some alphabaculoviruses (Fig. 1). As expected, no positively selected sites were detected, whereas negative or purifying selection was identified as the predominant evolutionary force acting on *sf58* gene (mean $d_N/d_S = 0.22$, $P < 0.05$). Interestingly, the sites involved in the major changes, residues 24-QLV-26 and 28-RN-29, are under negative selective pressure, suggesting strong selection against variation in these residues (Table 1).

3.2. Sf58 is a late transcribed gene in SfMNPV-G

Single RT-PCR products of the expected size were obtained following amplification of *sf58* mRNA obtained from larvae infected

Table 1
Ratio of nonsynonymous (d_N) and synonymous (d_S) substitutions ($P < 0.05$) and residues involved in the major changes of the SF58 protein of genotype SfMNPV-G compared to reference genotype SfMNPV-B.

Position	Residue SfMNPV-B	Residue SfMNPV-G	d_N/d_S	P-value
24	Q	I	0.17025	0.00099
26	V	E	0.11854	0.00204
27	S	S	0.25816	0.01570
28	R	T	0.13333	0.00195
29	N	Q	0.18844	0.00205

with SfMNPV-B or -G genotypes (Fig. 3). The amplification products were detected at a very low level at 12 hpi, increased at 48 hpi and remained at a steady-state up to 144 hp. Control amplifications, that were performed to ensure the absence of contaminant DNA, were negative. Similarly, a *sfpolh* amplification product was detected at a very low intensity at 24 hpi, increased at 48 hpi and remained at a steady-state level up to 96 hpi. This result indicates that the *sf58* gene of the SfMNPV-G genotype is a transcribed gene and is transcribed 12–24 h before that of *sfpolh*.

3.3. Generation of Sf58null and Sf58rescue viruses

The selected bacmid was expected to contain a deletion of the *sf58* gene, located between nt 54,983 and 55,327 in the SfMNPV-B genome. Replacement of the *sf58* gene with the kanamycin cassette in Sf58null was confirmed by restriction endonuclease analysis and PCR with specific primers targeted at the predicted recombinant junction regions (Simón et al., 2005). Sf58 rescue virus was generated by recombination between Sf58null DNA and a PCR product that covered *sf58* gene. Recombination occurred *in vivo* following intrahemocelic injection of larvae. In total, 2 of the 30 clones analyzed carried the restored *sf58* gene. The genomic arrangement of all recombinant viruses was verified by sequencing. Sfbac, Sf58null and Sf58null rescue bacmid OBs were produced by intrahemocelic inoculation with identical quantities of the respective DNAs. Injection of these DNAs resulted in 58–62% larval mortality. Infections were initiated by injection of viral DNAs on

three occasions and similar percentages of mortality were observed on each occasion.

3.4. Absence of oral infectivity of Sf58null OBs

The LC$_{50}$ values of Sfbac OBs were estimated at 1.89×10^5 and 2.13×10^6 OBs/ml in second and fourth instars, respectively. In contrast, Sf58null virus was found to be non-infectious orally; even when second instars fed on an extremely high concentration of OBs (10^9 OBs/ml) no mortality was observed, whereas OBs of the Sf58null rescue virus were orally infectious at the single concentration tested (90% mortality), and this virus was not subjected to further study. Sf58null ODVs retained their ability to replicate in Sf9 cells and could replicate and kill *S. frugiperda* larvae when injected into the hemocoel but not when larvae were inoculated *per os*. ODV infectivity assays in Sf9 cells revealed that Sf58null ODVs were as infective as those of Sfbac. No differences were found in ODV titers released from similar numbers of OBs of both viruses ($t = 0.091$, df = 22, $p = 0.928$) (Fig. 4A). ODVs released from samples of 5×10^8 OBs and injected into fourth instars resulted in similar percentages of mortality (±S.E.) between 72.9% and 74.7% ($t = 0.283$, df = 18, $p = 0.780$) in insects infected by each of the viruses (Fig. 4B).

3.5. Optical brightener treatment did not influence Sf58null OB infectivity

Addition of Tinopal UNPA-GX to OB inoculum had no effect on Sf58null OB infectivity; no NPV mortality was observed in *S. frugiperda* larvae that had fed on Sf58null OBs in the presence or absence of the optical brightener (Table 2). In contrast, virus mortality was consistently 100% in second instars that fed on Sfbac OBs in mixtures with Tinopal UNPA-GX compared to 52% and 90% mortality in insects that fed on Sfbac OBs alone at the lowest and highest concentrations tested, respectively. A similar pattern was seen in fourth instars; compared to insects that consumed Sfbac OBs alone, virus mortality increased from 92% to 100% and from 19% to 100% in mixtures comprising Sfbac OBs and Tinopal
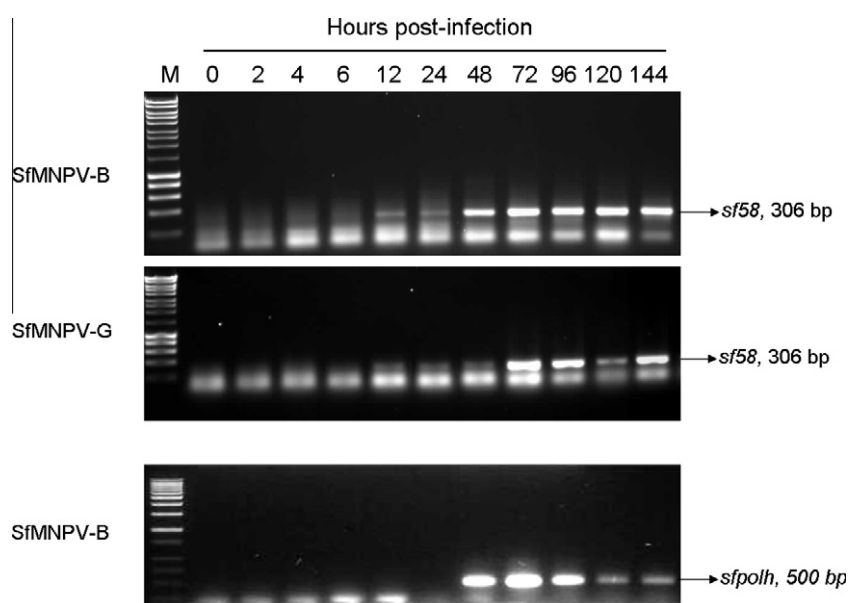
Fig. 3. Temporal expression of *sf58* genes of SfMNPV-B and SfMNPV-G genotypes. RT-PCR analysis of *sf58* genes were performed on total RNA extracted from orally infected larvae with SfMNPV-B OBs and in intrahemocelically infected larvae following injection of SfMNPV-G ODVs at indicated times post infection (hpi). Transcript amplifications were performed using Sf58.1 and Sf58.2 primers. For comparison, a temporal expression of SfMNPV-B *sfpolh* gene was performed on SfMNPV-B infected larvae with Sfpolh.1 and Sfpolh.2 primers. M1 indicates molecular DNA marker of 1 kb (Hyperladder I, Bioline).

Fig. 4. (A) ODV content, shown as ODV infectious units, of 5×10^8 OBs of SfMNPV bacmid (Sfbac) and Sf58null bacmid viruses. Sf9 cells were serially infected (1:10, 1:50. 1:250, 1:1250 and 1:6250) with ODVs released from OBs. ODV titers (ODV/ml) were calculated by end point dilution. (B) Percentage of mortality of *S. frugiperda* larvae injected with ODVs released from 5×10^8 OBs.

UNPA-GX in the highest and lowest OB concentrations, respectively. These results suggest that the mode of action of *sf58* does not involve degradation of the midgut peritrophic membrane.

3.6. Complementation assays

Mixtures of SfMNPV-B OBs and SfMNPV-G OBs did not result in infection by the defective SfMNPV-G genotype even in presence of Tinopal UNPA-GX. In all cases insects died exclusively from SfMNPV-B genotype and no SfMNPV-G profile was detectable by REN analysis or PCR using specific primers targeted around the deletion break point of the SfMNPV-G genotype (Simón et al., 2005). The activity of the OB mixture (genotypes B + G) in second

or fourth instars was significantly lower than that of SfMNPV-B OBs in the presence or absence of Tinopal UNPA-GX (Table 3). In second instars the relative potency of the mixture was 0.371 or 0.395 compared to that of pure genotype B OBs in the absence or presence of optical brightener, respectively. Both for second and fourth instars, equal mixtures of genotype B and genotype G OBs resulted in potencies that were less than 0.5 compared to genotype B OBs, probably because the relationship between inoculum concentration and potency is not additive but depends on the shape of the logarithmic concentration–response curve. These results are consistent with SfMNPV-B genotype OBs being the only infective OBs present in the inoculum and indicate that SfMNPV-B OBs did not have the capacity to rescue the non-infective phenotype of SfMNPV-G OBs.

4. Discussion

To determine the origin of the non-infective phenotype of SfMNPV-G OBs, the complete sequence of the SfMNPV-G genome was determined. A major deletion of 4860 kb that is completely included in the deletions already characterized in SfMNPV-C and -D genotypes, did not explain the SfMNPV-G phenotype (Simón et al., 2005). A second minor deletion in SfMNPV-G affected *sf131*, an ortholog of the *acp26* gene. This gene has been described as an auxiliary gene that does not influence key aspects of virus transmission such as budded virus or OB production, infectivity or speed-of-kill in insects infected by AcMNPV (Rodems and Friesen, 1993; Simón et al., 2008b). A SfMNPV bacmid in which *p26b* was disrupted due to the presence of a single *Asc*I restriction site had no influence on the biological activity of SfMNPV bacmid OBs compared to that of the SfMNPV-B virus (Simón et al., 2008a). This observation supports the results of the study on AcMNPV that deletion of *p26* has no substantial effects on the insecticidal properties of these viruses (Simón et al., 2008a, 2008b). A third difference was detected in *sf58*. The nucleotide sequence of this gene is conserved between the reference genotypes of two different SfMNPV strains from Nicaragua (SfMNPV-B) and Missouri (SfMNPV-3AP2) (Harrison et al., 2010; Simón et al., 2011), and other alpha- and betabaculoviruses. However, differences in 14 nucleotides resulted in major amino acid changes with clear implications in the functionality of the SF58 putative protein in the SfMNPV-G genotype.

A Sf58null bacmid was constructed in which the *sf58* gene was deleted by homologous recombination, a technique that has been used to delete baculovirus genes and to analyze their function (Nie and Thielmann, 2010; Pijlman et al., 2003; Wu et al., 2010). The results presented here provide genetic and biological evidence that deletion of *sf58* from the SfMNPV bacmid resulted in the pro-

Table 2
Percentage of virus mortality of *S. frugiperda* second and fourth instar following peroral infection with Sfbac and Sf58null OBs in the presence or absence of 1% Tinopal UNPA-GX. Two concentrations were used for each instar: 1.2×10^6 and 5.0×10^4 OBs/ml for second instars and 4.0×10^7 and 4.0×10^4 OBs/ml for fourth instars (three replicates were performed).

Instar	Virus	Concentration (OBs/ml)	Mortality (%)						Average mortality (%)	
			Rep. 1		Rep. 2		Rep. 3			
			− Tinopal	+ Tinopal	− Tinopal	+ Tinopal	− Tinopal	+ Tinopal	− Tinopal	+ Tinopal
Second	Sfbac	1.2×10^6	93	100	97	100	81	100	90	100
		5.0×10^4	56	100	43	100	58	100	52	100
	Sf58null	1.2×10^6	0	0	0	0	0	0	0	0
		5.0×10^4	0	0	0	0	0	0	0	0
Fourth	Sfbac	4.0×10^7	98	100	91	100	87	100	92	100
		4.0×10^4	15	95	23	93	18	87	19	92
	Sf58null	4.0×10^7	0	0	0	0	0	0	0	0
		4.0×10^4	0	0	0	0	0	0	0	0

Table 3
Probit regression of SfMNPV-B (B), SfMNPV-G (G) genotypes and the OB mixture between SfMNPV-B and SfMNPV-G (B + G) in a 1:1 proportion in second and fourth instars of *S. frugiperda* in the presence or absence of 1% Tinopal UNPA-GX.

Instar	Viruses	1% Tinopal UNPA-GX	LC$_{50}$ (OBs/ml)	Relative potency	Fiducial limits (95%)		P
					Low	High	
Second	B	–	7.04×10^4	1	–	–	–
	G	–	–	–	–	–	–
	(B + G)	–	1.90×10^5	0.371	0.219	0.628	<0.05
	B	+	2.25×10^4	1	–	–	–
	G	+	–	–	–	–	–
	(B + G)	+	5.69×10^4	0.395	0.255	0.611	<0.05
Fourth	B	–	3.02×10^5	1	–	–	–
	G	–	–	–	–	–	–
	(B + G)	–	2.13×10^6	0.142	0.078	0.258	<0.05
	B	+	1.84×10^4	1	–	–	–
	G	+	–	–	–	–	–
	(B + G)	+	7.26×10^4	0.253	0.127	0.503	<0.05

Probit regressions were fitted using the PoloPlus program. A test for non-parallelism was not significant for second instars in the absence ($\chi^2 = 0.01$, df = 1, $P = 0.908$) or in the presence ($\chi^2 = 0.83$, df = 1, $P = 0.362$) of Tinopal UNPA-GX, or in fourth instars in the absence ($\chi^2 = 0.04$, df = 1, $P = 0.842$) or in the presence ($\chi^2 = 0.14$, df = 1, $P = 0.712$) of Tinopal UNPA-GX. Relative potencies were calculated as the ratio of effective concentrations relative to the SfMNPV-B virus.

duction of Sf58null OBs that lost *per os* infectivity, while retaining ODV infectivity in cell culture or by intrahemocoelic injection. Sf58null ODVs were as infective as SfMNPV bacmid ODVs in larvae or cell culture, suggesting defects in ODV or occlusion body morphogenesis were unlikely explanations for the observed non-infectious OB phenotype. The behavior of the Sf58null virus was similar to that of SfMNPV-G, suggesting that the observed phenotype is due to the mutation of this gene. Similarly, OB infectivity was restored in the Sf58null rescue virus indicating that the only process significantly affected by the mutation in *sf58* is the primary infection process in host midgut cells. The addition of a stilbene brightener and/or the presence of orally infectious B-genotype OBs failed to enhance or recover the infectivity of the SfMNPV-G OBs, ruling out an effect involving disruption of the peritrophic membrane. In addition, transcriptional analysis revealed that *sf58* from SfMNPV-G is a transcribed gene and its pattern of transcription is identical to that of the SfMNPV-B *sf58* gene. The fact that *sf58* is transcribed 12 h before the *sfpolh* gene and the presence of a late promoter upstream from the start codon confirmed that this gene is a late transcribed gene.

Orthologs of SF58 are present in alpha- and betabaculoviruses that are lepidopteran-infecting baculoviruses. Recently, Rohrmann (2011) reported that an insertional mutation in the homolog of *sf58* (*bm91*), aborted the infectivity of *Bombyx mori* NPV. SF58 is also homolog to the SpltNPV ORF97 (Chen et al., 2006) or *Antheraea pernyi* NPV (AnpeNPV) ORF100 (Shi et al., 2007), for which the structural localization of SPLT97 and ANPE100 have been identified, but without evidence to date of the role of this protein in primary infection. In line with our findings, Chen et al. (2006) identified *splt97* as a late transcribed gene (by 6–96 hpi), localized in the ODV envelope. Shi et al. (2007) described ANPE100 in association with ODVs, but not with BVs, as expected for a protein involved in the primary infection process.

Computational analyzes revealed that SF58 presented an 11 kDa conserved domain. Other *per os* infectivity factors, such as *Autographa californica* MNPV ORF145 and 150 (*ac145* and *ac150*), also possess this domain. However deletion of *ac145* and *ac150* reduced, but did not abolish, the oral infectivity of OBs (Lapointe et al., 2004; Zhang et al., 2005). The deduced amino acid sequence of the SF58 protein of SfMNPV-G retains the 11 kDa conserved domain, suggesting that this domain might not be involved in the lack of oral infectivity of this genotype. In contrast, several significant changes in the secondary structure and other SF58 features were identified that could be responsible for loss of function. Based on these findings, mutations in the *sf58*

gene of SfMNPV-G are reflected in structural and functional changes that are likely deleterious for this genotype. The defective SfMNPV-G genotype is almost certainly maintained in the population as a result of co-occlusion and co-infection with the fully functional SfMNPV-B genotype, as occurs with the defective SfMNPV-C and -D genotypes (Simón et al., 2004). A prominent transmembrane domain is located in the central region of the protein that is conserved in alpha- and betabaculovirus SF58 homologs. Hydrophobic transmembrane motifs are involved in membrane insertion, anchoring, and are features of a number of ODV membrane proteins (Hong et al., 1997; Peng et al., 2010; Slack and Arif, 2007). The conservation of this motif in SF58 homologs suggests that membrane association is a highly conserved trait. Similarly, all other known PIFs are located to the ODV envelope and present characteristic transmembrane domains (Peng et al., 2010). The PIFs are necessary for primary virus infection that involves binding to microvilli receptors and fusion between the ODV membrane and the microvilli of midgut cells (Okhawa et al., 2005). This is a multistep process (Horton and Burand, 1993) that seems to involve a highly stable complex of PIF factors (PIF1, PIF2 and PIF3) from the ODV envelope in association with P74 (Faulkner et al., 1997; Haas-Stapleton et al., 2004; Peng et al., 2010; Slack et al., 2008; Zhou et al., 2005). Peng et al. (2010) postulated that the major internal region of PIF3 may interact with other PIFs. According to the predicted structure of the SfMNPV-G SF58 the transmembrane domain and the external region are conserved, which suggests that the regions potentially involved in binding and membrane fusion are maintained. However a significant modification was identified towards the amino terminal region (cytosol-located region) of the SF58 of SfMNPV-G, suggesting involvement in the absence of oral infectivity of this genotype. These modifications altered the predicted secondary structure and putative signal peptide sequence. The altered putative signal peptide sequence might not be functional in the SfMNPV-G genotype, resulting in SF58 being redirected to a secretory pathway or localized in an inappropriate membrane, both of which would disrupt its localization in the ODV membrane, leading to the loss of oral infectivity. Moreover, this gene is subjected to negative (purifying) selection that involves selection against amino acid changes that cause loss or reduction in function (Sharp et al., 2007). Consistent with this idea, the sites involved in this major change were identified as being under negative selection, indicating the importance of retaining these residues unchanged. Clearly, further studies are necessary to determine the mechanism and the target of this newly discovered PIF factor.

To date, all PIF proteins (P74, PIF1 to PIF5) are encoded by genes conserved among all sequenced baculovirus genomes (Braunagel and Summers, 2007; Fang et al., 2009; Herniou et al., 2003; van Oers and Vlak, 2007; Peng et al., 2010; Harrison et al., 2010). This suggests that the interactions among these proteins and mechanisms of primary midgut infection are highly conserved in members of the Baculoviridae family. In addition, p74 and pif1 to pif5 all have homologs in other DNA viruses including nudiviruses (Wang and Jehle, 2009) and salivary gland hypertrophy viruses, SGHVs, (García-Maruniak et al., 2009). The conservation of these genes suggests a similar basic cell entry mechanism for these viruses. Recently a new per os infectivity factor present only in group II alphabaculoviruses has been described (Luo et al., 2011). H. armigera nucleopolyhedrovirus occlusion-derived virus-associated protein, HA100, significantly affects the oral infectivity of OBs in host insects, suggesting that the association HA100 with the ODV contributes to the infectivity of OBs in vivo.

However, this is the first evidence of a new PIF protein present only in alpha- and betabaculoviruses, suggesting that homologs of sf58 play a specific role in the transmission of lepidopteran baculoviruses. These results open an intriguing new avenue of study into the initial entry mechanisms of these viruses and the mechanisms that determine host specialization in members of the lepidopteran-infecting baculoviruses. Characterization of the molecular mechanisms involved in primary infection are also likely to have clear applications for the improvement of these viruses as the basis for biological insecticides.

Acknowledgments

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