Sindbis Virus Induces Apoptosis through a Caspase-Dependent, CrmA-Sensitive Pathway

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Sindbis virus infection of cultured cells and of neurons in mouse brains leads to programmed cell death exhibiting the classical characteristics of apoptosis. Although the mechanism by which Sindbis virus activates the cell suicide program is not known, we demonstrate here that Sindbis virus activates caspases, a family of death-inducing proteases, resulting in cleavage of several cellular substrates. To study the role of caspases in virus-induced apoptosis, we determined the effects of specific caspase inhibitors on Sindbis virus-induced cell death. CrmA (a serpin from cowpox virus) and zVAD-FMK (N-benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone) inhibited Sindbis virus-induced cell death, suggesting that cellular caspases facilitate apoptosis induced by Sindbis virus. Furthermore, CrmA significantly increased the rate of survival of infected mice. These inhibitors appear to protect cells by inhibiting the cellular death pathway rather than impairing virus replication or by inhibiting the nsP2 and capsid viral proteases. The specificity of CrmA indicates that the Sindbis virus-induced death pathway is similar to that induced by Fas or tumor necrosis factor alpha rather than being like the death pathway induced by DNA damage. Taken together, these data suggest a central role for caspases in Sindbis virus-induced apoptosis.

Sindbis virus is an alphavirus of the Togaviridae family which causes encephalitis in mice and thus serves as a model for closely related human encephalitic viruses. Infection of a variety of cultured cell types with Sindbis virus triggers programmed cell death (33). The induction of apoptosis in neurons of mouse brains and spinal cords correlates with the neurovirulence of the virus strain and with mortality in mice, suggesting that induction of apoptosis may be a primary cause of death of young mice (34). In support of this hypothesis, overexpressed inhibitors of apoptosis, such as Bcl-2 and IAP, can protect cultured cells from Sindbis virus-induced apoptosis, and Bcl-2 efficiently reduces mortality in mice (17, 31, 32). These findings also raise the possibility that endogenous inhibitors of apoptosis inhibit Sindbis virus-induced cell death, leading to a persistent virus infection (33, 61). Encephalitis and/or a fatal stress response may be a consequence of neuronal apoptosis (21, 59). Alternatively, there may be multiple pathways that work independently to cause fatal disease.

A crucial role for the caspase family of cysteine proteases in the execution phase of programmed cell death is supported by genetic (24, 52, 66), biochemical (29, 57), and physiological (25) evidence. A current model proposes a cascade of events by which caspases proteolytically activate other caspases (35, 39, 46). More recent evidence suggests that different death stimuli trigger the activation of a subset of upstream caspases that possess long prodomains at their N termini (3, 41, 62). These prodomains serve to target proteases to specific protein complexes, where the prodomains are removed by proteolysis to produce active proteases. These caspases proteolytically activate other downstream caspases (with shorter prodomains) that cleave key substrates to ultimately produce the characteristic apoptotic phenotype of cell shrinkage, membrane blebbing, chromatin condensation, oligonucleosomal DNA fragmentation, and cell death (42, 53). A growing list of proteolytic substrates of the caspases have been identified, including protein kinase C delta (18), the retinoblastoma tumor suppressor (56), fodrin (12, 38), lamins (30, 47), the nuclear immunophilin FKBP46 (1), Bcl-2 (7), and several autoantigens (5), and they all are cleaved after an aspartate residue (P1 position). The precise role of these cleavage events is not known, but they may either inactivate key cellular functions or produce cleavage products with pro-death activity. The cleavage product of Bcl-2 is potently proapoptotic (7), and cleavage of a novel protein designated DFF was recently shown to trigger DNA fragmentation during apoptosis (36). These proteolytic events also serve as biochemical markers of apoptosis. Furthermore, cell death can be inhibited with pseudosubstrate inhibitors of the caspases, such as the cowpox virus serpin CrmA (19, 48), and synthetic peptides such as zVAD-FMK (67). The key feature of these inhibitors is an aspartate at the P1 position, consistent with their specificity for caspases.

A role for caspases in viral infections is suggested by the finding that baculovirus infection activates an apoptotic cysteine protease in insect cells that is inhibited by the virus-encoded caspase inhibitor p35 (2). Similar work with mutant adenoviruses has suggested that the adenovirus protein E1A activates caspase 3 (CPP32), generating cleaved products of poly(ADP-ribose) polymerase (PARP) (4). In addition, PARP cleavage is detected during infection of mouse neuroblastoma cells with Sindbis virus (60). To further study the role of these proteases in Sindbis virus-induced programmed cell death, we confirmed that Sindbis virus activates cellular caspases and demonstrated the participation of a subset of caspases in the execution of the apoptotic process.
was generating by cloning a Bst in Dulbecco's minimal essential medium supplemented with 10% fetal bovine...metabolically labeled three washes with 13 mHanks' balanced salt solution for plaque assay quantitation on BHK cells.

Cell lines and cell viability. Low-passage-number (<15) BHK and mouse neuroblastoma cells (N18) were maintained free of Mycoplasma contamination in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, 100 μg/mL of streptomycin, 100 μM of glutamine, 100 U/mL of insulin, and 2 mM of l-glutamine.

Detection of viral proteins. BHK cells were seeded at 5 × 104 per well in six-well dishes and infected with recombinant Sindbis viruses (MOI, 10). After three washes with 1× PBS, cells were starved in 1 mL of 10% serum-supplemented methionine- and cysteine-free medium for 1 h and metabolically labeled with 35S-methionine per well (ICN Biomedicals) at a final concentration of 10 μCi/mL. Before being harvested, the cells were washed three times with ice-cold PBS and lysed on ice with 300 μL of a buffer containing 1% Nonidet P-40, 1% sodium deoxycholate, 50 mM Tris (pH 7.5), 15 mM NaCl, and 25 μg/mL of aprotinin per mL. To detect Sindbis virus structural proteins, 20 μL of lysate was resolved by SDS–15% polyacrylamide gel electrophoresis (PAGE) and visualized with 1 M salicylic acid, and by autoradiography. For immunoprecipitation of Sindbis virus nonstructural proteins, labeled cell lysates were immunoprecipitated with rabbit anti-Sindbis virus antiserum raised against nsP2 (provided by Charlie Rice) at a 1:100 dilution in a total volume of 200 μL for each 60 μL of lysate as previously described (15).

Immunostaining. Lysates from BHK and N18 cells infected at an MOI of 10 were prepared as described above, analyzed by SDS-PAGE, and immunoblotted (ECL; Amersham) with anti-CrmA or anti-Bcl-x antibodies (provided by David Rice) at a 1:100 dilution in a total volume of 200 μL. To detect Sindbis virus structural proteins, 20 μL of lysate was resolved by SDS–15% polyacrylamide gel electrophoresis (PAGE), amplified with 1 M salicylic acid, and by autoradiography. For immunoprecipitation of Sindbis virus nonstructural proteins, labeled cell lysates were immunoprecipitated with rabbit anti-Sindbis virus antiserum raised against nsP2 (provided by Charlie Rice) at a 1:100 dilution in a total volume of 200 μL for each 60 μL of lysate as previously described (15).

Protease inhibitors. BHK or N18 cells were preincubated for 2 h with 5 to 50 μM zVAD-FMK (N-benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; Enzyme Systems Products) and infected with Sindbis virus strain AR339 (MOI, 10) in the presence of zVAD-FMK. The inhibitor was replenished at 24 h postinfection. Cells were harvested at ~48 h postinfection to determine their viability. zFA-FMK, a fluoromethyl ketone which is inactive against caspases, was used as a negative control.

In vitro protease assays. A glutathione S-transferase (GST)-CrmA fusion protein expressed from a modified pGEX2T plasmid (provided by Emily Cheng) was purified from bacteria, and protein concentrations were determined by the bicinchoninic acid protein assay (Pierce). Pro-interleukin-1β (pIL-1β) (provided by Jennifer Lewis and Henry George) was in vitro translated by use of the TNT System (Promega). Inhibition of caspase 1 (IL-1β-converting enzyme) protease activity by GST-CrmA was performed as described previously (48): 50 μg of caspase 1 (provided by Susan Molineaux) was incubated for 30 min on ice with 1 or 5 μL of purified GST-CrmA (0.5 to 2.5 μg) or GST (2.3 to 11.5 μg) in 100 mM HEPES (pH 7.5)–10% sucrose–0.1% 3-[3-cholamidopropyl(dimethyl-ammonio)]-1-propanesulfonate (CHAPS)–10 mM dithiothreitol and then incubated at 37°C for 30 min with 2 μL of [35S]methionine-labeled, in vitro-translated pIL-1β in a total reaction volume of 20 μL with a final concentration of GST-CrmA or GST >1 μM. The whole reaction product was analyzed by SDS-PAGE and autoradiography.

To evaluate the effect of GST-CrmA on the Sindbis virus cytosine proteinase nsP2, unlabeled, in vitro-translated Sindbis virus polyprotein nsP132 with Gly-
fected with a recombinant virus encoding CrmA were approximately 55% viable at 48 h postinfection, while those infected with a virus in which a stop codon was inserted into the crmA open reading frame had a viability of 8% (Fig. 2A). Similar results were obtained with Bcl-xL, a Bcl-2-related protein which was previously demonstrated to protect cells from Sindbis virus-induced apoptosis (8, 33). Similar results were obtained with N18 cells (data not shown), indicating that caspase activation is a general mediator of Sindbis virus-induced cell death. Immunoblot analysis verified the occurrence of CrmA and Bcl-xL protein expression in recombinant virus-infected cells, with increasing levels of protein from 6 to 24 h postinfection (Fig. 2B). Although overexpressed CrmA is presumed to affect caspases other than caspase 1, these results suggest that CrmA-insensitive proteases may not be involved in triggering Sindbis virus-induced apoptosis (see Discussion).

The synthetic peptide inhibitor zVAD-FMK is a broad inhibitor of cysteine proteases with a specificity for Asp in the P1 position (67). BHK cells treated with zVAD-FMK were resistant to wild-type (AR339) Sindbis virus-induced cell death in a dose-dependent manner (Fig. 2C). Infected cells treated with a 50 μM concentration of a peptide inhibitor had 37% (sevenfold) higher viability than cells treated with a control compound, zFA-FMK, lacking an aspartate residue. Treatment with zVAD-FMK also protected N18 cells infected with Sindbis virus (data not shown).

Apoptosis in mouse brains is easily detected by 24 h after intracranial inoculation with Sindbis virus (34). The mortality induced by Sindbis virus infection in mice correlates with apoptotic death of virus-infected neurons (34). To determine if caspases contribute to a fatal infection in vivo, 1-day-old mice were inoculated intracranially with recombinant viruses encoding crmA. Mice were partially protected by CrmA, as indicated by an increase in the time until death and by the survival of 25% of the animals. In contrast, recombinant viruses expressing CrmA with a premature stop codon resulted in 100% mortality by day 12 of the experiment (Fig. 3A). Taken together, the antideath effects of the serpin CrmA and the peptide inhibitor zVAD-FMK suggest that caspases have an exer-
E2, and E1. To determine if CrmA or Bcl-xL altered the ac-
processed by cellular proteases to yield the glycoproteins E3,
giving rise to the transmembrane proteins, which are further
autocatalytically cleaves itself from a polyprotein precursor,
by others (15, 22).

The viral capsid protein and two cellular proteins, p95 and p52,
were coprecipitated with the nonstructural proteins, as reported
acceptor role during Sindbis virus infection both in vitro and in
vivo.

Effects of caspase inhibitors on Sindbis virus replication. To
determine if CrmA protected mice from a fatal Sindbis virus
infection by suppressing virus replication, plaque assays of
brain homogenates were performed. Although a modest re-
duction in the level of CrmA-expressing virus compared to that
of the CrmA-stop construct was detected at 1 day postinfection,
no differences in these levels were observed between days
2 and 10 postinfection, indicating that CrmA did not signifi-
cantly alter progeny virus production in mouse brains (Fig.
3B).

For a more detailed analysis, the effects of caspase inhibitors
on Sindbis virus replication were studied in the BHK cell line.
A one-step growth curve demonstrated that progeny recombi-
nant viruses encoding either CrmA or CrmA-stop were first
detectable at 4 h postinfection (Fig. 4). Therefore, CrmA did
not delay the first round of virus replication.

Cleavage of the nonstructural polyprotein precursor
nsP1234 into the individual nsP proteins, which include the
polymerase and other proteins required for plus- and minus-
strand viral RNA synthesis, is accomplished by the viral cyto-
eine protease located in nsP2. Mutations that disrupt nsP2
protease function inactivate the virus (55). Because nsP2
cleaves at sites containing a Gly at the P2 position and not at
sites with an Asp at the P1 position, CrmA (or zVAD-FMK)
would not be expected to inhibit the nsP2 protease.

To verify that CrmA did not alter the production of Sindbis virus
structural proteins, the nsP protein complexes were immuno-
precipitated with anti-nsP2 antibodies from lysates of pulse-
labeled cells. No differences in the ratios of precursor nsP123
and individual proteins nsP1, nsP2, and nsP3 were detected in
the presence of CrmA (Fig. 5A, lane A) or Bcl-xL (lane X) (detectable at 11 h postinfection). It is unlikely that either the
Capsid protease or the relevant cellular proteases are inhibited
(directly or indirectly) by the caspase inhibitor CrmA or the
antiapoptotic Bcl-xL protein, since the capsid and glycoprotein
levels were equivalent.

The effect of CrmA on progeny virus production in BHK
cells was determined by measuring the titer of virus produced
FIG. 4. One-step growth curves of with recombinant viruses with and without
CrmA show no differences. BHK cells were infected with CrmA or CrmA-stop
recombinant virus (MOI, 10), and progeny viruses produced during a 1-h period
were collected from the supernatants and titered by plaque assay. The function of
CrmA and CrmA-stop was confirmed by determining cell viability in control
wells. The results represent the means of two independent experiments plaqued
in duplicate for each time point. Bars for standard error of the mean (SEM) are
hidden by the symbols, and the dashed horizontal line marks the limit of detec-
tion.

FIG. 5. CrmA does not alter production of nonstructural and structural Sind-
bis virus proteins. (A) Sindbis virus nonstructural proteins (P123, P1, P2, and P3)
were immunoprecipitated from equal volumes of labeled lysates, prepared 1 h
after infection, with recombinant viruses encoding crmA (A), crmA-stop (AS),
bcl-xL (X), and bcl-xL-stop (XS) or from mock-infected cells (M) with rabbit
antiserum raised against nsP2. The nonstructural proteins were identified by
their molecular masses (the positions of molecular size markers [in kilodaltons]
are shown on the left), by comparison to published protein patterns (15), and by
comparison to nonstructural proteins immunoprecipitated with a mixture of
antibodies to nsP2 and nsP3 (X2) provided by M. Gorrell and D. Griffin. Proteins
were resolved by SDS–PEG PAGE and processed with salicylic acid prior to
autoradiography. The results are representative of three independent experi-
ments. (B) Sindbis virus structural proteins were analyzed by SDS–15% PAGE
and autoradiography of labeled whole-cell lysates harvested at the indicated
times (in hours) after infection of BHK cells. These results are representative of
six independent time course experiments. The arrows indicate the precursor viral
glycoproteins (pE3E2E1 and pE2), the mature glycoproteins (E1 and E2), and
the capsid protein (C). Molecular mass standards (in kilodaltons) are indicated.
determined. The cells were pretreated with 50 μM zVAD-FMK or zFA-FMK for 2 h, and the inhibitors were replenished after 24 h. Each datum point is the mean of values for three independent wells harvested on the same day. These results are consistent with the observation that similar levels of viral proteins (both structural and nonstructural) were detected in infected cells with the observation that similar levels of viral proteins (both structural and nonstructural) were detected in infected cells.

Cell viability (Fig. 2A). These results are consistent with the observation that similar levels of viral proteins (both structural and nonstructural) were detected in infected cells (Fig. 5). Likewise, treatment with zVAD-FMK had no effect on wild-type virus production in BHK cells (Fig. 6B). It has been pointed out that the caspase active site has greater re-
similarity to viral cysteine proteases than to other cellular proteases (58). Therefore, to eliminate the possibility that the proteolytic activity of viral nsP2 for its target substrate was not inhibited by CrmA in vitro.

DISCUSSION
Caspase 1 is a homolog of the Caenorhabditis elegans protease CED-3, which mediates programed cell death in nem-

CrmA does not inhibit the viral nsP2 protease activity in vitro. It has been pointed out that the caspase active site has greater resemblance to viral cysteine proteases than to other cellular proteases (58). Therefore, to eliminate the possibility that CrmA could impair the function of the viral nsP2 protease, purified CrmA protein was assessed for its ability to inhibit the nsP2 protease in an in vitro assay (14). 35S-labeled, in vitro-translated Sindbis virus precursor nsP1234 served as a substrate. This construct contains mutations of the protease cleavage sites between nsP1 and nsP2 and between nsP2 and nsP3 to prevent processing of the precursor because nsP2 alone (detached from its flanking proteins) is an inefficient protease. Incubation of the protease nsP1*2*3 with the substrate nsP1234 resulted in partial cleavage of nsP4, produc-

Unlabeled in vitro-translated nsP123 served as the source of active nsP2 enzyme. This construct contains mutations of the protease cleavage sites between nsP1 and nsP2 and between nsP2 and nsP3 to prevent processing of the precursor because nsP2 alone (detached from its flanking proteins) is an inefficient protease. Incubation of the protease nsP1*2*3 with the substrate nsP1234 resulted in partial cleavage of nsP4, produc-

during a 1-h interval at various times after infection (Fig. 6A). Although the CrmA-stop virus produced approximately two-fold higher titers than virus expressing CrmA, this small difference was deemed unlikely to account for the observed differences in cell viability (Fig. 2A). These results are consistent with the observation that similar levels of viral proteins (both structural and nonstructural) were detected in infected cells (Fig. 5). Likewise, treatment with zVAD-FMK had no effect on wild-type virus production in BHK cells (Fig. 6B).

FIG. 6. Caspase inhibitors CrmA and zVAD-FMK do not significantly inhibit Sindbis virus replication. (A) Recombinant viruses produced in BHK cells supernatants (MOI, 10) during 1-h intervals were collected, and plaque assays were performed in duplicate. Each datum point is the mean of values for three independent wells harvested on the same day, and the results are representative of seven independent experiments. (B) Production of progeny Sindbis virus in independent wells harvested on the same day, and the results are representative of three independent wells harvested on the same day. FIG. 7. CrmA inhibits caspase 1 but does not inhibit nsP2 protease activity in vitro. (A) In vitro-translated pIL-1b was digested with caspase 1 in the presence (1 or 5 μM) or absence (–) of purified CrmA protein and then analyzed by SDS–10% PAGE. Cleavage of pIL-1b to the 17.5-kDa mature form (mIL-1b) was inhibited by GST-CrmA but not by GST protein. (B) Labeled in vitro-translated nsP1234 (C481G) was digested with unlabeled in vitro-translated nsP2 protease (P12*3) in the presence (3 or 5 μM) or absence (–) of purified GST-CrmA or GST protein alone. Labeled nsP123 and molecular mass standards (in kilodaltons) serve as markers.
CrmA inhibits cell death induced by Fas, TNF, and nerve growth factor withdrawal that do not involve CrmA-resistant proteases (Fig. 8). However, consistent with our study on Sindbis virus, transfection of CrmA and zVAD-FMK treatment do not block cell death indefinitely, which has been assumed to mean that these inhibitors are unable to stave off all of the intracellular caspases. Nevertheless, this observation leaves open the possibility that caspase-independent pathways are also operational in Sindbis virus-infected cells (Fig. 8).

Although CrmA is a cysteine protease inhibitor, its specificity for caspases, as dictated by the presence of an Asp at the CrmA reactive site, strongly suggests that CrmA could not interfere with either the serine protease in the Sindbis virus capsid protein or the cysteine protease found in nsP2. This is an important issue because these proteases are essential for viral replication (55). Furthermore, a mutation in the protease domain of the nsP2 gene, resulting in a single amino acid change (Pro726Ser), produces a virus that fails to kill cells and establishes a persistent infection of BHK cells (16). However, because CrmA does not appear to have a direct or indirect effect on nsP2 or capsid, CrmA is not likely to impair cell killing by interfering with viral protease activity. Several pieces of information support this conclusion. (i) The viral protease cleavage sites do not contain an aspartate at the P1 position. The capsid protease of Sindbis virus cleaves after the sequence TEEW, which has no resemblance to caspase cleavage sites (e.g., DEVD or YVAD). Based on the sequences of 10 alphaviruses, the nsP2 protease cleaves following the consensus sequence XAG(A/G) (55), and substitution of the P1 glycine for valine at the nsP3-nsP4 cleavage site in Sindbis virus abolishes cleavage (14). (ii) Experimental results presented here demonstrate that CrmA did not cause an accumulation of viral nonstructural precursors, and the viral structural proteins accumulated normally between 0 and 11 h postinfection. (iii) Purified CrmA protein failed to interfere with cleavage of the nsP3-nsP4 site by nsP2 in vitro.

The role of CrmA in cowpox virus infections is to reduce the host immune response by blocking production of IL-1β, and recent evidence suggests that CrmA may also prevent apoptosis of cowpox virus-infected cells (48, 49). Likewise, other antiapoptotic genes encoded by large DNA viruses appear to be required for completion of the virus replication cycle. Deletion of p35 from baculovirus or deletion of E1B 19K from adenovirus severely reduces progeny virus production because the cells die prematurely from apoptosis (11, 64). Several herpesviruses encode homologs of the cellular bcl-2 gene, a potent inhibitor of a wide variety of death stimuli (9, 23, 43). In contrast to these viruses, Sindbis virus appears to thrive in apoptotic cells, presumably in part because the replication cycle of Sindbis virus is as short as 4 h (Fig. 4), allowing abundant virus production prior to cell death. In fact, overexpression of the viral Bcl-2 gene can slow the replication of influenza virus, Semliki Forest virus, and human immunodeficiency virus (45, 51, 54). Thus, some viruses may prefer to replicate in the milieu of an apoptotic cell. In contrast to these findings with Bcl-2, we detected only slight reductions in Sindbis virus replication when caspase inhibitors were present (Fig. 2 to 4 and 6). One possible explanation for this discrepancy is that caspase inhibitors function downstream of Bcl-2 (10). (The effect of Bcl-2 overexpression on viral replication in mouse brains is currently under study.) Thus, it remains possible that viral persistence in mouse brains is facilitated not only by the presence of endogenous apoptosis inhibitors but also by a reduction in virus replication.

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REFERENCES


