An Apoptosis-Inhibiting Gene from a Nuclear Polyhedrosis Virus Encoding a Polypeptide with Cys/His Sequence Motifs

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Two different baculovirus genes are known to be able to block apoptosis triggered upon infection of Spodoptera frugiperda cells with p35 mutants of the insect baculovirus Autographa californica nuclear polyhedrosis virus (AcMNPV): p35 (P35-encoding gene) of AcMNPV (R. J. Clem, M. Fechheimer, and L. K. Miller, Science 254:1388-1390, 1991) and iap (inhibitor of apoptosis gene) of Cydia pomonella granulosis virus (CpGV) (N. E. Crook, R. J. Clem, and L. K. Miller, J. Virol. 67:2168-2174, 1993). Using a genetic complementation assay to identify additional genes which inhibit apoptosis during infection with a p35 mutant, we have isolated a gene from Orgyia pseudotsugata NPV (OpMNPV) that was able to functionally substitute for AcMNPV p35. The nucleotide sequence of this gene, Op-iap, predicted a 30-kDa polypeptide product with approximately 58% amino acid sequence identity to the product of CpGV iap, Cp-IAP. Like Cp-IAP, the predicted product of Op-iap has a carboxyl-terminal C3HC4 zinc finger-like motif. In addition, a pair of additional cysteine/histidine motifs were found in the N-terminal regions of both polypeptide sequences. Recombinant p35 mutant viruses carrying either Op-iap or Cp-iap appeared to have a normal phenotype in S. frugiperda cells. Thus, Cp-IAP and Op-IAP appear to be functionally analogous to P35 but are likely to block apoptosis by a different mechanism which may involve direct interaction with DNA.

The ability of cells to undergo apoptotic cell death in response to viral infection and the ability of viruses to block cellular apoptosis constitute a virus-host interaction which is crucial in determining the outcome of cell of virus infection at both the cellular and organisinal levels (4, 22, 34). Because apoptosis is also involved in a wide variety of normal and abnormal organismal processes, including tissue homeostasis, immune system function, embryonic development, and cancer (31), the molecular pathways leading to apoptosis are important to define.

Large DNA-containing viruses are proving to be valuable sources of genes which block apoptosis. Some members of the families Adenoviridae, Baculoviridae, and Herpesviridae have genes involved in blocking cellular apoptosis or programmed cell death during infection (2, 3, 6, 13, 25, 34) or share homology (24, 26) with bcl-2, a mammalian proto-oncogene which blocks normal apoptosis when overexpressed (17, 33). Adenovirus E1B proteins are thought to abrogate the function of p53 (7, 34), a tumor suppressor which accumulates in response to DNA damage (23), arresting the cell cycle at G1/S for DNA repair (18, 19) and possibly triggering apoptosis when DNA damage is severe (7). Thus far, baculoviruses appear to use genes distinctively different from those of mammalian viruses to block apoptosis. Characterizing these genes and their function(s) is likely to provide insight into the pathways governing this evolutionarily conserved process.

Two different baculovirus genes, p35 and iap, block apoptosis induced after infection of Spodoptera frugiperda cells (SF-21) with p35 mutants of Autographa californica nuclear polyhedrosis virus (AcNPV) (3, 6). AcMNPV p35 mutants, such as vAcAnh (also known as the annihilator), induce widespread apoptosis in SF-21 cells between 12 and 24 h postinfection (3).

Premature cell death by apoptosis results in the shut off of all protein synthesis, a 100- to 1,000-fold decrease in budded virus production, and the complete absence of occlusion bodies (3, 4, 15). The predicted sequence (10) of the p35 product, P35, reflects no obvious homology to other sequences in GenBank. Our interest in searching for apoptosis-inhibiting genes in baculoviruses other than AcMNPV was stimulated by the observation that the Orgyia pseudotsugata NPV (OpMNPV) genome, which is largely colinear with the AcMNPV genome (1), appears to lack a p35 homolog (11, 21). Although the genes flanking AcMNPV p35 and its upstream neighbor p94 (10) are similarly positioned in OpMNPV, these two genes are not present in this position of the OpMNPV genome, and no hybridization to OpMNPV DNA was observed with AcMNPV p35 DNA as a probe (11).

With the expectation of locating distantly related homologs of p35, we developed a genetic complementation assay to identify apoptosis-blocking genes in which SF-21 cells are cotransfected with vAcAnh and the test baculovirus DNA and transfected cells are subsequently monitored for occluded virus production. Several baculovirus DNAs, including OpMNPV DNA and Cydia pomonella granulosis virus (CpGV) DNA, were able to complement the p35 defect in this assay. We first examined the more distantly related CpGV genome and discovered that a single CpGV gene, Cp-iap (inhibitor of apoptosis), was able to block apoptosis in vAcAnh-infected cells (6). The product of Cp-iap, Cp-IAP, has no sequence homology to the AcMNPV p35 gene product, P35. Cp-IAP may be a DNA binding protein, on the basis of the presence of a C-terminal zinc finger-like motif, referred to as a C3HC4 motif, which is also found in a number of proteins encoded by virus regulatory genes (e.g., herpes simplex virus ICP0 and AcMNPV ie-n), proto-oncogenes (e.g., mel-18 and FMI), insect developmental regulatory genes [e.g., su(2)2 and pse] (5), and other genes encoding DNA-interactive proteins (9, 29). A homolog of Cp-iap also exists in the AcMNPV genome, but this homolog, Ac-iap, is nonfunctional in the complementation assay (6).

In this study, we found that OpMNPV has a gene homolo-
FIG. 1. Genomic, cosmid, and subclone maps of OpMNPV as well as the nucleotide sequence of Op-iap. (A) HindIII map of the OpMNPV genome is shown at the top (21). The thick bars beneath the map represent overlapping cosmid clones used in the complementation assay. The map at the bottom shows restriction sites surrounding the iap ORF, which is represented by the cross-hatched box; the arrow above the box indicates the direction of the ORF. (B) Nucleotide sequence of a 1.4-kb active fragment of clone 13 containing iap. Below the DNA sequence is the predicted amino acid sequence of IAP. Key restriction potential regulatory sites are indicated. A late transcriptional start site (ATAAG), early TATA box, and polyadenylation signals downstream of the ORF are underlined. The C3HC4 motif is double underlined.
gous to Cp-iap, which was able to block vAcAnh-induced apoptosis in SF-21 cells. The OpMNPV homolog, Op-iap, was in a genomic position roughly equivalent to the position of the Ac-iap homolog and was more closely related to Cp-iap than to Ac-iap. In addition to the C-terminal C3HC4 motif, all three IAPs contain two tandemly repeated Cys/His motifs.

**MATERIALS AND METHODS**

**Viruses and insect cells.** Wild-type (wt) AcMNPV (L-1) and recombinant viruses (vASB6-1 and vAnhR11) were propagated in cell lines derived from either *S. frugiperda* IPLB-SF-21 (SF-21) cells (32) or *Trichoplusia ni* TN-368 cells (16), and titers were determined as described previously (6, 27). The mutant vAcAnh was propagated in TN-368 cells only, and its titer was determined. Cells were maintained at 27°C in TC-100 medium supplemented with 10% fetal bovine serum and 0.26% tryptose broth by standard methods (27).

DNA cloning, sequencing, and analysis. Cosmid clones, generated by cloning partially digested HindIII fragments (21) and spanning the entire genome, were kindly provided by George Rohrmann (Oregon State University, Corvallis). Only cosmid 13 showed activity in the complementation assay (Fig. 1A). An active 3.9-kb *SalI-PstI* fragment from cosmid 13 was gel purified, subcloned into the *SalI-PstI* site of pBluescript KS+, and analyzed further. After digestion of the *SalI-PstI* fragment with *HincII*, an active 3.2-kb fragment was further subcloned into the *EcoRV* site of pBluescript KS+. Deletion clones were generated in both directions with exonuclease III and mung bean nuclease (14) and were assayed with the complementation assay, revealing an active 1.4-kb subclone (pOpiap). Subclones were sequenced in both directions by the dideoxy chain termination method and were analyzed with the Intelligenetics and Genetics Computer Group packages (8).

**Complementation assay and isolation of recombinant viruses.** DNA (1 to 2 µg each) of vAcAnh and test DNA (i.e., OpMNPV DNA, cosmid DNA, or plasmid subclones) were cotransfected into SF-21 cells (6). Three to 4 days later, the plates were visually screened by light microscopy. Plates with cells containing occlusion bodies were scored as positive. Recombinant viruses were isolated by plaque purification of budded viruses released from three independent cotransfections of vAcAnh DNA with either the subclone containing the 3.2-kb fragment described above or pOpiap. The recombinants were further purified by two rounds of plaque purification in SF-21 cells before characterization.

**Metabolic labeling of infected cells.** Protein pulse-labeling of infected cells was carried out as previously described (4, 27) with minor modifications. Briefly, either SF-21 or TN-368 cells (10⁶) were infected at a multiplicity of infection of 20 PFU per cell (as assayed in TN-368 cells) or mock infected. After 1 h, the inoculum was replaced with TC-100 (time zero). Two hours before each time point, the cells were resuspended in medium, centrifuged at 1,000 × g for 5 min, and resuspended in TC-100 medium lacking supplements, methionine, and cysteine and placed at 27°C. One hour before each time point, the cells were centrifuged again, and the medium was replaced with 0.5 ml of methionine-deficient medium containing 25 µCi of Tran35S-label (>1,000 Ci/mmol [ICN]). At the time point, the cells were centrifuged, the radioactive medium was removed, and the cell pellets were washed, lysed, and stored at −80°C (27). These lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (20) and autoradiography (27).

**Nucleotide sequence accession number.** The nucleotide sequence presented in this report has been submitted to GenBank and was assigned the number L224564.
RESULTS

Mapping and sequencing of an OpMNPV gene complementing an AcMNPV p35 mutant, vAcAnh. Cotransfection of vAcAnh and OpMNPV genomic DNA resulted in polyhedral occlusion body formation. Control experiments with either viral DNA transfected individually did not produce polyhedra (data not shown). Thus, a gene or genes in the OpMNPV genome could functionally substitute for p35. To identify the complementing gene, overlapping cosmid DNAs encompassing the entire OpMNPV genome (21) were individually cotransfected along with vAcAnh DNA (Fig. 1A). Only cosmid 13 displayed activity in our assay. Two adjacent cosmid clones, 1 and 54, completely overlapped clone 13 but were negative in the assay, suggesting that the expression of the gene responsible for rescue required sequences spanning the HindIII site separating clones 1 and 54.

To further define the gene responsible for rescue, subclones of cosmid 13 were tested for complementation. A 3.2-kb HindII subclone displayed apoptosis-inhibiting activity, and selected deletions of this clone were tested in the complementation assay (see Materials and Methods). One subclone, pOpIap, contained an active 1.4-kb insert, which was sequenced (Fig. 1B).

Computer analysis of the sequence revealed an open reading frame (ORF) capable of encoding a 30-kDa polypeptide (Fig. 1B) sharing 58% amino acid identity with Cp-IAP. Cotransfection assays with several other subclones indicated that this ORF was essential to the rescuing activity of pOpIap (data not shown). Like Cp-IAP, the OpMNPV IAP (Op-IAP) has a C3HC4 finger motif at its carboxy terminus.

To determine the orientation and location of Op-iap with respect to the restriction map, Southern blots of HindIII-digested OpMNPV DNA (Fig. 2A) were hybridized to a 0.9-kb TaqI probe encompassing the entire iap ORF (Fig. 2B). This TaqI fragment hybridized to the OpMNPV HindIII-K fragment (Fig. 2, lane 14) and to cosmid clones 1 and 13 (Fig. 2, lanes 11 and 13), but it did not hybridize to cosmid 54 (lane 12). A HindIII site is located approximately 50 nucleotides upstream of the putative translational start site of the 30-kDa ORF and is close to the upstream TaqI site. The inserts in cosmids 1 and 54 are separated in the OpMNPV genome by a HindIII site (21). Collectively, the data indicate that Op-iap is located at the left end of HindIII-K and would be expected to be transcribed in the leftward (counterclockwise) direction with respect to the OpMNPV map (Fig. 1A). Because the HindIII site is located between the translational start site of the iap ORF and two putative transcriptional regulatory signals, a late consensus TAAG sequence at -112 bp and a putative early TATA box at -64 bp from the ATG, it is likely that the lack of activity of cosmid 1, which contains the intact iap ORF, is due to the separation of the iap ORF from these 5’ regulatory sequences. We confirmed this by testing a subclone of Op-iap truncated at the HindIII site in the complementation assay; the subclone did not result in formation of polyhedra. The region 3’ of the ORF contains two AATAAA sequences which may serve as polyadenylation signals for iap transcripts.

Characterization of vAcAnh-OpMNPV iap recombinant virus DNA. Cotransfection of vAcAnh DNA with clones containing Op-iap resulted in the presence of viruses with an occlusion-positive phenotype in the extracellular media. Six independent recombinants were characterized: three were isolated from vAcAnh cotransfection with pOpIap, and the other three were isolated with the plasmid containing the 3.2-kb iap-containing insert. To show that each of the recombinants contained an intact iap ORF, genomic DNA from these recombinants was digested with TaqI, Southern blotted, and hybridized to the 0.9-kb TaqI fragment comprising the entire Op-iap ORF (Fig. 2). While viral DNAs isolated from wt AcMNPV and vAcAnh did not hybridize to the probe (Fig. 2B, lanes 1 and 2), all six recombinant DNAs contained the 0.9-kb
were studied were fragments some bands, however, the inserts more wt AcMNPV, vAcAnh, vASB6-1, a recombinant rescued with Cp-iap), vOpIAPR-10, a recombinant rescued with Op-iap. At selected times (in hours, as shown above the lanes), cells were pulse-labeled and then lysed. Samples were subjected to SDS-PAGE followed by autoradiography. Mock-infected cells (lane M) were treated in parallel as a control. The positions of the molecular mass markers are shown on the left.

FIG. 4. Kinetics of protein synthesis in cells infected with iap-containing recombinants. SF-21 cells (A) or TN-368 cells (B) were infected with wt AcMNPV, vAcAnh, vASB6-1 (a recombinant rescued with Cp-iap), or vOpIAPR-11 (a recombinant rescued with Op-iap). At selected times (in hours, as shown above the lanes), cells were pulse-labeled and then lysed. Samples were subjected to SDS-PAGE followed by autoradiography. Mock-infected cells (lane M) were treated in parallel as a control. The positions of the molecular mass markers are shown on the left.

fragment (Fig. 2B, lanes 3 to 8), showing that this region remained intact in the recombinants. It is not known why lanes 3, 4, and 6 show less hybridization to the probe DNA; it is likely that these viruses are genetically unstable (see below).

The sites of Op-iap integration into the vAcAnh genome were studied by digesting DNA of the three pOpiaq-derived recombinants with selected restriction endonucleases (Fig. 3A), which were Southern blotted and hybridized to the 0.9-kb TaqI fragment (Fig. 3B). In two recombinants (vOpIAPR-9 and vOpIAPR-10), a complex but reproducible pattern of bands, some of which were submolar, suggested that the recombinants had more than one copy of iap. In vOpIAPR-10 (lane 3), the intensities of both the HindIII-I and PstI-E fragments were decreased, indicating the presence of one or more inserts in the region between 33 and 38 map units; however, the submolar nature of the bands suggested genomic instability and/or persisting impurity of the virus despite three plaque purifications. In vOpIAPR-9 (lane 2), the probe hybridized weakly to new submolar bands in the PstI and HindIII digests and to a large fragment in the XhoI pattern, possibly XhoI-C, -D, or -E. Recombinant vOpIAPR-11 DNA (lane 4) displayed alterations in HindIII-B1 and the XhoI-B fragments, indicating a single iap insertion between 68 and 75 AcMNPV map units.

Kinetics of protein synthesis in cells infected with p35 mutant Op-iap+ and p35 mutant Cp-iap+ recombinants. Protein synthesis profiles of vOpIAPR-11 and vASB6-1, a recombinant derived from cotransfections of vAcAnh and Cp-iap (6), were compared with those from the parent vAcAnh and wt AcMNPV (Fig. 4). In SF-21 cells, the rate, nature, and quantity of protein synthesized in cells infected with either recombinant were virtually indistinguishable from that observed in wt
FIG. 5. Sequence alignments of known iap homologs. (A) Alignment of the predicted amino acid sequences from the iap homologs of OpMNPV, CpGV, AcMNPV, and Chilo iridescent virus (CIV). Identical residues are enclosed in shaded boxes, and dots indicate gaps introduced to optimize the alignment. The CIV-IAP nucleotide sequence (12) was frameshifted in two places to obtain the amino acid sequence shown. The alignment was carried out with the Genetics Computer Group PILEUP computer program (8). (B) Alignment of the conserved and repeated amino acid sequences (BIR motifs) found at the N-terminal end of the ORFs. The sequences are compared both among and within the different iap homologs. Identical residues conserved in four or more of the sequences are enclosed in shaded boxes. Dots indicate gaps introduced to optimize the alignment. Numbers above the amino acid sequence designate the location of these residues within each corresponding IAP homolog. The first BIR motifs within the Op-iap, Cp-iap, and Ac-iap homologs are indicated as Op-1, Cp-1, and Ac-1, respectively; the second BIR repeats within these iap homologs are indicated as Op-2, Cp-2, Ac-2, and CIV-2, respectively.
AcMNPV infections (Fig. 4A). As expected (4, 15), vAcAnh displayed protein synthesis profiles characteristic of early viral infection through 12 h, but protein synthesis diminished as the cells completed apoptosis (4). In TN-368 cells (Fig. 4B), a cell line that does not undergo apoptosis upon infection with vAcAnh, all four viruses showed similar patterns, except that both recombinants expressed higher quantities of several low-molecular-weight proteins than were observed in the wt AcMNPV or vAcAnh protein profiles at 24 and 48 h postinfection. The identity of these peptides was not determined, but they are likely to be breakdown products of polyhedrin. The significance of this observation is not clear; the appearance and quantity of these peptides varied among experiments. The 48-h lane in the wt AcMNPV infection in TN-368 cells shown in Fig. 4B was apparently mishandled during this particular experiment; AcMNPV normally synthesizes significant quantities of polyhedrin at this time.

**DISCUSSION**

We identified a gene of OpMNPV which blocks apoptosis induced during infection of SF-21 cells with AcMNPV mutants lacking functional p35. This gene, Op-iap, is a homolog of both Cp-iap and Ae-iap. Op-iap and Ac-iap appear to be located in similar positions on the relatively colinear genomic maps of OpMNPV and AcMNPV, although the correspondence of genes flanking Op-iap has not been determined. Op-iap shares 58% amino acid identity with Cp-iap but only 28% identity with Ae-iap, which is functionally inactive in the complementation assay. All three IAP homologs possess a C3HC4 motif (Fig. 5A [specifically CX2CX1GXH2CX2, CX2, uCPXCR, where X is any amino acid]) at their carboxyl terminus. This motif is a subset of a more general C3HC4 motif (CX2CX1GXH2CX2, GXH2CX2, CX2, uCPXCR) found in approximately 30 other proteins (5), one of which is known to have DNA binding properties. Unlike most of the proteins in this group, the C3HC4 motifs of the IAPs are C terminal rather than N terminal and have a central CXH2CX2 sequence rather than CXH2CX2, C-terminal C3HC4 motifs are also found in the gene encoding peroxisome activating factor (30) and the Drosophila neuralized (nen) gene which is involved in restricting the number of neural progenitor cells during embryonic neurogenesis (28).

Two tandem repeats of the sequence GX2, YX2D3, CX2, CX2, WX2, HX2, C are present in the N-terminal and central portion of baculovirus IAPs (Fig. 5B). We have named the repeat unit a BIR (baculovirus iap repeat) motif; the spacing of cysteines and histidine within the BIR suggests the possibility of metal ion coordination and nucleic acid binding. Additional sequence identities can be found if Ac-iap, which is inactive in the assay, is excluded from the comparison. The Chilo iridescent virus homolog, CIV-iap (12), is not complete but appears to have at least one BIR motif as well as the C3HC4 motif. The activity of this gene in our assay has not been tested.

Consistent with the apparent nonhomologous integration of Cp-iap into vAcAnh (6), Op-iap appeared to integrate at multiple sites in the genome of AcMNPV. The sequence identity between Ac-iap and the other baculovirus iaps may not be enough to favor recombination at this site. Alternatively, the Ac-iap iap homolog may have an additional function. Since OpMNPV appears to lack a p35 homolog, it may rely solely on Op-iap to block apoptosis. Further genetic analysis of baculovirus iaps and p35 is likely to provide insight into the molecular mechanism(s) of apoptosis.

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**ADDITION IN PROOF**

The amino acid sequence in Fig. 1B is incorrect in three locations: residue 28 should be W instead of Y, residue 50 should be D instead of N, and residue 120 should be E instead of Q.

**REFERENCES**


