Spodoptera frugiperda Caspase-1, a Novel Insect Death Protease That Cleaves the Nuclear Immunophilin FKBP46, Is the Target of the Baculovirus Antiapoptotic Protein p35*

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Employing the degenerate primer-dependent polymerase chain reaction approach used recently to clone human Mch2, we have identified and cloned the insect Spodoptera frugiperda target of the baculovirus antiapoptotic protein p35. This protein named Sf caspase-1 belongs to the family of caspases and is highly related to human Mch3 and CPP32 in sequence and specific activity. The proenzyme of Sf caspase-1 is 299 amino acids in length and can undergo autocatalytic processing in Escherichia coli to an active enzyme heterocomplex. Autoprocessing occurs at Asp-28, Asp-184, and Asp-195 to generate the large p19/p18 and small p12 subunits. Sf caspase-1 is able to induce apoptosis in Sf9 cells and is capable of cleaving p35 to similar sized fragments as observed with extracts from p35 null mutant baculovirus-infected Sf9 cells. Sf caspase-1 activity is potently inhibited by p35, suggesting that it is an important target of this antiapoptotic protein. Finally, the Sf nuclear immunophilin FKBP46 was identified as a death-associated substrate for Sf caspase-1.

Aspartate-specific cysteine proteases (caspases) (1) play a central and evolutionarily conserved role in transducing the apoptotic signal and final execution of apoptosis (2–6). In the central and evolutionarily conserved role in transducing the apoptotic signal and final execution of apoptosis (2–6). In the

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/Eup Data Bank with accession number(s) U15140.†

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† The abbreviation used is: ICE, interleukin 1β converting enzyme; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; Tricine, N-tris(hydroxymethyl)methylglycine.

Materials and Methods

Cloning of Sf Caspase-1 Proenzyme—An aliquot (10 μl) of Sf9 α Uni-ZAP XR cDNA library (22) containing ~10^9 plaque-forming units was denatured at 99 °C for 5 min and then subjected to two PCR amplification steps in the presence of degenerate primers encoding the pentapeptide GSWFI/GSWYI and QACRG as described (6, 23). The secondary PCR products were then used as probes to obtain full-length Sf caspase-1 clones.

Expression of Sf Procaspase-1 and p35—The open reading frame of Sf procaspase-1 was subcloned into the bacterial expression vector pET21b in-frame with a N-terminal T7-tag and a C-terminal His-tag and transformed into BL21(DE3) bacteria. The mature protease was purified on a Ni^2+—affinity resin and microsequenced by automated Edman degradation (Applied Biosystems 477A equipped with a data analyzer). ProCPP32 and proMch3 were expressed and purified in a similar fashion. p35 was expressed in the same system without T7-tag. Baculovirus encoding T7-Sf procaspase-1-His6 under the late polyhedrin promoter was generated as described previously (22). Because baculovirus replication occurs early during infection, late Sf caspase-1 synthesis has no effect on baculovirus propagation.

In Vitro Transcription/Translation and Cleavage Assays—p35 and Sf caspase-1 cDNAs were in vitro transcribed and translated in the presence of ['³⁵S]methionine as described recently (7–9). Two μl of translation mixture was incubated with pure enzymes or cell extracts in a 10-μl volume for various times at 37 °C. The products were analyzed by Tricine-SDS-PAGE.

Preparation of Sf9 Apoptotic Extracts—p35 null mutant baculovirus (vp35-3) was propagated in TN-368 insect cells which are resistant to

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Preparation of Sf9 Apoptotic Extracts—p35 null mutant baculovirus (vp35-3) was propagated in TN-368 insect cells which are resistant to
baculovirus-induced apoptosis. Sf9 cells were infected with wild type or vp35 and harvested 24 h after infection. The cells were suspended in ICE buffer (7) and lysed by a 2-3 cycle of freeze-thaw followed by homogenization. The cell lysates were centrifuged at 16,000 × g for 15 min, and the supernatants were collected and then used for the enzymatic assays.

**Western Blot Analysis of Sf FKBP46 and Sf Caspase-1**—Sf FKBP46 and the T7-tagged Sf caspase-1 were analyzed by Western blotting using a rabbit polyclonal antibody raised against Sf9 FKBP46 (22) or T7-antibody (Novagen), respectively.

**RESULTS AND DISCUSSION**

**Sf Procaspase-1 Belongs to the CED-3 Subfamily of Caspsases**—Using a PCR approach developed recently in our laboratory to identify and clone novel members of the caspase family from different species (23), a 2.4-kilobase cDNA was cloned from an Sf9 cDNA library. This cDNA encodes a 299-amino acid protein named Sf caspase-1 proenzyme (Sf procaspase-1) (Fig. 1), with a predicted molecular mass of 32 kDa. Sequence alignment of Sf procaspase-1 with all known caspases revealed that it has highest homology to the human downstream apoptotic effectors Mch3 (42% identity) (24), Mch2 (38% identity) (25), and Mch2a (38% identity) (23), followed by other family members. Additionally, Sf procaspase-1 belongs to the Ced-3 subfamily (7, 8), which includes the proenzymes of Ced-3, CPP32, Mch2, Mch3, Mch4, Mch5, and Mch6 (7, 8). Sf procaspase-1 is also structurally similar to other caspases. A mature Sf caspase-1 could be derived from the precursor proenzyme by cleavage at Asp-195 to generate against Sf FKBP46 (21) or T7-antibody (Novagen), respectively.

**Expression, Purification, and Microsequencing of Sf Caspase-1**—To determine the enzymatic activity and primary structure of Sf caspase-1 and the exact autocatalytic processing sites in its proenzyme, it was expressed in bacteria, purified, and microsequenced. This is because bacteria do not contain any caspase activity, and mutant Cys → Ala active site caspases are not autoprocessed in bacteria. Expression of Sf procaspase-1, containing N-terminal T7-tag and C-terminal His6-tag, produced soluble mature enzyme. As shown in Fig. 2A, purified mature Sf caspase-1 migrates in SDS-gels as three bands of apparent molecular masses of 19, 18, and 13 kDa. The N terminus of the 13-kDa band starts with Gly-196, indicating that processing occurred after Asp-195 of Sf procaspase-1. The calculated molecular mass of this peptide excluding the C-terminal His6-tag is ~12 kDa. The N terminus of the 19-kDa and 18-kDa bands start with Ala-29, indicating that processing occurred after Asp-28 of Sf procaspase-1. Processing at these residues removes a 4-kDa prodomain. Site-directed mutagenesis of Asp-184 and Asp-195 revealed that the difference in size between the two polypeptides is due to processing at Asp-184 in the case of the 18-kDa polypeptide and Asp-195 in the case of the 19-kDa band (data not shown).

Similar results were also obtained after incubation of 35S-labeled Sf procaspase-1 with mature recombinant Sf caspase-1 (Fig. 2B). Sf caspase-1 was able to process its proenzyme in a time-dependent fashion to generate the p19, p18, and p12 species. Based on these data, Sf procaspase-1 can autoprocess after Asp-28, Asp-184, and Asp-195 to generate the two subunits (p19/p18 and p12) and is indicated by horizontal arrows.

FIG. 1. Predicted amino acid sequence of Sf procaspase-1. The active site pentapeptide QACQG is boxed. Cleavage sites after Asp-28, Asp-184, and Asp-195 are indicated by vertical arrows. The two subunits, and Asp-15 and Asp-28 which would remove the precursor proenzyme by cleavage at Asp-195 to generate and Mch6 (7, 8). Sf procaspase-1 is also structurally similar to CPP32 (38% identity) (25), and Mch2 (38% identity) (24), caspases revealed that it has highest homology to the human caspase-1) (Fig. 1), with a predicted molecular mass of 32 kDa. The N-terminal sequence of p19 and p18 is ALGSNS, and the T7-tagged Sf caspase-1 were analyzed by Western blotting using a rabbit polyclonal antibody raised against Sf9 FKBP46 (22) or T7-antibody (Novagen), respectively.

**p35 Is a Substrate and a Potent Inhibitor of Sf Caspase-1**—Sf insect cells respond to baculovirus infection by activating a novel caspase to initiate apoptosis (21). This process is counteracted by expression of the baculovirus-encoded protein p35 which is a substrate for, and a potent inhibitor of, members of the caspase family (19, 20).

To determine whether p35 is a substrate for Sf caspase-1, purified recombinant Sf caspase-1 was incubated with 35S-labeled p35 for various times (Fig. 3A). This generated the expected 10- and 25-kDa fragments, indicative of cleavage at Asp-87 (Fig. 3A). Similar results were also obtained after incubation of 35S-labeled Sf procaspase-1 with mature recombinant Sf caspase-1 enzyme. (2)S Methionine-labeled Sf procaspase-1 was incubated with pure mature Sf caspase-1 (1 ng/µl) for the indicated times (min) at 37°C. The reaction products (2 µl/lane) were then analyzed by Tricine-SDS-PAGE and autoradiography. Full-length Sf procaspase-1 and the cleavage products are indicated.

**Western Blot Analysis of Sf FKBP46 and Sf Caspase-1**—Similar results were also obtained after incubation of 35S-labeled Sf procaspase-1 with mature recombinant Sf caspase-1 (Fig. 2A). Sf caspase-1 was able to process its proenzyme in a
Fig. 3. Cleavage of p35 by Sf caspase-1, CPP32, and Mch3. 

[35S]Methionine-labeled p35 was incubated with pure mature Sf caspase-1 (1 ng/μl) for the indicated times (min) at 37 °C (A) or buffer (lane 1), apoptotic extract from vp35Δ baculovirus-infected Sf9 cells (1 μg/μl) (lane 2), Sf caspase-1 (5 ng/μl) (lane 3), recombinant hCPP32 (3 ng/μl) (lane 4), or recombinant hMch3 (2 ng/μl) (lane 5) for 1 h at 37 °C (B). The reaction products were then analyzed by Tricine-SDS-PAGE and autoradiography. The full-length p35 and the 25-kDa and 10-kDa cleavage products are indicated. The 25-kDa product is less radioactive and autoradiography. The full-length p35 and the 25-kDa and 10-kDa cleavage products are indicated. The 25-kDa product is less radioactive and autoradiography. The full-length p35 and the 25-kDa and 10-kDa cleavage products are indicated. The 25-kDa product is less radioactive and autoradiography. The full-length p35 and the 25-kDa and 10-kDa cleavage products are indicated.

[Image: Fig. 3]

Fig. 4. Inhibition of Sf caspase-1 by recombinant p35. Equivalent protease activity of recombinant Sf caspase-1 (30 pg/μl) or apoptotic lysate from vp35Δ baculovirus-infected Sf9 cells (1 μg/μl) was incubated with the indicated concentrations of recombinant p35 and then assayed for protease activity using the tetrapeptide substrate DEVD-AMC (50 μM). The enzymatic activities were expressed as a percentage of protease activity in the absence of p35.

[Image: Fig. 4]

Fig. 5. Cleavage of the Sf nuclear immunophilin FKBP46 by Sf caspase-1 and during vp35Δ baculovirus-induced apoptosis. Purified recombinant Sf FKBP46 (20 ng/μl) (lanes 1 and 2) or nuclei from wild type virus-infected Sf9 cells (−0.5 μg/μl) (24 h postinfection, lanes 4 and 5) were incubated with (+) or without (−) recombinant Sf caspase-1 (5 ng/μl) for 2 h at 37 °C and then analyzed by SDS-PAGE and immunoblotting with an FKBP46-specific polyclonal antibody. Nuclei from vp35Δ baculovirus-infected Sf9 cells (Δp35, lane 3) were isolated and directly analyzed by SDS-PAGE and immunoblotting. Full-length and 25-kDa cleavage products of FKBP46 are indicated.

[Image: Fig. 5]

Fig. 6. Overexpression of Sf procaspase-1 in Sf9 cells results in its activation, cleavage of FKBP46, and DNA cleavage. Sf9 cells were infected with wild type baculovirus or recombinant baculovirus encoding T7-tagged Sf procaspase-1. At the indicated times (A, B, and C), the cells were harvested, fractionated into cytosolic and nuclear fractions, and 20 μg of each cytosolic fraction was analyzed by Western blotting with a T7-antibody (A) or by enzymatic assay with the DEVD-AMC (50 μM) peptide substrate (B). The nuclear fractions (20 μg/lane) were analyzed by Western blotting with the FKBP46 antibody (C). D, induction of DNA cleavage by overexpressed Sf caspase-1. Total DNA was isolated from Sf9 cells infected with wild type baculovirus (negative control, lane 1) or recombinant baculoviruses encoding ProICEγ (positive control, lane 2) or Sf procaspase-1 (lane 3) and then analyzed by 1.8% agarose-gel electrophoresis.

[Image: Fig. 6]
Sf9 nuclear DNA binding immunophilin recently discovered in our laboratory. We demonstrated that FKBP46 is cleaved specifically during vp35 baculovirus-induced apoptosis of Sf9 cells and by the death effector component Sfcaspase-1. Because the basic apoptosis program has been highly conserved during evolution, the identification of these two components should facilitate the efforts to elucidate the molecular mechanisms and the physiological significance of apoptosis in diverse organisms ranging from insects to mammals.

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