Dual mutations in the Autographa californica nucleopolyhedrovirus FP-25 and p35 genes result in plasma-membrane blebbing in Trichoplusia ni cells

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Spodoptera frugiperda cells infected with Autographa californica nucleopolyhedrovirus (AcMNPV) lacking a functional anti-apoptotic p35 protein undergo apoptosis. However, such mutants replicate normally in Trichoplusia ni (TN-368) cells. An AcMNPV plaque isolate (AcdefrT) was identified during propagation of a virus deficient in p35 in TN-368 cells. This virus exhibited enhanced budded-particle formation in TN-368 cells, but was partially defective for polyhedra production in the same cells. Virus replication in AcdefrT-infected TN-368 cells was accompanied by extensive plasma-membrane blebbing and caspase activation late in infection, both features of apoptosis. Rescue of the p35 locus of AcdefrT continued to result in a reduction in polyhedra and increase in budded virus production in TN-368 cells, but no plasma-membrane blebbing was observed. The mutation was mapped to the FP-25 gene locus. This gene mutation combined with the non-functional p35 was found to be responsible for the cell-blebbing effect observed in AcdefrT-infected TN-368 cells.

Infection of insect cells with the Autographa californica nucleopolyhedrovirus (AcMNPV) results in the production of two virus forms. In late stages of infection, from about 8 h post-infection (p.i.), nucleocapsids bud from the plasma membrane and acquire a lipoprotein envelope. Production of this budded virus (BV) continues until approximately 24 h p.i., whereafter nucleocapsids are retained within the nucleus and become enveloped. These are subsequently occluded to form polyhedra (Funk et al., 1997). Infection of Spodoptera frugiperda (Sf21) cells with wild-type AcMNPV causes transient plasma-membrane blebbing at 12 h p.i. (Clem et al., 1991). In Sf21 cells infected with an AcMNPV p35 mutant, blebbing intensifies, resulting in disintegration of cells into apoptotic bodies. Cell blebbing is not observed in Trichoplusia ni (TN-368) cells infected with either wild-type virus or AcMNPV p35 mutants (Clem et al., 1991). We isolated a spontaneous AcMNPV mutant from a plaque assay conducted in TN-368 cells, during construction of a virus deficient in p35 function. The virus was supposed to have a 488 bp deletion from positions 116395 to 116883 of the AcMNPV C6 genome (Ayres et al., 1994). Thus, it lacked the p35 basal promoter region and the first 391 bases of the p35 coding region.

After infection of TN-368 cells (uninfected; Fig. 1a) with the p35-negative virus, an aberrant phenotype was observed at 3 days p.i. Instead of normal polyhedra production, as seen in AcMNPV-infected TN-368 cultures (Fig. 1b), cells contained few occlusion bodies (Fig. 1c). Furthermore, virus-infected cells exhibited extensive blebbing of vesicles (Fig. 1c). The virus was designated AcdefrT, as it was considered to be defective for replication in TN-368 cells, presumably owing to a spontaneous mutation.

The p35 locus was restored to observe its effect on the phenotype of AcdefrT-infected Sf21 cells. Plasmid pAcEcoRI-S, containing p35 (Ayres et al., 1994), was digested with EcoRI. This was mixed with AcdefrT DNA and used to transfect Sf21 cells (King & Possee, 1992). Medium containing virus was harvested at 4 days post-transfection and screened for cell blebbing and polyhedra production. Virus from a homogeneous polyhedra-producing recombinant plaque of wild-type appearance was amplified in Sf21 cells. It was designated AcdefrT*p35* and exhibited a plaque phenotype indistinguishable from that of AcMNPV in Sf21 cells (Fig. 1f, g). This suggested that the spontaneous

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Cultures of TN-368 cells were infected with AcdefrT to determine the phenotype of the modified virus. As with AcdefrT, TN-368 cells infected with AcdefrTp35 showed a reduction in the number of polyhedra within each cell compared with wild-type infection, as judged by visual inspection of the cultures. However, the extensive cell blebbing seen with AcdefrT infection was not observed in cells infected with AcdefrTp35 (Fig. 1d), suggesting that this feature of the mutant phenotype was due to a combination of the unknown mutation and the non-functional p35 gene.

Induction of apoptosis in Sf21 cells by AcMNPV p35-negative viruses is associated with reduced BV formation (Hershberger et al., 1992; Clem & Miller, 1993). Therefore, BV production in AcdefrT-infected Sf21 or TN-368 cells was examined. Either cell type was infected with AcMNPV, AcdefrT or AcdefrTp35 (10 p.f.u. per cell). Medium was harvested at 72 h p.i. and viruses were titrated three times by plaque assay in the appropriate cell line. The mean titre for AcMNPV in Sf21 cells was $2.93 \times 10^8$ p.f.u. ml$^{-1}$. As expected, AcdefrT showed greatly reduced levels of BV production in Sf21 cells compared with AcMNPV, owing to the absence of p35. The AcdefrT titres only reached a maximum of $1.24 \times 10^4$ p.f.u. ml$^{-1}$. AcdefrTp35, however, showed similar BV titres to the wild type, reaching $6.63 \times 10^8$ p.f.u. ml$^{-1}$ at 72 h p.i. Similar experiments were done using TN-368 cells. In these cells, whilst the mean BV titre for the wild-type virus at 72 h p.i. was $4.8 \times 10^6$ p.f.u. ml$^{-1}$, titres of both AcdefrT and AcdefrTp35 were found to reach $2.9 \times 10^8$ and $2.7 \times 10^8$ p.f.u. ml$^{-1}$, respectively.

The AcdefrT-infected TN-368 cells exhibited extensive plasma-membrane blebbing, suggesting that apoptosis was
induced. Apoptosis is associated with caspase activation from procaspases in cells. Therefore, caspase activation was monitored in AcMNPV-, AcdefrT- and AcdefrT$p35^r$-infected Sf21 and TN-368 cells (Fig. 2). Cell cytosolic extracts were assayed by using a caspase-3 colorimetric activity assay kit (Chemicon International, Inc.). The kit provides a means of assaying the activity of caspases that recognize the DEVD motif. It is based on spectrophotometric detection of the chromophore $p$-nitroaniline ($p$NA) after cleavage from the labelled substrate DEVD-$p$NA. Thus, levels of free $p$NA reflect levels of caspase activity within the cell. In Sf21 cells, levels of caspase activity were five times higher in AcdefrT-infected cultures than with AcMNPV or AcdefrT$p35^r$ (Fig. 2a). This was consistent with the absence of $p35$ in AcdefrT. In TN-368 cells infected with the same three viruses, extracts were prepared at 24, 36 and 48 h p.i. (Fig. 2b). At 24 h p.i., caspase activity in AcdefrT-infected cells was marginally higher than in extracts from mock-infected cells or those infected with AcMNPV or AcdefrT$p35^r$. By 36 h p.i., the levels of caspase activity in AcdefrT-infected TN-368 cells were nearly double those observed in any of the other samples. At 48 h p.i., caspase activity in AcdefrT-infected cells was over sixfold higher than that in mock- or AcdefrT$p35^r$-infected cells. Levels of caspase activity in AcMNPV-infected cells were also higher at 48 h p.i., but a two-tailed Student’s $t$-test confirmed the difference in activity between AcdefrT and AcMNPV samples to be statistically significant.

A strategy to identify the gene responsible for the abnormal phenotype in AcdefrT-infected TN-368 cells was developed based on recreation of the mutant virus. An AcdefrT cosmid library was made for use in this experiment (Fig. 3a). A partial digest was carried out on AcdefrT genomic DNA by using the restriction enzyme MboI. Digests producing fragments of approximately 30 kbp were pooled and ligated into the SuperCos 1 vector (Stratagene). The identity of cloned AcdefrT genomic fragments was ascertained by sequencing the ends of each insert and alignment with the complete AcMNPV sequence (Ayres et al., 1994). Clones were selected spanning most of the AcMNPV genome (Fig. 3a).

Individual AcdefrT cosmid clones were added to AcMNPV$p35^r$ DNA (Griffiths et al., 1999), containing the Escherichia coli lacZ gene inserted into the $p55$ locus, and used to transfect TN-368 cells. The progeny virus was titrated in the same cell type after 5 days. Cell blebbing in AcdefrT-infected TN-368 cells was due to a combination of the unknown mutation and the absence of $p35$. Thus, by using AcMNPV$p35^r$ DNA in this experiment, mutant plaques could be identified on the basis of both a reduction in number of polyhedra and the occurrence of plasma-membrane blebbing.

Plaque screening revealed possible AcdefrT-like plaques in those dishes infected with medium harvested from cells transfected with AcMNPV$p35^r$ genomic DNA and cosdef 4 (Fig. 3a). In the same experiment, none of the other cosmids resulted in the production of AcdefrT-like plaques. The AcMNPV gene complement within cosdef 4 was analysed by PCR amplification of $p35$ DNA in this experiment, mutant plaques could be identified on the basis of both a reduction in number of polyhedra and the occurrence of plasma-membrane blebbing.

![Fig. 2. Caspase-3-like activity in virus-infected insect cells. Insect cells in monolayer culture were infected with AcMNPV, AcdefrT or AcdefrT$p35^r$ at an m.o.i. of 10 p.f.u. per cell or mock-infected. Cell cytosolic extracts were analysed for caspase-3-like activity. The reactions were incubated for 2 h at 37°C. Spectrophotometric detection of the chromophore $p$-nitroaniline ($p$NA) after cleavage from the labelled substrate DEVD-$p$NA was performed by using a microtitre plate reader at 405 nm. (a) Caspase activity in virus-infected and mock-infected Sf21 cells at 24 h p.i. (b) Caspase activity in virus-infected and mock-infected TN-368 cells at 24, 36 and 48 h p.i.](http://vir.sgmjournals.org)
7 adenines between positions +181 and +188 of FP-25K (Fig. 3c). The insertion of this base produced a frameshift mutation resulting in the premature occurrence of a stop codon and subsequent truncation of the FP-25 protein from the wild-type 214 aa to 63 aa.

Confirmation that this mutation was responsible for the observed phenotype of each virus and that no other mutation present in the viral genome was responsible was provided by reconstructing both AcdefrT and AcdefrT p35 r. A 366 bp region of the AcdefrT FP-25 locus containing the point mutation was PCR-amplified by using Pfu polymerase and purified by using a Qiagen PCR purification kit. This fragment was mixed with either AcMNPV or AcMNPV p35 DNA and used to cotransfect TN-368 cells. Medium was harvested and screened for AcdefrT- and AcdefrT p35 r-type plaques. Mutant plaques were identified successfully and the reconstructed viruses (recAcdefrT and recAcdefrT p35 r) were amplified.

Both recAcdefrT (Fig. 1h) and recAcdefrT p35 r (data not shown) produced the same plaque phenotypes as the original AcdefrT and AcdefrT p35 r viruses, respectively, in TN-368 cells and Sf21 cells (data not shown). Sequencing FP-25 from each of the two recreated viruses confirmed the presence of only the inserted adenine in the same position as in AcdefrT and AcdefrT p35 r. An analysis of BV titres, similar to that described above, was carried out to confirm that recAcdefrT produced levels of BV similar to AcdefrT in TN-368 cells. As expected, both AcdefrT and recAcdefrT produced higher levels of BV than did AcMNPV in this cell line. The mean BV titre for the wild-type virus was found to be 4.6 × 10^5 p.f.u. ml^-1 at 72 h p.i. Consistent with earlier analyses of BV titres, AcdefrT BV levels were higher than those observed for the wild type, reaching 2.6 × 10^6 p.f.u. ml^-1 at 72 h p.i. Titres of recAcdefrT were also found to be greater than those of AcMNPV, as well as exceeding those of AcdefrT, reaching 1.2 × 10^9 p.f.u. ml^-1 by 72 h p.i. Together, these results confirmed that the reduction in polyhedral and increase in BV production observed in AcdefrT p35 r-infected TN-368 cells was due to a mutation in the FP-25 gene alone.

Mutations in the AcMNPV FP-25 would be expected to result in the production of FP mutants in both Sf21 and TN-368 cells. This was not the case, however, with the FP-25
mutation found here. Although truncation of the FP-25 protein was predicted to be severe, reducing the 214 aa protein to only 63 residues, the difference in phenotype observed in these two cell types suggests that it did not affect its ability to function normally in Sf21 cells.

The work described in this report indicates that infection of TN-368 cells with an AcMNPV mutant possessing mutations in FP-25 and p35 induces plasma-membrane blebbing in TN-368 cells. An assay of caspase-3-like activity in insect cells showed that elevated levels of caspase activity were present in AcdefrT-infected TN-368 cells. This indicated that an apoptotic response had been generated in these cells after infection with a virus lacking functional p35 and 25K. The TN-368 cells have been found to be more resistant to apoptosis than Sf21 cells. Whilst infection with AcMNPV p35 mutants or treatment with the RNA-synthesis inhibitor actinomycin D induced apoptosis in Sf21 cells, cell death did not occur in TN-368 cells exposed to these same stimuli (Clem et al., 1994). The observation of a unique phenotype associated with mutations in FP-25 and p35 was unexpected. The characteristics associated most commonly with the FP genotype are a reduced number of polyhedra per cell compared with the wild type, occlusions containing no virions or virions of altered morphology, altered intranuclear envelopment and the production of more BV than by the wild type (Ramoska & Hink, 1974; Potter et al., 1976; Wood, 1980). Fraser et al. (1983) found a common feature of many AcMNPV and Galleria mellonella (Gm) MPV mutants to be insertion of moderately repetitive host-DNA sequences into a region of the genome encoding a 25 kDa protein. These were later correlated with large insertions of host-cell DNA or deletions of viral DNA, detectable by restriction-endonuclease analysis, in this region of the genome (Beames & Summers, 1988, 1989). Targeted mutation of AcMNPV 25K confirmed alterations to this gene to be sufficient to result in these complex characteristics of the FP phenotype (Harrison & Summers, 1995). Although FP mutants of Lymantria dispar (Ld) MNPV and Helicoverpa armigera (Ha) SNPV have been identified (Slavicek et al., 1995; Bischoff & Slavicek, 1997; Chakraborty & Reid, 1999; Lu et al., 2002), these mutations were the result of point changes or small insertions or deletions in the FP-25K coding region (Bischoff & Slavicek, 1997; Lu et al., 2002). This suggests that LdMNPV and HaSNPV mutants may arise through a mechanism different from that involved in the formation of most AcMNPV and GmMNPV FP mutants. However, the frameshift mutation within AcdefrT is clearly more like the minor changes observed to occur within the LdMNPV and HaSNPV genomes. This feature, linked with the absence of p35 in AcdefrT-infected cells, must be responsible for the apparent induction of apoptosis in TN-368 cells infected with this virus. Whether or not such dual mutations accumulate in the genomes of baculoviruses in insect larvae is unclear. However, we did note an increase in caspase-3-like activity in AcMNPV-infected TN-368 cells, which might indicate accumulation of these mutations in a proportion of the wild-type virus population.

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References


