Suppression of Apoptosis in Insect Cells Stably Transfected with Baculovirus *p35*: Dominant Interference by N-Terminal Sequences *p35*^{1–76}

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Expression of *p35* from the DNA genome of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) suppresses virus-induced apoptosis and promotes virus replication in *Spodoptera frugiperda* (SF21) cells. To examine the molecular mechanism by which *p35* prevents apoptosis in insects, SF21 cells were stably transfected with *p35*. Neomycin-resistant cell lines that synthesized protein P35 were identified. Stable transfection with *p35* protected SF21 cells from apoptosis induced by actinomycin D concentrations that caused apoptotic death of untransfected cells. Cellular expression of *p35* also blocked apoptosis induced by infection with *p35* null mutants and restored mutant replication to levels comparable to those of wild-type virus. In contrast, stable expression of the mammalian death suppressor *bcl-2* failed to block actinomycin D- or AcMNPV-induced apoptosis. Thus, *p35* was sufficient to prevent apoptosis, whereas *bcl-2* was not, suggesting that the activities of the two nonhomologous death regulators are functionally distinct. Stable expression of the truncation mutant *p35*^{1–76}, containing the N terminus of *p35*, failed to block apoptosis. However, *p35*^{1–76} interfered with *p35* antiapoptotic activity, since stably transfected cells underwent apoptosis upon infection with wild-type AcMNPV. Despite normal levels of viral *p35* transcription, P35 levels were selectively reduced during infection. Thus, *p35*^{1–76} acted as a dominant inhibitor by directly or indirectly affecting the synthesis or stability of viral P35. These results suggested that the N terminus of P35 constitutes a functional domain which is required to interact with other proteins, possibly host invertebrate death regulators or P35 itself.

Apoptosis is an active death process that plays a significant role in viral pathogenesis. Cell destruction by apoptosis involves a highly regulated, multistep suicide program that often culminates in the degradation of chromosomal DNA into oligonucleosome-sized fragments, plasma membrane blebbing, and eventual cytolysis (for recent reviews, see references 39 and 47). Both DNA and RNA viruses have the capacity to induce apoptotic death of vertebrate and invertebrate cells (7, 21, 26, 29, 33, 38, 44). Since apoptosis and the resulting loss of host cell integrity are correlated with decreased virus replication (5, 8, 18, 21, 38), this host response may represent an antiviral defense mechanism (reviewed in references 47 and 49). In support of the hypothesis that viruses have evolved to counter such a host defense, diverse viral proteins with apoptotic suppressor activity have been identified (3, 5, 7, 9, 12, 15, 38). As yet, the molecular mechanisms by which viruses induce and subsequently suppress apoptosis are not fully understood.

The baculovirus *p35* gene is a *trans*-dominant suppressor of programmed cell death (7, 18, 37, 43). Encoded by *Autographa californica* nuclear polyhedrosis virus (AcMNPV) and *Bombyx mori* nuclear polyhedrosis virus (7, 11, 27), the 35-kilodalton product (P35) of *p35* exhibits no obvious sequence similarity to known death suppressor proteins. Synthesized both early and late during infection, P35 accumulates in the cytosol of AcMNPV-infected cells (19). Upon infection of cultured *Spodoptera frugiperda* (SF21) cells, AcMNPV mutants lacking *p35*

cause intracellular DNA degradation, premature cytolysis, and a dramatic reduction in yields of infectious virus (8, 18), including budded virus (BV) and occluded virus (for a recent review of baculoviruses, see reference 35). In contrast, *p35* null mutants replicate normally in *Trichoplusia ni* (TN368) cells which do not undergo apoptosis upon infection (7, 8, 18). By directly or indirectly facilitating virus replication, *p35* provides a selective advantage to AcMNPV in apoptosis-sensitive cells (31) and affects host range (8).

When overexpressed in vertebrate cells, the mammalian proto-oncogene *bcl-2* prevents apoptosis that is induced by virus infection and thus promotes cell survival (21, 33, 38). Since *bcl-2* also blocks programmed cell death induced by other diverse stimuli, this regulator functions at a central step in the cell death pathway (reviewed in reference 39). Although the mechanism by which the membrane-associated protein BCL2 functions is unknown, current evidence suggests that its death repressor activity is mediated in part through interaction with other death regulators, including the BCL2-related protein Bax (50). When independently expressed in mammalian neural cells or transgenic nematodes (*Caenorhabditis elegans*), *bcl-2* and *p35* prevent programmed cell death induced by the same signals (37, 43, 48, 51). Although P35 and BCL2 act in the same highly conserved death pathway, their lack of homology and different subcellular distribution suggest that they function by distinct mechanisms.

To examine the mechanism of apoptotic suppression by *p35* in insects, we generated neomycin-resistant SF21 cell lines that were stably transfected with AcMNPV *p35* or mammalian *bcl-2*. This approach enabled us to compare the functions of the two death regulators in the absence or presence of other baculovirus proteins. Unlike the case for *bcl-2*, stable expression of *p35* conferred resistance to apoptosis induced by the

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transcriptional inhibitor actinomycin D, indicating that *p35* was sufficient to block apoptosis in SF21 cells. In addition to preventing virus-induced apoptosis, cellular expression of *p35* also restored replication of *p35* null mutants and thus is the first example of phenotypic complementation of a baculovirus mutation by stable introduction of the viral gene into the host cell. We also report that cells stably transfected with N-terminal *p35* sequences (*p35*¹⁻⁷⁶) underwent AcMNPV-induced apoptosis despite normal *p35* transcription early in infection. Thus, cellular expression of *p35*¹⁻⁷⁶ inhibited the antiapoptotic activity of viral *p35*. The identification of this dominant negative mutant suggests that apoptotic suppression by *p35* requires that P35 interact with other host proteins and that the N terminus of P35 may be important for mediating such interactions.

MATERIALS AND METHODS

Cells and viruses. *S. frugiperda* IPLB-SF21 (46) and *T. ni* TN368 (20) cell lines were propagated in TC100 growth medium (GIBCO Laboratories) supplemented with 2.6 mg of tryptose broth per ml and 10% heat-inactivated fetal bovine serum (HyClone Laboratories). Viruses wild-type AcMNPV (L-1 strain) (30), wt/lacZ, vΔ35K, vΔ35K/lacZ, vΔ35K/lacZ/35K⁺ (18), v(Δ3'-162/Δ5'-154)35K-CAT (10), and AcNPV-BCL2 (1) were described previously. Infection of cell monolayers commenced at time zero upon addition of extracellular BV at the indicated multiplicity of infection (MOI). Following 1 h of gentle rocking at room temperature, the virus was removed and replaced with supplemented TC100. All cells were incubated at 27°C. Virus titers were obtained by plaque assay using TN368 cells. *lacZ*-containing viruses produced blue plaques that were visualized by including 100 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml of agarose overlay.

Construction of plasmids. The 1,051-bp *Bgl*II-*Sst*I fragment containing the *p35* open reading frame (ORF) and polyadenylation signal from plasmid pPRM-35K-ORF (19) was inserted at the corresponding sites located downstream of the *ie-1* promoter in plasmid pIE1_{Bgl} (36). The 495-bp *hr5*-containing *Bam*HI fragment from p_{hr5}^B (40) was then inserted at the *Bam*HI site of the resulting plasmid to generate pIE1^{hr+}-*p35* and pIE1^{hr-}-*p35* (Fig. 1) in which the AcMNPV *hr5* enhancer was placed in either orientation (genome sense [+]) or opposite [-] upstream of the *ie-1* promoter. pIE1^{hr+}-*p35*^{fs} (Fig. 1) was generated by limited *Bcl*II digestion of pIE1^{hr+}-*p35*, end repair with Klenow fragment, and intramolecular ligation; the resulting 4-bp insertion created a +1 frameshift at codon 76 and fused 16 out-of-frame codons to the *p35* ORF. pIE1ΔXS^{hr}-*p35* was generated by deleting the *Xho*I-*Sma*I vector fragment upstream from *hr5* in pIE1^{hr+}-*p35*, followed by end repair and intramolecular ligation. pIE1^{hr+}-*p35*⁷⁶ (Fig. 1) was generated by inserting the amber stop linker CTAGCTAGCTAG into pIE1ΔXS^{hr}-*p35* at the *Bcl*II site (end repaired with Klenow fragment) which terminated the *p35* ORF two codons after codon 76. pIE1^{hr+}-*p35*^{ΔHSp} is identical to pIE1^{hr+}-*p35*^{fs} except that the aforementioned stop linker replaced the deleted sequences between the *Hind*III and *Spe*I sites of *p35* (amino acid residues 132 to 287) and the parental vector was pIE1ΔXS^{hr}-*p35*, not pIE1^{hr+}-*p35*.

To facilitate the insertion of genes under control of the *ie-1/hr5* promoter, plasmid pIE1^{hr}/PA was constructed by eliminating an upstream *Spe*I site of pIE1ΔXS^{hr}-*p35* and replacing *p35*, extending from its 5' noncoding leader to the *Spe*I site with a polylinker containing unique *Bgl*II, *Pst*I, *Hind*III, and *Sal*I sites. Plasmids pIE1^{hr}-*bcl2*^{CL} and pIE1^{hr}-

FIG. 1. Genetic organization of *p35* and *bcl-2* plasmids used to transfect SF21 cells. *p35* and *bcl-2* were placed under control of the AcMNPV *ie-1* promoter (prm), which included sequences from nucleotides -561 to +76 relative to the +1 RNA start site (→). The AcMNPV *hr5* enhancer (positive or negative orientation) was inserted upstream from the *ie-1* promoter. Each ORF (open arrow with the translational start site indicated (▼)) is followed by a polyadenylation signal (PA). Plasmids pIE1^{hr+}-*p35* and pIE1^{hr-}-*bcl2* contain the full-length ORFs of *p35* and *bcl-2*, respectively. pIE1^{hr+}-*p35*^{fs} contains a +1 frameshift at the *Bcl*II site which truncates the *p35* ORF at codon 76 and adds 16 out-of-frame amino acids (shaded); the translational stop site is indicated (●). pIE1^{hr+}-*p35*⁷⁶ contains a stop linker inserted at the *Bcl*II site that truncates the ORF at codon 76 and fuses two noncoded amino acids to the C terminus. Plasmid pIE1-neo/PA contains *neo* under control of the *ie-1* promoter sequences from -161 to +11. Restriction site abbreviations: B, *Bam*HI; Bc, *Bcl*II; Bg, *Bgl*II; C, *Cla*I; H, *Hind*III; Hc, *Hinc*II; M, *Mlu*I; P, *Pst*I; Sp, *Spe*I; Ss, *Sst*I.

bcl2^{CR} were generated by inserting the 0.87-kb *Xba*I-*Sal*I fragment from pbluebcl2 (6) or the 1.0-kb *Xba*I-*Sal*I fragment from pbcl-2 into the *Bgl*II and *Sal*I sites, respectively, of pIE1^{hr}/PA; the *Xba*I and *Bgl*II overhangs were repaired prior to ligation. Plasmid pbcl-2 contains the 0.95-kb *Eco*RI fragment of the *bcl-2* cDNA pB4 (45) inserted into the *Eco*RI site of pBluescript (Stratagene).

Plasmid pIE1-neo/PA was generated by inserting the *neo*-containing 1,014-bp *Bgl*II-*Sma*I fragment from pSV2Neo (42) into the *Sma*I site of pIE1_{prm}/BS; the *Bgl*II overhang was repaired with Klenow fragment. pIE1_{prm}/BS contains the *ie-1* promoter, extending from nucleotides -161 to +1 relative to the +1 RNA start, within pBluescript. A polyadenylation signal contained on a 148-bp *Sau*3A-*Hind*III fragment (derived from 85.1 map units of the AcMNPV genome [10]) was subsequently inserted downstream from the *neo* ORF.

Isolation of stably transfected cell lines. SF21 cells were stably transfected and selected by G418 resistance essentially as described by Jarvis et al. (25). Briefly, 2 μg of a *p35*- or *bcl-2*-encoding plasmid and 1 μg of pIE1-neo/PA were mixed with 30 μl of Lipofectin (Bethesda Research Laboratories) and added dropwise to 10⁶ SF21 cells in a 60-mm-diameter plate. Transfections with *bcl-2* plasmids included 2 μg of carrier salmon sperm DNA. After 6 h, the cells were washed and overlaid with supplemented TC100. The cells were gently scraped into the growth medium 18 h later and diluted sixfold into 100-mm-diameter plates or twofold into 60-mm-diameter

plates. The medium was replaced 48 h after transfection with supplemented TC100 containing 1 mg of Geneticin (G418 sulfate; GIBCO-BRL) per ml. Cells were maintained in G418-containing medium for 2 weeks and then in supplemented TC100 without G418 for approximately 1 week, at which time colonies were visible. Pooled cell lines (designated $p35^{\text{pool}}$ and $p35^{\text{fs/pool}}$) were obtained by scraping the cells from the 60-mm-diameter plates and transferring them into a tissue culture flask. Cloned cell lines were isolated from the 100-mm-diameter plates by using cloning cylinders.

β -Galactosidase assays. Infected cells were harvested, washed with ice-cold phosphate-buffered saline (140 mM NaCl, 40 mM KCl, 50 mM Na_2HPO_4 , 2 mM KH_2PO_4 [pH 6.2]), and suspended in 0.25 M Tris (pH 8.0). Cells were subjected to three freeze-thaw cycles, and clarified lysates were assayed for β -galactosidase activity by using the substrate *p*-nitrophenyl- β -D-galactopyranoside as described previously (18).

DNA fragmentation assays. Low-molecular-weight DNA was extracted from SF21 cells as described previously (19, 23). Briefly, cells were scraped gently and collected by low-speed centrifugation. Apoptotic vesicles were collected from the medium supernatant by centrifugation at $14,000 \times g$ for 15 min, combined with the intact cells, and suspended in lysis buffer (10 mM Tris [pH 7.5], 10 mM EDTA, 0.2% Triton X-100). DNA was isolated from clarified lysates by high-salt precipitation (100 mM NaCl, 64% ethanol) and subjected to electrophoresis on 2% agarose-Tris-borate-EDTA (TBE) gels in the presence of ethidium bromide.

Immunoblot analysis. Total cell lysates were prepared by suspending cells in equal volumes of phosphate-buffered saline and lysis buffer (2% sodium dodecyl sulfate [SDS], 5% β -mercaptoethanol) and then boiling them for 5 min. Lysates were subjected to electrophoresis on SDS-12.5% polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked for 1 h at room temperature with 3% bovine serum albumin (BSA) or 5% dried milk, both in TBST (10 mM Tris [pH 7.4], 150 mM NaCl, 0.05% Tween 20). To detect P35, membranes were incubated for 1 h with rabbit antiserum α -P35NF (19) diluted 1:10,000 in TBST plus 0.3% BSA, followed by goat anti-rabbit immunoglobulin G (IgG) conjugated to alkaline phosphatase (Jackson ImmunoResearch) diluted 1:10,000 in the same. To detect gp64, murine tissue culture supernatant containing monoclonal antibody AcV5 (22) diluted 1:100 and goat anti-mouse IgG conjugated to alkaline phosphatase (Jackson ImmunoResearch) diluted 1:10,000 were used as primary and secondary antibodies, respectively. To detect BCL2, anti-BCL2 rabbit polyclonal serum (14) diluted 1:1,000 in TBST plus 5% dried milk and goat anti-rabbit IgG conjugated to alkaline phosphatase (Jackson ImmunoResearch) diluted 1:10,000 in the same were used. All membranes were washed 1 h after addition of the secondary antibody and incubated with a substrate solution containing 0.33 mg of nitroblue tetrazolium chloride and 0.17 mg of BCIP (5-bromo-4-chloro-3'-indolylphosphate *p*-toluidine salt) per ml until color development.

Primer extension of RNA. Total intracellular RNA was isolated by using guanidine isothiocyanate-cesium chloride and quantitated by primer extension assays using reverse transcriptase as described previously (40). An excess of 5'-end-labeled oligonucleotide primers complementary to *p35* (+15 to +53 relative to the ATG initiation codon), *ie-1* (+99 to +130), and the chloramphenicol acetyltransferase (CAT) gene (+53 to +90) were used. After denaturation, the extension products were subjected to electrophoresis on 6% polyacrylamide-TBE gels. Dideoxy-chain termination sequencing ladders were gen-

erated by using [^{35}S]dATP (Amersham) and a CAT-specific primer (34) annealed to a plasmid containing the *p35* promoter fused to the CAT gene.

RESULTS

Constitutive expression of *p35* in stably transfected cells. To generate SF21 cell lines that were stably transfected with *p35*, we constructed plasmids in which *p35* or mutants thereof were placed under control of the AcMNPV *ie-1* promoter and the *hr5* enhancer (Fig. 1). The *ie-1* promoter is active in uninfected *S. frugiperda* cells and is further stimulated by *cis* linkage to the *hr5* enhancer (13, 25, 36, 40). As a nonfunctional control, a frameshift was introduced at codon 76 of the *p35* ORF, resulting in premature termination of P35 (Fig. 1). When assayed in a recombinant virus, the *p35* frameshift mutation ($p35^{\text{fs}}$) is phenotypically indistinguishable from a *p35* null mutant (18). G418-resistant cell lines were generated by cotransfection of either the *p35*-containing plasmid $\text{pIE}^{\text{hr+}}\text{-p35}$ or the mutant plasmid $\text{pIE}^{\text{hr+}}\text{-p35}^{\text{fs}}$ and plasmid pIE1-neo/PA , in which *neo* was placed under the control of a truncated *ie-1* promoter (Fig. 1). Cloned and pooled G418-resistant cell lines were propagated in the absence of G418. The growth properties and morphology of stably transfected cells did not differ from those of untransfected SF21 cells. No G418-resistant cells were recovered when pIE1-neo/PA was omitted from the transfections.

To measure the levels of P35 synthesized in stably transfected cells, whole cell lysates were subjected to immunoblot analysis using P35-specific antiserum. Wild-type P35 was detected in six of seven cloned cell lines, including $p35^{\text{H1}}$, $p35^{\text{H3}}$, and $p35^{\text{H4}}$ (Fig. 2A, lanes 3 to 5). The cell line $p35^{\text{pool}}$, derived from a pool of *p35* stably transfected cells, also synthesized P35 (lane 6). P35 levels were approximately 25 to 200% of that produced by SF21 cells infected with wild-type AcMNPV (lane 2). In contrast, no P35 was detected in the cloned cell line $\text{neo}^{\text{p35-}}$ (lane 7). Southern blot and PCR analyses of genomic DNA confirmed the presence of *p35* fused to the *ie-1* promoter in $p35^{\text{H1}}$ and $p35^{\text{pool}}$ cells but not $\text{neo}^{\text{p35-}}$ cells, whereas *neo* was detected in all three cell lines (data not shown). No P35-related proteins were detected in $p35^{\text{fs/pool}}$ cells (Fig. 2A, lane 8) despite the presence of $p35^{\text{fs}}$ as detected by Southern blot and PCR analyses of genomic DNA from these cells (data not shown). The failure to detect the truncated protein derived from $p35^{\text{fs}}$ -transfected cells may have been due to inefficient recognition by our P35 antiserum that was generated against P35 amino acid residues 43 through 132 (19).

***p35* increases resistance to actinomycin D-induced apoptosis in stably transfected cells.** When synthesized during AcMNPV infection, P35 blocks apoptosis that is induced by treatment of SF21 cells with the transcriptional inhibitor actinomycin D (9). Thus, to assess the ability of cellularly expressed *p35* to block apoptosis, we first measured the sensitivity of stably transfected cells to actinomycin D-induced apoptosis. Apoptotic degradation of DNA into oligonucleosome-sized fragments was most pronounced in cell lines that lacked *p35*, including SF21, $p35^{\text{fs/pool}}$, and $\text{neo}^{\text{p35-}}$ cells (Fig. 3). In these cells, DNA fragmentation was induced by 75 ng of actinomycin D per ml of growth medium and was extensive at 100 ng/ml. In contrast, *p35*-expressing cell lines $p35^{\text{H1}}$ and $p35^{\text{pool}}$ were resistant to DNA fragmentation induced by the same concentrations of actinomycin D; no fragmentation was detected with 75 ng of actinomycin D per ml, and only minor degradation was detected with 100 ng/ml. There was a direct correlation between the level of apoptotic blebbing and the extent of DNA fragmentation (data not shown). Taken to-

FIG. 2. Immunoblot analysis of stably transfected SF21 cells. Total protein lysates from the indicated cell lines stably transfected with plasmids containing *p35* (A) or *bcl-2* (B) were subjected to SDS-polyacrylamide gel electrophoresis and immunoblot analysis using P35 and BCL2 antisera, respectively. Lysates from mock-infected (mi) or virus-infected (inf) SF21 cells 48 h after infection are shown. Cells were infected with wild-type AcMNPV (A, lane 2) or recombinant AcMNPV-BCL2 (B, lane 2). Each lane in panel A contains total protein from 0.8×10^5 cells except lane 8, which contains twofold more protein. Each lane in panel B contains total protein from 2×10^5 cells. The positions of molecular weight standards (sizes in kilodaltons) are indicated at the left.

gether, these results indicated that *p35* expression in stably transfected cells increased cellular resistance to actinomycin D-induced apoptosis and therefore is sufficient to block apoptosis in SF21 cells.

Constitutive expression of *p35* blocks virus-induced apoptosis and restores replication of a *p35* null mutant. AcMNPV mutants lacking *p35* replicate poorly in SF21 cells and cause

FIG. 3. Resistance of *p35*-transfected SF21 cells to actinomycin D-induced DNA fragmentation. The indicated cell lines (3×10^6 cells per plate) were incubated at 27°C in growth medium containing from 0 to 100 ng of actinomycin D per ml. After 12 h, low-molecular-weight DNA was isolated from the apoptotic bodies and remaining intact cells and then subjected to electrophoresis on a 2% agarose-TBE gel containing ethidium bromide. Molecular weight (MW) markers are indicated (sizes in base pairs) at the left.

intracellular DNA fragmentation and premature cytolysis (7, 18). To determine whether *p35* expression in stably transfected cells blocked virus-induced apoptosis and restored mutant replication, we measured BV production and very late gene expression in cells infected with vΔ35K/lacZ; this *p35* deletion mutant contains the *lacZ* gene under control of the polyhedrin promoter as a reporter of very late gene expression (18). The yield of vΔ35K/lacZ BV from the *p35*-expressing cell lines p35^{H1}, p35^{H3}, p35^{H4}, and p35^{pool} was on average 12-fold higher than that from cells lacking *p35* (SF21 and neo^{p35-}) or cells stably transfected with the frameshift mutant (p35^{fs/pool}) (Fig. 4A). β-Galactosidase levels were also 110- to 150-fold higher in p35^{H1}, p35^{H3}, and p35^{H4} cells than in cells lacking functional *p35* (Fig. 4A). Thus, cellular expression of *p35* was sufficient to restore very late gene expression and BV production of the *p35* null mutant. Although p35^{pool} cells produced higher levels of vΔ35K/lacZ BV, β-galactosidase levels were lower than in the cloned cell lines. This difference may be due to disparate levels of P35 synthesis in individual cells of pooled lines which are genetically heterogeneous compared with cloned lines. Consistent with this conclusion was the observation that vΔ35K/lacZ caused apoptotic cytolysis in a small fraction of p35^{pool} cells but not in cloned cells expressing *p35* (data not shown).

Constitutive expression of *p35* also blocked virus-induced degradation of intracellular DNA. Infection of p35^{H1} cells with vΔ35K/lacZ caused little or no DNA fragmentation (Fig. 5, lane 13). In contrast, DNA fragmentation was extensive in vΔ35K/lacZ-infected SF21 cells and neo^C cells (lanes 12 and 14); the cloned cell line neo^C was obtained by stable transfection with *neo* alone. No oligonucleosome-like DNA fragmentation was detected in SF21, p35^{H1}, and neo^C cells upon mock infection (lanes 2 to 4) or infection with recombinant virus wt/lacZ, which contains a functional copy of *p35* (lanes 7 to 9). The lack of virus-induced DNA fragmentation and the restoration of near-wild-type levels of *p35* mutant replication indicated that stable expression of *p35* from the host cell was as effective in suppressing apoptosis as that from the AcMNPV genome during infection.

Transfected cell lines were infected with recombinant wt/lacZ to determine if constitutive *p35* expression affected wild-type AcMNPV replication. Although all lines tested produced BV, yields were approximately twofold lower in *p35*-expressing cells than in SF21 or neo^{p35-} cells (Fig. 4B). Similarly, β-galactosidase levels from the polyhedrin promoter of wt/lacZ were two- to threefold lower in p35^{H1}, p35^{H3}, and p35^{H4} cells than in SF21, neo^{p35-}, or p35^{pool} cells. These results indicated that stably transfected cells retained the ability to support virus replication and that cellular expression of full-length *p35* had only a minimal effect on wild-type AcMNPV replication.

Inhibition of viral *p35* activity in cells stably transfected with *p35*¹⁻⁷⁶. p35^{fs/pool} cells that were transfected with the N-terminal frameshift mutation p35^{fs} exhibited apoptotic cytolysis when infected with wt/lacZ (data not shown). This finding suggested that cellular expression of N-terminal *p35* sequences inhibited AcMNPV-expressed *p35*. To examine this possibility, SF21 cells were stably transfected with p35¹⁻⁷⁶. Plasmid pIE1^{hr+}-p35⁷⁶ was constructed by introducing consecutive termination codons into the *p35* ORF at codon 76 (Fig. 1). G418-resistant cells stably transfected with pIE1^{hr+}-p35⁷⁶ transcribed p35¹⁻⁷⁶ RNA from the *ie-1* promoter (see below). Since pooled and cloned cells were phenotypically indistinguishable, the cloned cell line p35^{76D} was chosen for further analysis.

Infection of p35^{76D} cells with wild-type AcMNPV caused extensive apoptotic blebbing and cytolysis (Fig. 6A). In con-

FIG. 4. Effect of cellular *p35* expression on BV production and polyhedrin-driven late gene expression. The indicated cell lines were infected with vΔ35K/lacZ (A) or wt/lacZ (B) at an MOI of 5 PFU per cell. The growth medium and cells were collected 48 h after infection. The yield of extracellular BV was determined by plaque assay on TN368 cells; intracellular β-galactosidase activity was measured spectrophotometrically. Infections were performed in triplicate, and the error bars indicate standard deviation.

trast, untransfected SF21 and p35^{H1} cells remained intact and accumulated normal levels of occluded virus upon infection (Fig. 6C and D). Apoptotic DNA fragmentation in p35^{76D} cells was extensive when infected either with *p35*-containing wt/lacZ or *p35* deletion mutant vΔ35K/lacZ (Fig. 5, lanes 10 and 15). DNA degradation was not detected in mock-infected p35^{76D} cells (lane 5), indicating that *p35*¹⁻⁷⁶ expression per se does not induce apoptosis. Wild-type AcMNPV also caused apoptosis of SF21 cells stably transfected with plasmid pIE1^{hr+}-p35^ΔHSp (data not shown). Since this plasmid lacks the 3' half of *p35*

(from *Hind*III to *Spe*I sites; Fig. 1), the sensitivity of these cells to virus-induced apoptosis was due to *p35* N-terminal sequences. Taken together, these results indicated that cellular expression of *p35*¹⁻⁷⁶ was sufficient to block the antiapoptotic activity of virus-expressed *p35*, suggesting that N-terminal P35 sequences can act as a *trans*-dominant inhibitor of P35.

***bcl-2* expression is not sufficient to block apoptosis in SF21 cells.** Despite their lack of sequence similarity, both *p35* and the mammalian proto-oncogene *bcl-2* block apoptotic death in vertebrates and invertebrates (37, 39, 43). To compare the mechanisms of action of these genes, SF21 cells were transfected with plasmids containing *bcl-2* under control of the *ie-1/hr5* promoter (Fig. 1); two independently isolated *bcl-2* cDNAs (6, 45) differing in their 5' noncoding leader regions were used. Immunoblot analysis demonstrated that cell lines stably transfected with *bcl-2* synthesized the 26-kDa BCL2 protein (Fig. 2B). Cellular BCL2 was electrophoretically indistinguishable from that produced by recombinant virus AcMNPV-BCL2 in which *bcl-2* was expressed under control of the polyhedrin promoter (lane 2). Of the G418-resistant cell lines examined, *bcl-2*^H and *bcl-2*^A (lanes 3 and 4) synthesized the highest steady-state levels of BCL2 and were therefore chosen for further analysis. BCL2 was not detected in lysates from mock-infected cells or neo^C cells (lanes 1 and 5).

To determine if *bcl-2* prevented apoptotic death of SF21 cells, *bcl-2*^H and *bcl-2*^A cells were treated with actinomycin D or infected with the apoptosis-inducing mutant vΔ35K/lacZ. The extent of actinomycin D-induced DNA fragmentation in both *bcl-2* cell lines (Fig. 7A, lanes 4 to 9) was comparable to that in neo^C cells (lanes 1 to 3) and untransfected SF21 cells (Fig. 3, lanes 2 to 5). Thus, unlike P35, BCL2 did not increase cellular resistance to actinomycin D-induced apoptosis. BCL2 also failed to suppress virus-induced apoptosis, since DNA

FIG. 5. Resistance of SF21 cells stably transfected with *p35* to AcMNPV-induced DNA fragmentation. The indicated cell lines (3×10^6 cells per plate) were mock-infected (mi) or infected with wt/lacZ or vΔ35K/lacZ at an MOI of 5. Low-molecular-weight DNA was isolated 48 h after infection and subjected to agarose gel electrophoresis as described in the legend to Fig. 3. Molecular weight (MW) markers are indicated (sizes in base pairs) at the left.

FIG. 6. AcMNPV-induced apoptosis in SF21 cells stably transfected with *p35*¹⁻⁷⁶ or *bcl-2*. The indicated cell lines were infected with wild-type AcMNPV at a low MOI (L) of 5 PFU per cell or a high MOI (H) of 30 PFU per cell and photographed 24 h later, using a phase-contrast microscope (magnification, $\times 228$).

fragmentation (Fig. 7B, lanes 5 and 8) and premature cytolysis were extensive in $\Delta 35K/lacZ$ -infected *bcl-2*^H and *bcl-2*^A cells. Likewise, BCL2 failed to stimulate BV production or very late gene expression of $\Delta 35K/lacZ$ (data not shown).

Unexpectedly, infection with *p35*-containing virus also caused DNA fragmentation in *bcl-2*^H and *bcl-2*^A cells (Fig. 7B, lanes 6 and 9). However, apoptosis was dependent on the multiplicity of input virus; apoptotic blebbing and cytolysis

FIG. 7. Intracellular DNA fragmentation in SF21 cells stably transfected with *bcl-2*. Low-molecular-weight DNA was isolated from the indicated cell lines 12 h after treatment with 0 to 100 ng of actinomycin D per ml of growth medium (A) or 24 h after infection with $\Delta 35K$, which lacks (–) *p35*, or $\Delta 35K/lacZ/35K$ ⁺, which contains (+) *p35* (MOI of 5) (B). DNA from mock-infected (mi) cells was included. The DNA from 10^6 cells (A) or 3×10^6 cells (B) was subjected to agarose gel electrophoresis as described in the legend to Fig. 3.

FIG. 8. Levels of virus-synthesized P35 and gp64 in cells stably transfected with $p35^{1-76}$ or $bcl-2$. Total protein lysates were prepared from cells at the indicated times after infection with the $p35$ -containing virus wt/lacZ (MOI of 5) and subjected to polyacrylamide gel electrophoresis and immunoblot analysis using antiserum specific for P35 (A) or glycoprotein gp64 of AcMNPV (B). Each lane contains the protein from 10^5 cells and associated apoptotic bodies from the indicated cell line. The positions of molecular weight standards (sizes in kilodaltons) are indicated at the left.

were evident in $bcl-2$ -expressing cells infected with a low MOI of 5 but not with a higher MOI of 30 (Fig. 6B). In contrast, wild-type virus-induced apoptosis of $p35^{76D}$ cells was independent of virus multiplicity, since cytolysis was comparable at high and low doses of wild-type AcMNPV (Fig. 6A).

Apoptosis in cells transfected with $p35^{1-76}$ and $bcl-2$ correlates with loss of viral P35. The apparent inhibition of $p35$ function in $p35^{76D}$ and $bcl-2^A$ cells after infection with wild-type virus suggested that viral $p35$ expression was repressed or the antiapoptotic activity of newly synthesized P35 was blocked. To distinguish these possibilities, we used immunoblot analysis to measure the intracellular levels of P35 during infection with the $p35$ -containing virus wt/lacZ (Fig. 8A). As expected from previous studies (19), P35 accumulated in SF21 cells from 12 to 48 h after infection (lanes 1 to 4). In contrast, P35 was not detected at any time after infection of $p35^{76D}$ cells (lanes 5 to 8). The lack of P35 in $p35^{76D}$ cells was not due to general inhibition of viral protein synthesis, since accumulation of AcMNPV glycoprotein gp64 in these cells was temporally and quantitatively comparable to that in infected SF21 cells (Fig. 8B; compare lanes 1 to 4 and lanes 5 to 8). Intracellular levels of P35 were also reduced in $bcl-2^A$ cells (Fig. 8A, lanes 9 to 12); at an MOI of 5, low levels of P35 were present at 12 and 24 h but disappeared later. The temporal accumulation of gp64 in $bcl-2^A$ cells was unaffected (Fig. 8B, lanes 9 to 12). Thus, the propensity of $p35^{76D}$ and $bcl-2^A$ cells to undergo AcMNPV-induced apoptosis was associated with a specific reduction in intracellular levels of P35.

Early viral transcription of $p35$ is unaffected in apoptotic $p35^{76D}$ cells. To determine if the lack of P35 was due to a reduction in $p35$ transcription from the AcMNPV genome, the steady-state level of viral $p35$ RNA was examined during infection of $p35^{76D}$ cells. RNA levels were compared by primer extension assays of total RNA isolated from cultures infected with an AcMNPV recombinant that contains $p35$ at its native position and the CAT gene inserted at the polyhedrin locus under control of the $p35$ promoter (10). Primers specific for the RNAs of $p35$, CAT, and the AcMNPV $ie-1$ and $ie-0$ genes were used (Fig. 9). The extension products generated from early (4 h) RNA indicated that $p35$ was transcribed in infected

$p35^{76D}$ cells (lane 9) and that the level of native $p35$ RNA was similar to that in infected SF21 cells (lane 8). The levels of early $ie-1$, $ie-0$, and $p35$ promoter-directed CAT RNAs in both cell lines were also comparable. Thus, the $p35$ promoter was fully active in directing early transcription of $p35$ and the heterologous CAT gene. Using mixed primers, the only RNA detected in mock-infected cells was specific to $p35^{1-76}$ transcribed in $p35^{76D}$ cells (lane 6). Upon infection, the steady-state level of this RNA (lane 9; see $ie-p35$ arrow) was significantly higher than that of native $p35$.

By 24 h, $p35$ promoter-directed RNAs switched to the late $p35$ initiation site as expected (10, 34). Compared with untransfected SF21 cells, the level of late $p35$ RNA was reduced three- to fourfold in $p35^{76D}$ cells (Fig. 9, lanes 11 and 12). However, it is unlikely that this small decrease caused apoptosis, since fivefold reductions in P35 levels have no effect on apoptotic suppression (19). Since the levels of late CAT and $ie-1$ RNAs were similar in $p35^{76D}$ and SF21 cells, the decrease in native $p35$ RNA levels was attributed to an instability of this RNA and not to reduced transcription from the $p35$ promoter. Such instability of late $p35$ RNA may be due indirectly to apoptosis, which was extensive in $p35^{76D}$ cells at this time. Consistent with this possibility was the presence of higher levels of late $p35$ RNAs in nonapoptotic $bcl-2^A$ cells (lane 13) infected at a high MOI. The $bcl-2^A$ cells also produced normal levels of $p35$, CAT, $ie-1$, and $ie-0$ RNAs early and late in infection (lanes 10, 13, 16, and 19). Taken together, the data indicate that the lack of viral P35 accumulation was not due to selective repression of $p35$ transcription, suggesting that $p35^{1-76}$ affected the synthesis or stability of viral P35.

DISCUSSION

$p35$ functions as a general regulator of programmed cell death. By generating stably transfected SF21 cell lines, we have demonstrated for the first time that constitutive expression of baculovirus $p35$ protects insect (lepidopteran) cells from apoptosis that is induced by either infection with AcMNPV or exposure to the transcriptional inhibitor actinomycin D. The acquired resistance to actinomycin D-induced apoptotic death also establishes that $p35$ is sufficient to suppress apoptosis in SF21 cells. Although it is unknown whether actinomycin D and AcMNPV cause apoptosis by the same mechanism, our data provide the strongest evidence yet that $p35$ acts directly within a cellular pathway to prevent virus-induced apoptosis rather than activating other virus-encoded antiapoptotic genes. The collective findings that stable expression of $p35$ blocks programmed cell death in cells from diverse organisms, including insects, nematodes (43), and mammals (37), indicates that $p35$ is a general regulator of cell death in a highly conserved pathway. Moreover, the analogous death suppressor activity of mammalian $bcl-2$ in these same organisms (1, 28, 48) suggests that $bcl-2$ and $p35$ function in the same death pathway, but most likely at different points.

Complementation of AcMNPV $p35$ mutants by cellular expression of $p35$. Although BV production and late gene expression of $p35$ null mutants are impaired in SF21 cells (8, 18), stable expression of $p35$ in transfected cells restored mutant replication to near wild-type levels (Fig. 4). This confirms that $p35$ is a *trans*-dominant effector of baculovirus replication (7, 18). Stable expression of $p35$ from the host cell was as effective in preventing virus-induced apoptosis and promoting virus replication as $p35$ expressed from the AcMNPV genome (Fig. 4 and 5). Since synthesis of cellular proteins under control of the $ie-1$ promoter in stably transfected cells is strongly inhibited late after AcMNPV infection (24), our

FIG. 9. Quantitation of AcMNPV RNAs in cells stably transfected with *p35*¹⁻⁷⁶ and *bcl-2*. The indicated 5'-end-labeled primers were annealed to total RNA isolated from SF21, *p35*^{76D}, and *bcl-2*^A cells 4 and 24 h after infection with the CAT-containing AcMNPV recombinant v(Δ 3'-162/ Δ 5'-154)35K-CAT (MOI of 10) and then extended with reverse transcriptase. The primers were annealed simultaneously (mixed) or independently (*p35*, CAT, or *ie-1*) to RNA samples. The extension products generated from equivalent amounts of RNA (17 μ g) were subjected to polyacrylamide gel electrophoresis and autoradiography. The position of the products derived from primers specific for *p35*, *ie-1/ie-0*, and CAT RNAs are indicated at the right. The products derived from RNA initiated from the early (E) or late (L) *p35* promoter motifs are indicated. No products were detected with RNA isolated from mock-infected (mi) cells except that derived from the *p35* primer (see *ie-p35* arrow) annealed to *p35*¹⁻⁷⁶ RNA from *p35*^{76D} cells (lane 6). A dideoxy-chain termination ladder (lanes 1 to 4) was generated by using a CAT-specific primer and a plasmid containing the *p35* promoter fused to the CAT gene present in recombinant virus v(Δ 3'-162/ Δ 5'-154)35K-CAT.

findings indicate that P35 synthesized prior to or immediately after infection is sufficient to prevent virus-induced apoptosis. This is consistent with the conclusion that early synthesis of P35 is required for efficient virus replication (19).

Cellular expression of full-length *p35* under control of the *ie-1/hr5* promoter had little or no effect on wild-type AcMNPV replication; apoptosis was repressed, and BV yields were only slightly reduced (Fig. 4B). Moreover, 3 to 5 days after AcMNPV infection, cells stably transfected with *p35* lost viability in a manner that was morphologically and temporally comparable to that of untransfected cells (4). Thus, cellular expression of *p35* did not extend the life span of virus-infected cells. In contrast, other studies have shown that stable expression of *bcl-2* renders permissive murine cells susceptible to persistent infection by Sindbis virus and extends the life of infected cells (33). Whether *p35* expression enables persistent AcMNPV infection of SF21 cells remains to be determined.

trans-dominant interference of *p35* antiapoptotic activity. Stable transfection with *p35*¹⁻⁷⁶, containing the first 76 codons of *p35*, did not protect cells from virus-induced or actinomycin D-induced apoptosis. However, cellular expression of *p35*¹⁻⁷⁶ interfered with virus-expressed *p35*, since infection with *p35*-

containing viruses caused apoptosis in these stably transfected cells (Fig. 5 and 6A). Dominant interference of *p35* by *p35*¹⁻⁷⁶ was investigated by measuring the level of viral *p35* expression in *p35*¹⁻⁷⁶-expressing cells. Despite normal accumulation of the abundant virus glycoprotein gp64, P35 was not detected in cloned *p35*^{76D} cells upon infection with a *p35*-containing virus (Fig. 8). Thus, the absence of antiapoptotic activity was due in part to selective loss of viral P35. Primer extension quantitation of total RNA indicated that viral transcription of *p35* was not repressed in apoptotic *p35*^{76D} cells (Fig. 9). Thus, *p35*¹⁻⁷⁶ either directly or indirectly reduced the synthesis or stability of new viral P35.

Different mechanisms could account for *p35*¹⁻⁷⁶ inhibition of P35 antiapoptotic activity. Changes in posttranscriptional processing, nuclear transport, or translation of *p35* mRNA could reduce or eliminate de novo synthesis of P35. Alternatively, the putative protein product of *p35*¹⁻⁷⁶ (*P35*¹⁻⁷⁶) might competitively block the interaction of P35 with a factor(s) that is required for antiapoptotic function and protein stability. The specific loss of viral P35, but not gp64, in *p35*^{76D} cells (Fig. 8) is consistent with a mechanism involving competition between *P35*¹⁻⁷⁶ and P35 for limiting factors important for protein

function and stability. Although we have yet to detect P35¹⁻⁷⁶ (probably because of inefficient recognition by our P35 antiserum), intracellular P35¹⁻⁵¹ was a stable protein when fused to β -galactosidase and detected by β -galactosidase antiserum (4). The higher steady-state level of p35¹⁻⁷⁶ RNA compared with viral p35 RNA (Fig. 9) also suggests that if stable, P35¹⁻⁷⁶ was more abundant than viral P35. Accumulation of P35¹⁻⁷⁶ prior to infection may also facilitate competition for limiting factors. Additional studies are required to distinguish these possibilities.

The identification of p35¹⁻⁷⁶ as a dominant negative effector suggests a model wherein suppression of apoptosis by p35 requires P35 interaction with other death regulators. Moreover, this protein interaction can be blocked by overexpression of a domain at the N terminus of P35. Candidates for such interacting factors include host proteins in the cell death pathway or P35 itself if oligomerization is required for function. It is relevant in this respect that the death repressor activity of mammalian BCL2 is regulated by direct interaction with Bax, a BCL2-related protein that forms heterodimers with BCL2 (50). The absence of viral P35 in p35¹⁻⁷⁶ cells implies that the lack of proper protein interactions contributes to P35 instability. Consistent with such instability is the finding that the N terminus of P35, including amino acid residues 1 to 76, is especially intolerant of amino acid insertions and that such mutations cause reduced P35 accumulation (2). Comparable to P35¹⁻⁷⁶-mediated destabilization of P35, it has been shown that transforming protein E6 of human papillomavirus targets the tumor suppressor protein p53 for proteolytic degradation by direct protein interaction (41). Thus, p53 is an example of an apoptotic effector that is regulated by protein-mediated alterations in the rate of protein turnover (32).

Lack of *bcl-2* antiapoptotic function in stably transfected SF21 cells. Although cells stably transfected with *bcl-2* synthesized readily detected levels of BCL2 protein (Fig. 2), such expression failed to increase resistance to apoptosis induced by actinomycin D or AcMNPV. Moreover, cellular expression of *bcl-2* did not promote replication of p35 null mutants even though BCL2 levels increased upon infection (data not shown). Thus, *bcl-2* failed to complement the p35 null mutation. It is unlikely that improper BCL2 targeting was responsible for lack of antiapoptotic activity in stably transfected cells, since BCL2 associates with intracellular membranes when overexpressed in infected *S. frugiperda* cells (1). Taken together, our results indicate that *bcl-2* is not sufficient to prevent apoptosis in SF21 cells and thus suggest that p35 does not function indirectly by stimulating the expression or activity of an insect *bcl-2* homolog. This conclusion is supported by the finding that p35 rescues the embryonic lethality of a loss-of-function mutation in *ced-9* (43), the *C. elegans* homolog to *bcl-2* (16, 17). Although p35 and *bcl-2* function in the same death pathway, current evidence suggests that they function by distinct mechanisms.

Infection of *bcl-2*-transfected cells with p35-containing AcMNPV caused conditional apoptosis (Fig. 6B and 7). However, our data indicated that the induction of apoptosis in these cells is mechanistically distinct from that of p35¹⁻⁷⁶-transfected cells. First, the apoptotic response of *bcl-2*-transfected cells was abrogated by infection with higher doses of virus (Fig. 6B), suggesting that the corresponding higher expression of viral p35 overcomes the inhibitory effect. Second, preliminary experiments indicated that replication of p35 mutants is reduced in cells transfected with *bcl-2* compared with untransfected SF21 cells (4). In contrast, mutant replication in p35¹⁻⁷⁶-transfected cells was equivalent to that in SF21 cells. Thus, expression of *bcl-2* may indirectly interfere with AcMNPV

replication but only at low multiplicities. Further studies are required to test such possibilities.

Future applications of stably transfected SF21 cells. Our studies which indicate that cellular expression of p35 restored replication of p35 null mutants suggest that the generation of stably transfected cell lines will be useful in the characterization of other baculovirus mutants defective in essential replication genes. The high-level, constitutive expression of p35 provided by the *ie-1/hr5* promoter has also provided a means to investigate the function of p35 in the absence of other virus proteins and may enable further identification and characterization of dominant negative mutants. Finally, the availability of a culture system in which death suppressor activity is provided by p35, but not *bcl-2*, should facilitate studies to distinguish the molecular mechanisms by which both genes regulate apoptosis and mediate viral pathogenesis.

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