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Homologous p35 proteins of baculoviruses show distinctive anti-apoptotic activities which correlate with the apoptosis-inducing activity of each virus

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Abstract **The anti-apoptotic activity of p35s from two baculoviruses, *Autographa californica* nucleopolyhedrovirus (AcNPV) and *Bombyx mori* NPV (BmNPV), was compared in mammalian cells. AcNPV p35 efficiently blocked apoptosis induced by caspase overexpression, but BmNPV p35 did so very poorly. Analysis of chimeric p35s and in vitro cleavage of wild type p35s suggest that the cleavage efficiency of p35 correlates with the blocking activity. Single amino acid substitutions of BmNPV p35 with those observed in AcNPV p35, however, resulted in significant loss of its anti-apoptotic activity. We speculate that sequences flanking the cleavage site have uniquely evolved during baculovirus evolution.**

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Key words: Apoptosis; Baculovirus; p35; Caspase; Evolution

1. Introduction

The molecular mechanisms of the execution phase of the apoptotic process are well conserved in various organisms and recent intensive studies have identified the central component of apoptosis, a family of cysteine proteases called caspases (see [1–3]). Caspases have been isolated from various organisms from nematodes to humans and are represented by caspase-1 (interleukin-1β converting enzyme, ICE), caspase-2 (ICH-1), and caspase-3 (CPP32) [4,5].

Vertebrate and invertebrate viruses often block apoptosis induced by viral infection in infected cells; this process is believed to be a defense mechanism by host cells attacked by viruses (see [6]). Some viral proteins have been shown to be inhibitors of mammalian caspases (see [6,7]). The caspase inhibitors include crmA of a cowpox virus [8,9] and p35 of the baculoviruses *Autographa californica* NPV (AcNPV) [10] and *Bombyx mori* NPV (BmNPV) [11].

AcNPV p35 has been used extensively in the study of apoptosis in *Caenorhabditis elegans* [12], *Drosophila melanogaster* [13], and mammals [14] because of its potent anti-apoptotic activity in these organisms. In vitro studies have shown that AcNPV p35 is a suicide substrate for caspases and that it is cleaved at a peptide bond between Asp^{87} and Gly^{88}. Cleaved products of p35 form a stable complex with caspases, which results in the loss of proteolytic activity, at least in vitro [15], followed by the termination of the apoptotic process in cells. Efficient inhibition of apoptosis by p35 indicates the existence of caspase homologs in many organisms including insects. Sf caspase-1 has recently been isolated from *S. frugiperda*, a susceptible host for AcNPV replication [16]. p35 cleavage experiments show that p35 is an efficient substrate for Sf caspase-1 in vitro [16].

There are interesting observations in terms of the pro-apoptotic activities of baculoviruses. An AcNPV p35-deletion mutant replicates very poorly and induces extensive apoptosis in *S. frugiperda* cells (SF-21) [10]. Infection of *B. mori* cells (BmN) with BmNPV p35-deletion mutant (BmNPV p35 shows 89.6% amino acid sequence identity to AcNPV p35 [11]) also induces the initial apoptotic response in BmN cells such as membrane blebbing, but BmN appear to recover afterward [11]. These observations suggest that the anti-apoptotic activity of BmNPV p35 is not necessarily as potent as AcNPV p35, because the apoptosis induced by BmNPV infection seems to be much milder than that of AcNPV. Nucleotide sequence analysis of BmNPV p35 shows that it has a potential cleavage site for caspases at Asp^{87}-Gly^{88}, corresponding to that of AcNPV p35. However, the BmNPV p35 protein has 28 amino acid substitutions including several amino acid residues very close to the putative cleavage site. Although the differences in amino acid sequence seem to affect the anti-apoptotic activity of p35, a direct comparison of the p35 activities between these viruses has not yet been made partly because of their different host range specificity [17]. We examined BmNPV p35 for its anti-apoptotic activity in mammalian cells in comparison with that of AcNPV p35. Our data show that BmNPV p35 is a less efficient inhibitor of mammalian apoptosis and also a poorer substrate for caspases when compared with AcNPV p35. The evolution of p35 is also discussed in light of the relationship between anti- and pro-apoptotic activities of baculovirus.

2. Materials and methods

2.1. Expression plasmid

The expression constructs of the caspase family members fused with *Escherichia coli lacZ* were used for the transfection of cells [18,19]. The caspase-1-lacZ and caspase-2-lacZ constructs were kindly provided by Masayuki Miura (Osaka University, Japan). All the expression constructs used in this study had the same pcDNA3 (Invitrogen, Carlsbad, CA) vector backbone.

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The last author, Professor Maeda, has passed away after the acceptance of the paper. The rest of the authors would like this paper to be a memorial to him.

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Unless otherwise stated standard techniques of molecular cloning were used as described previously [20]. The coding region of p35 of AcNPV was amplified using Vent DNA polymerase (New England Biolabs, Beverly, MA) and the following primers for the polymerase chain reaction (PCR): NTOP61, 5′-CTCGGATCCATGTGTGTA-ATTTTTCCGGTAG-3′; NBOT52, 5′-CTCCTCGAGTTATTTAA-TTGTGTTTAATATTACATT-3′. The amplified fragments were digested with *Bam*HI and *Xho*I and then cloned into pcDNA3 cleaved with *Bam*HI and *Xho*I to generate pNB131.

The expression construct pNB124, the BmNPV p35 cDNA in pcDNA3, was generated by the same strategy as described above, using NBOT53 (5′-CTCCTCGAGTTATTTAATCATGTCTAATAT-TACATT-3′) instead of NBOT52 as a primer for PCR.

The chimeric plasmid DNAs were constructed by exchanging the *Bcl*I-*Bgl*I (Ac-b1, Bm-a1) or *Bgl*I-*Aat*II fragments (Ac-b2, Bm-b2) between AcNPV and BmNPV p35.

For single residue replacements, amino acid residues Gly^{81}, Lys^{85}, and Ile^{86}, of BmNPV p35 were respectively replaced with Asp^{81}, Glu^{85}, and Met^{86} through PCR mutagenesis with the following mutagenic primers. Mut-81D, 5′-TTTGATCAACTAGAACGCGATTA-CAGCGATAAAATTGATGGATTTCAC-3′; Mut-85Q, 5′-TTTGA-TCAACTAGAACGCGAATACAGCGATCAAATTGATGGATTT-CAC-3′; Mut-86M, 5′-TTTGATCAACTAGAACGCGAATACAG-CGATAAAATGGATGGATTTCAC-3′. These primers were used with a primer which contained *Aat*II sequence, GACGTC: 5′-TAAGCTACGACGTCGTACTCGTAAAGTCC-3′. PCR was done with the BmNPV p35 gene as a template. The amplified fragments were digested with *Bcl*I and *Aat*II, and then exchanged with the corresponding *Bcl*I-*Aat*II fragment of the coding region of BmNPV p35. The mutagenized p35 was subcloned into pcDNA3 to generate pcBm-mut81, pcBm-mut85, pcBm-mut86.

2.2. Cell culture and transient cotransfection

Rat-1 fibroblast cells were maintained in culture at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum (BioWhittaker, Walkersville, MD) and 50 U/ml penicillin and 50 μg/ml streptomycin (Gibco BRL, Rockville, MD). The day before transfection, cells were seeded at a density of 1.3×10^4 cells/cm^2. Expression constructs were transferred to cells with lipofectamine reagent (Gibco BRL) according to the manufacturer's protocol. In total 2.0 μg of DNA was transfected in a 35 mm diameter culture dish: 0.5 μg of *lacZ* fusion constructs (either *casp-1-lacZ*, *casp-2-lacZ* or control *lacZ*) and 1.5 μg of the test plasmid (either p35, CD20 or pcDNA3). The expression of chimeric genes in cells was detected by staining with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) [18,19].

2.3. In vitro cleavage

For in vitro synthesis of p35 proteins, the p35 genes were recloned into pBluescript II SK(−) vector after amplification of the p35 coding

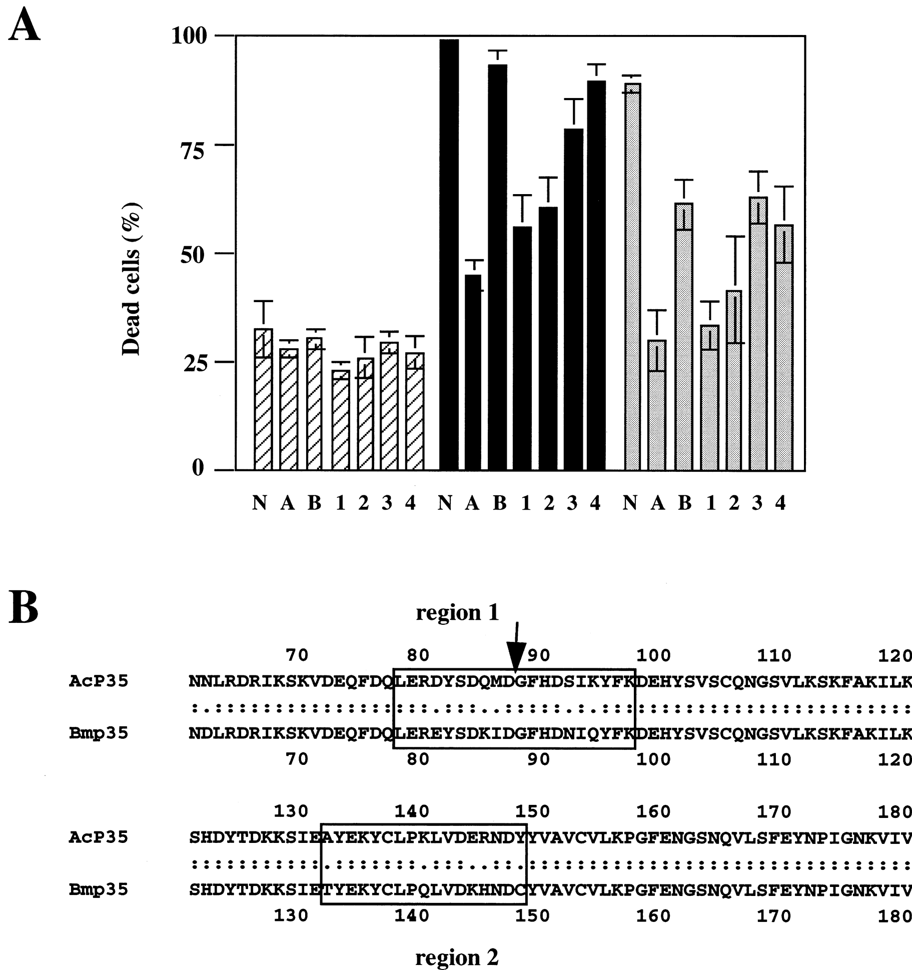

Fig. 1. A: Cotransfection assay for suppressor activity of BmNPV and AcNPV p35. Rat-1 cells were transiently transfected with the expression constructs, fixed 24 h later, and stained with X-gal solution for 3 h. The data shown are the percentage of round blue cells among the total number of blue cells counted. In each transfection, over 200 blue cells were randomly chosen and counted. The data were collected from three independent experiments. Either *lacZ* (hatched bars), *casp-1-lacZ* (black bars) or *casp-2-lacZ* (gray bars) were transfected with either CD20 (N), AcNPV p35 (A), BmNPV p35 (B), Bm-a1 (1), Ac-b2 (2), Ac-b1 (3), or Bm-a2 (4). B: Comparison of BmNPV p35 and AcNPV p35. Amino acid sequences covering region 1 and region 2 are compared between AcNPV p35 and BmNPV p35. Conserved amino acid residues are indicated with colons (:), sequence differences are marked with dots (.). The cleavage site of AcNPV p35 by caspase-1 is indicated by an arrow.

region. The conditions of the PCR were as described above, except that the upstream primer, 5′-TCGGATCCGCCACCATGTGTG-TAATTTTTCCGGTA-3′, contained a consensus Kozak sequence, GCCACCATG. ^{35}S-Labeled proteins were prepared by in vitro transcription and translation using the T7-coupled TNT reticulocyte lysate system (Promega, Madison, WI) and [^{35}S]methionine (Amersham, Buckinghamshire, UK). Labeled proteins were incubated at 30°C with *E. coli* lysates containing either caspase-1 or caspase-2 [21]. The reaction mixture contained 25 mM HEPES (pH 7.5) with 5 mM EDTA, 5 mM dithiothreitol, 10% sucrose, 10 μg/ml leupeptin and 250 μM phenylmethylsulfonyl fluoride. Amounts of proteins used for cleavage reactions were 0.5 μl of the labeled protein and 23–28 μg of proteins in bacterial lysates. Cleavage products were analyzed by 15% SDS-polyacrylamide gel electrophoresis. Detection of proteins was done by autoradiography with a BAS2000 Bioimaging analyzer (Fuji Film, Japan).

3. Results

3.1. Anti-apoptotic activity of BmNPV p35

To analyze the anti-apoptotic activity of BmNPV p35 in comparison with AcNPV p35, a transient expression system was used to overexpress p35 and caspase in mammalian cells. Either AcNPV p35 or BmNPV p35 with either caspase-1 (ICE) [18] or caspase-2 (ICH-1) [19] was examined, since transfection assay and in vitro proteolysis analysis suggest that caspase-1 and caspase-2 have different specificities for substrates [19,22], although both enzymes can cleave AcNPV p35 in vitro [15]. Expression constructs of *casp-1* and *casp-2* fused with *E. coli lacZ* have been successfully used to demonstrate that their overexpression induces apoptosis in mammalian cells [18,19]. Transfected cells can easily be detected by staining with X-Gal due to the β-galactosidase activity of the *lacZ* gene product. Dead cells are identified by their round shape and small size as compared to healthy cells, which are flat and well-attached to the culture plate (data not shown) [18].

Fig. 1A shows that the overexpression of either *casp-1* or *casp-2* induces apoptosis of Rat-1 fibroblast cells when they are introduced into cells with a negative control, CD20 cell surface marker [23]. Dead cells stained with anti-β-galactosidase antibody and Hoechst 33258 had fragmented nuclei, one of the hallmarks of cells undergoing apoptosis (data not shown). The background level of cell death induced by transfection with *lacZ* (control) was around 25%. The percentages of cell death induced by the caspases are over 90% of transfected cells (X-gal positive cells). With the cotransfection of the caspases and AcNPV p35, the cell-killing activities of both caspase-1 and caspase-2 were efficiently suppressed and the percentage of dead cells decreased to 45% (for caspase-1) and 30% (for caspase-2). These results were consistent with the in vitro data which have shown that the AcNPV p35 is an efficient suicide substrate for both caspases [15].

With the overexpression of BmNPV p35, however, the percentages of dead cells after cotransfection with the caspases were around 90% for caspase-1 and 65% for caspase-2. The percentages of cell killing in the presence of BmNPV p35 were reproducibly higher than those with AcNPV p35 (30–45%), although the percentage was lower than those of CD20 controls (nearly 100% for caspase-1 and 90% for caspase-2). Although caspase-2-induced apoptosis was significantly inhibited by BmNPV p35, these results indicate that BmNPV p35 can suppress the cell killing activity of the caspases at a lower efficiency than AcNPV p35.

3.2. Location of the region which affects the anti-apoptotic activity of p35

BmNPV p35 and AcNPV p35 are both 299 amino acid long polypeptides and possess 28 amino acid substitutions between them [11]. Among these substitutions, there are two highly variable regions, region 1 and region 2 (see Fig. 1B). Region 1, from the 81st residue through the 94th residue, possessed five amino acid substitutions (35% mismatch). This region of AcNPV p35 contains the cleavage site (Asp^{87}-Gly^{88}) for the caspases [15,24]. Region 2 (between the 132nd residue and the 148th residue) contained five amino acid substitutions (29% mismatch) (see [11]). Taking advantage of identical restriction enzyme sites flanking the region 1 DNA fragments, we made chimeric constructs: a BmNPV p35-based chimera whose Leu^{78} through Lys^{97} was replaced with its counterpart of AcNPV p35 (Bm-a1) and, vice versa, an AcNPV p35-based chimera which had the probable cleavage site of BmNPV (Ac-b1). These chimeric constructs were used for cotransfection experiments in mammalian cells to examine whether or not region 1 was involved in the difference in the anti-apoptotic activities of BmNPV and AcNPV p35s observed in the transient assay. In addition, region 2 was switched between AcNPV and BmNPV p35 proteins, generating two more chimeras (Ac-b2 and Bm-a2).

Fig. 1A shows the comparison of anti-apoptotic activities of the chimeric constructs with that of AcNPV p35 or BmNPV p35. p35 chimeras Bm-a1 and Ac-b2, both of which contained region 1 of AcNPV p35, showed suppressor activities similar to those observed in the transfection of the original AcNPV p35 (about 60% cell death in the case of caspase-1, and 30–45% cell death for caspase-2). However, p35 chimeras Ac-b1 and Bm-a2, both of which contained region 1 of BmNPV p35, showed suppressor activities as weak as the original BmNPV p35 (over 80% cell death for caspase-1, over 60% cell death for caspase-2). Sequences flanking the cleavage site (Asp^{87}-Gly^{88}) thus appear to be a principal determinant of the suppressor activity of p35s. These results indicated that (1) BmNPV p35 possessed significantly lower anti-apoptotic activity against caspases compared to AcNPV p35 and (2) the difference in anti-apoptotic activity was due to the amino acid substitutions surrounding the cleavage site of caspases located in region 1.

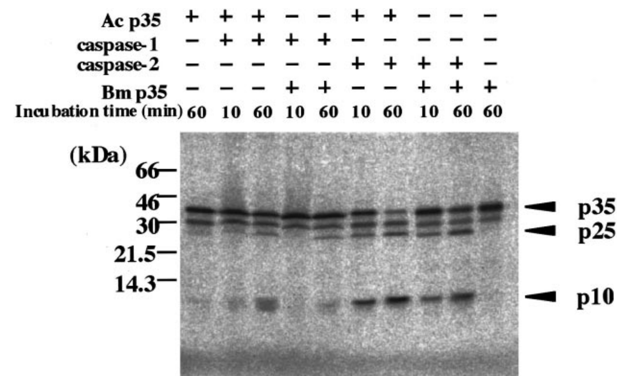

Fig. 2. In vitro cleavage of p35s by caspase-1 and caspase-2. [^{35}S]Methionine-labeled p35 was incubated with an *E. coli* lysate containing either caspase-1 or caspase-2 for 10 or 60 min at 30°C. Arrowheads indicate intact p35 protein and its cleaved products of 25 kDa and 10 kDa. Note that the in vitro translated p35 sample contains a smaller protein of about 30 kDa, which is presumably generated by non-specific transcription.

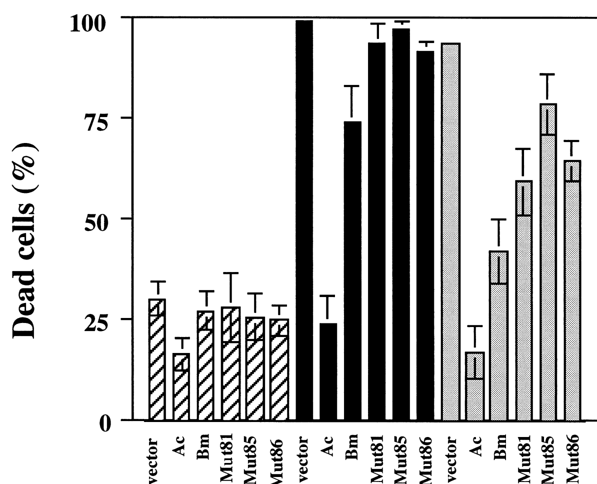

Fig. 3. Cotransfection assay for suppressor activity of mutated BmNPV p35. Rat-1 cells were transiently cotransfected with caspase-1 or caspase-2 and the mutant versions of BmNPV p35 (Mut81, Mut85, Mut86), and cell death was assayed as described in Fig. 1A. Bars represent the percentages of dead cells. pcDNA3 vector was used for the control plasmid. *lacZ* (hatched bars), *casp-1-lacZ* (black bars), *casp-2-lacZ* (gray bars).

3.3. In vitro cleavage of p35s

Xue and Horvitz [15] suggest the possibility that AcNPV p35 could inhibit caspases by its prolonged association with caspases as a poor substrate (by being cleaved inefficiently). To examine the efficiency of cleavage of AcNPV and BmNPV p35s by caspases in vitro, p35 polypeptides were labeled by in vitro translation using reticulocyte lysates and [^{35}S]methionine. Fig. 2 shows that p35 proteins of both AcNPV and BmNPV were cleaved by bacterial lysates containing either caspase-1 or caspase-2. The sizes of the proteolytic fragments are approximately 25 kDa (p25) and 10 kDa (p10), suggesting that both caspases cleave the p35 proteins at the same site in region 1. In addition to p35, there was another protein of about 30 kDa, which may have been a non-specific product, since the band intensity of the 30 kDa protein remained unchanged following the cleavage reaction (Fig. 2).

Quantitative analysis of the cleaved products showed that 32% of AcNPV p35 was cleaved by caspase-1 after incubation for 60 min, while only 12% of BmNPV p35 was cleaved under the same conditions. Caspase-2 cleaved 87% of AcNPV p35 and 61% of BmNPV p35 proteins during a 60 min incubation. Since anti-apoptotic activity of the AcNPV p35 was stronger than that of BmNPV p35 for both caspases, the results of the in vitro cleavage assay suggest that the more efficiently cleaved caspase substrate has a stronger anti-apoptotic activity.

3.4. Anti-apoptotic activity of artificially mutated p35s

To examine if a particular amino acid substitution between the two p35 proteins is mainly responsible for the difference in the anti-apoptotic activity, we introduced a series of mutations into BmNPV p35 so that each mutant sequence was more similar to the amino acid sequence of AcNPV p35 than the wild type BmNPV p35. Since four amino acid residues around the cleavage site (P4 through P1) and a following P1′ residue in substrates determine the cleavage efficiency by caspases [22,25], we focused on the sequence differences at P3 and P2 positions between BmNPV p35 and AcNPV p35 (see Fig. 1B). Mutant versions of BmNPV p35s, Mut85(K/Q) and Mut86(I/M), as well as a control mutant protein, Mut81(E/D), were subjected to the cotransfection assay with caspases as described above. We expected that Mut85(K/Q) and/or Mut86(I/M) would show higher anti-apoptotic activity than BmNPV p35 of wild type. All the mutant proteins (Mut81, Mut85, Mut86), however, were less effective in suppressing apoptosis induced by either caspase-1 or caspase-2, although expression of these mutant proteins made cells a little more resistant to caspases than cells transfected with a vector control (Fig. 3). We could not therefore attribute the difference in anti-apoptotic activity to a single amino acid substitution between these p35 proteins. This result suggests the possibility that the amino acid sequences, especially within the region 1, of either BmNPV p35 or AcNPV p35 is not tolerant to mutation, which would result in great decrease of its anti-apoptotic activity (see Section 4).

4. Discussion

p35 is found in two baculoviruses, BmNPV and AcNPV, which share high (90–95%) nucleotide sequence homology between their genomes [11]. Extensive functional analysis has only been done using AcNPV p35, which efficiently blocks caspases from various organisms. In this study, we compared BmNPV and AcNPV p35 in the same cell line with two different caspases. Both p35s had anti-apoptotic activities against both caspase-1 and caspase-2, suggesting that BmNPV p35 as well as AcNPV p35 can block different subspecies of caspases. We showed that BmNPV p35 is weaker than AcNPV p35 in terms of the suppression of either caspase-1- or caspase-2-induced apoptosis in mammalian cells. We also observed similar results for the different activities of the suppression of apoptosis using mouse myoblast cells, C2C12 (data not shown). Therefore, our system using mammalian cell lines appear to be useful for insect apoptosis studies involving caspases.

The in vitro cleavage study of p35 showed that AcNPV p35 appeared to be more efficiently cleaved by either caspase-1 or caspase-2 than BmNPV p35. This result suggests that the cleavage efficiency of p35 correlated with its anti-apoptotic activity, i.e. the better substrate for caspases is a more efficient inhibitor of apoptosis (by blocking caspase activities more efficiently). It has been found that AcNPV p35 is cleaved into two fragments, p25 and p10, by caspases and that the cleaved product(s) subsequently form a stable complex with caspases, resulting in the loss of activity of the caspases [15]. In addition to the cleavage efficiency, the stability of the p35-protease complex could also affect the inhibitory activity of p35 over caspases. It is possible that the stability of complexes formed between cleaved AcNPV p35 and the proteases is different from those formed with BmNPV p35, as these p35 proteins have 28 amino acid residue differences from each other.

Biochemical studies regarding the substrate specificity of caspases have shown that caspases can cleave oligopeptides with different sequences, although it definitely requires an Asp residue at the P1 position [5,22,25]. Four amino acid residues including the cleavage site Asp residue (P4 through P1 position) and a following P1′ residue determine the cleavage efficiency [22,25]. Among these residues, the P4 residue is

the key determinant of the distinct substrate preferences of the various caspase homologs [22,25]. The P2 and P3 residues have been thought to be less important for the substrate specificity, since X-ray crystallographic analyses of caspases have shown that there are less extensive contacts of caspases with P2 and P3 amino acids [26–28]. The sequence of P4 through P1′ in AcNPV p35 is $DQMD^{87}G^{88}$ while the corresponding sequence in BmNPV p35 is $DKID^{87}G^{88}$. It is interesting to note that the differences at these P2 (Met in AcNPV p35, Ile in BmNPV p35) and P3 (Gln and Lys) positions appear to be the major determinant for the difference in their anti-apoptotic activities which balance the apoptosis-inducing activities of respective viruses.

Our cotransfection assay has revealed that a single amino acid mutation introduced into BmNPV p35 in an artificial manner results in significant decrease of the anti-apoptotic activity. The mutant versions of p35s all have a common sequence of $DxxD^{87}G^{88}$ at the cleavage site for caspases. In vitro cleavage studies of these mutant proteins showed that they were cleaved by caspases not less efficiently than BmNPV p35 of wild type (data not shown). It is likely that such a low suppressor activity is not due to their cleavage efficiencies, but to either instability of the mutant proteins themselves in cells or reduced stability of pre- or postcleavage association of them with caspases. As for AcNPV p35, site-directed mutagenesis of the protein has revealed that p35 stability is unusually sensitive to mutagenesis, as most of p35 mutant proteins showed much decreased level of accumulation in insect cells [29].

Induction of apoptosis during baculovirus replication suggests an important role of the induction of apoptosis for successful replication of baculoviruses in insect cells. Baculoviruses can efficiently replicate in cells during the initial stages of apoptosis in which normal functions of host cells are abrogated [11]. In cells with normal metabolism, synthesis of viral DNAs and proteins should compete with those of host cells. If an NPV has a weak activity for induction of apoptosis, the anti-apoptotic proteins encoded by the NPV would be moderate accordingly. Although factors of apoptosis induced by NPVs are not well characterized, we presume that apoptosis in *B. mori* cells induced by BmNPV infection is weaker than that induced by AcNPV in *S. frugiperda* (see Section 1). The different anti-apoptotic activity observed between BmNPV p35 and AcNPV p35 is thus consistent with the idea that the anti-apoptotic activity of p35 is well balanced with the apoptosis-inducing activity of NPV.

AcNPV has the widest host range among baculoviruses (more than 30 host species) while BmNPV has a very narrow (one species) host range. Phylogenetic studies of insects and viruses have suggested that BmNPV has evolved in last 1000 years from its ancestors including AcNPV. Amino acid substitutions found in BmNPV p35 may be an important evolutionary process for adaptation to a host so that the virus can express an appropriate level of anti-apoptotic activity. A single amino acid substitution in the AcNPV p35 protein which could have occurred during evolution would result in loss of suppressor function as discussed above. It is more likely that only a set of mutations can modulate the p35 activity without severe decrease of the suppressor activity. This may explain why BmNPV p35 has acquired 28 amino acid substitutions for only 1000 years of its evolution. We have examined the suppressor activity of p35 in mammalian cells. In order to study the function of p35 during baculoviral replication in cell lines and insect larvae, we are currently constructing AcNPV and BmNPV mutants possessing chimeric p35s which will be used to determine the importance of regions 1 and 2.

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