Inhibition of Translation and Induction of Apoptosis by Bunyaviral Nonstructural Proteins Bearing Sequence Similarity to Reaper

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Members of the California serogroup of bunyaviruses (family *Bunyaviridae*) are the leading cause of pediatric viral encephalitis in North America. Significant cell death is observed as part of the infection pathology. We now report that a Bunyaviral nonstructural protein termed NSs shows sequence similarity to Reaper, a proapoptotic protein from *Drosophila*. Although NSs proteins lack the Reaper N-terminal motif critical for IAP inhibition, they do retain other functions of Reaper that map to conserved C-terminal regions. Like Reaper, NSs proteins induce mitochondrial cytochrome c release and caspase activation in cell-free extracts and promote neuronal apoptosis and mortality in a mouse model. Independent of caspase activation, Bunyavirus NSs proteins also share with Reaper the ability to directly inhibit cellular protein translation. We have found that the shared capacity to inhibit translation and induce apoptosis resides in common sequence motifs present in both Reaper and NSs proteins. Data presented here suggest that NSs induce apoptosis through a mechanism similar to that used by Reaper, as both proteins bind to an apoptotic regulator called Scythe and can relieve Scythe inhibition of Hsp70. Thus, bunyavirus NSs proteins have multiple Reaper-like functions that likely contribute to viral pathogenesis by promoting cell death and/or inhibiting cellular translation.

INTRODUCTION

Programmed cell death, or apoptosis, is a physiological process required in metazoans for normal development and tissue homeostasis (reviewed by Bergmann *et al.,* 1998; Vaux and Korsmeyer, 1999; Bangs and White, 2000; Meier *et al.,* 2000). In response to diverse apoptotic signals, both internally and externally generated, most cells activate a specific set of proteases, the caspases, to cleave intracellular substrates essential for the orderly dismantling of the dying cell (reviewed by Zimmermann and Green, 2001; Shi, 2002).

Caspases are ubiquitously expressed, but their enzymatic activity can be regulated by a family of proteins known as Inhibitors of Apoptosis, or IAPs (reviewed by Richter and Duckett, 2000; Shi, 2002). Although IAPs can inhibit caspase activity through direct binding, it has also been reported that RING domain-containing IAP proteins can act as E3 ligases to catalyze caspase ubiquitination

and degradation (Yang *et al.,* 2000; Suzuki *et al.,* 2001). Acting in opposition to IAPs is the RHG protein family, which can bind to IAPs and disrupt the IAP-caspase interaction. These IAP inhibitors, which include the *Drosophila* proteins Reaper, Grim, Hid, Jafrac2, and Sickle and the mammalian proteins Smac/Diablo and Omi/HtrA2, share a short N-terminal motif that can physically contact IAPs and inhibit their function (Wang *et al.,* 1999; Goyal *et al.,* 2000; Christich *et al.,* 2002; Holley *et al.,* 2002; Martins *et al.,* 2002; Srinivasula *et al.,* 2002; Tenev *et al.,* 2002; Verhagen *et al.,* 2002; Verhagen and Vaux, 2002; Wing *et al.,* 2002; Yoo *et al.,* 2002).

Although the Reaper, Grim, and Hid proteins inhibit IAPs through their similar N-termini, they do not share marked homology outside of the N-terminus. In the case of Reaper, a truncated protein lacking the N-terminus is unable to bind IAPs but can still induce apoptosis, suggesting that the C-terminal 2/3 of the protein mediates IAP-independent functions (Evans *et al.,* 1997a; Vucic *et al.,* 1997; McCarthy and Dixit, 1998). One suggested function of the C-terminal 2/3 of Reaper is induction of cytochrome c-release from mitochondria. Although the involvement of mitochondria in

Drosophila apoptosis is controversial, ectopic expression of Reaper clearly induces release of mitochondrial cytochrome c in cells of vertebrate origin, leading to Apaf-1–mediated caspase 9 activation (Evans *et al.,* 1997a). Furthermore, the Trp/GH3 motif of Grim, a block of sequence conserved in Sickle and Reaper, is critical for Grim's localization to mitochondria and its capacity to induce programmed cell death in flies (Claveria *et al.,* 2002).

The ability of Reaper to induce apoptosis in intact cells of both fly and vertebrate origin has been well documented (Evans *et al.,* 1997a; Vucic *et al.,* 1997; McCarthy and Dixit, 1998). We have shown in particular that recombinant Reaper can induce apoptotic changes in a cell-free extract derived from *Xenopus* eggs. Specifically, addition of either GST-Reaper or a peptide encoding the entire 65 amino acids of Reaper can trigger both mitochondrial cytochrome c release and caspase activation (Evans *et al.,* 1997a; Holley *et al.,* 2002). Although the IAP inhibitory properties of the full-length untagged Reaper most likely contribute to apoptotic induction, in the egg extract system mitochondria are absolutely required for measurable caspase activation in response to Reaper (Evans *et al.,* 1997a). While searching for Reaper-interacting proteins that mediate the observed mitochondrial effects, we identified Scythe, a member of the Bag family of Hsc 70/Hsp 70 regulators (Thress *et al.,* 1998, 1999). As shown for Bag 1, the founding member of the family, Scythe can inhibit Hsc/Hsp 70–mediated protein folding (Thress *et al.,* 2001). However, Scythe is unique in that this inhibition can be reversed by Reaper binding. Because removal of Scythe from egg extracts or addition of the recombinant Bag domain from Scythe prevented Reaper-induced caspase activation, we speculated that regulation of protein folding by Reaper might underlie its ability to control mitochondrial cytochrome c release (Thress *et al.,* 2001).

In addition to Reaper's ability to promote mitochondrial cytochrome c release and inhibit IAP function, we and others have recently reported that Reaper can destabilize IAPs (most likely through promotion of auto-ubiquitination) and perhaps more surprisingly, can suppress general protein translation (Thress *et al.,* 2001; Hays *et al.,* 2002; Holley *et al.,* 2002; Ryoo *et al.,* 2002; Yoo *et al.,* 2002). Regions outside of the Reaper N-terminus are necessary for these effects and the C-terminal 2/3 of the protein appears to be sufficient for the translational inhibition. Although the mechanism of translational inhibition by Reaper is not yet understood, it is attractive to speculate that translational inhibition, coupled to IAP destabilization, leads to effective IAP elimination.

Given that multiple activities of Reaper reside in a region of the protein with no known homologues, we decided to search sequence databases for proteins with similarity to the C-terminal 2/3 of Reaper. In this report we show that the nonstructural (NSs) protein encoded by some members of the *Bunyaviridae* family is similar at the sequence level to *Drosophila* Reaper. The *Bunyaviridae* are a large family of mainly arthropod-transmitted viruses characterized by a tripartite, negative-sense RNA genome. Several members of the family cause human disease including febrile illness, encephalitis, and hemorrhagic fevers (Elliott, 1997). Viruses in the California serogroup of the *Orthobunyavirus* genus, the bunyaviruses in which these Reaper-homologous NSs proteins are found, are the major cause of viral pediatric encephalitis in North America (Gonzalez-Scarano *et al.,* 1992). Although the precise function of the bunyavirus NSs protein is not known, studies on the prototype Bunyamwera virus in which NSs was deleted indicate that it is important for both

viral pathogenesis and suppression of host cell protein translation (Bridgen *et al.,* 2001).

We demonstrate here that sequence similarities between Reaper and the NSs proteins extend to functional similarities. Specifically, we show that bunyaviral NSs protein can act in the absence of other viral proteins to inhibit protein synthesis. Moreover, we find that NSs, like Reaper, can induce mitochondrial cytochrome c release and caspase activation. The NSs protein, like Reaper, can reverse Scythe-mediated inhibition of Hsc 70, suggesting that Reaper and NSs share a common mechanism of apoptotic induction. Interestingly, the shared capacity of NSs proteins and Reaper to inhibit translation and induce apoptosis appear to reside in common motifs present in both proteins. These results raise the intriguing possibility that induction of apoptosis by the NSs protein contributes to the pathological cell death observed during bunyaviral encephalitis.

MATERIALS AND METHODS

Secondary Structure Prediction

Secondary structure predictions for NSs and Reaper proteins were made using the PredictProtein server (http://www.embl-heidelberg.de/predict-protein/submit_def.html), specifically, the SSpro program (Baldi *et al.,* 1999). The DAS program was used to generate the hydrophobicity profiles (Cserzo *et al.,* 1997; Server address: http://www.sbc.su.se/~miklos/DAS/main-das.html).

Extract Preparation

Preparation of crude interphase *Xenopus* egg extracts (CS) was performed as previously described (Evans *et al.,* 1997a). Crude interphase translation extracts (CSt) were prepared the same way as CS, but no cycloheximide was added.

Production of GST Recombinant Proteins

GST and GST-Reaper were prepared as previously described (Evans *et al.,* 1997b). GST-NSsCe, GST-NSsLac, and GST-NSsSa were prepared the same as GST-Reaper.

In Vitro Translation Assays

In vitro translation assays using *Xenopus* extracts were conducted by adding 1 μCi μl^{-1} of Trans label (ICN, Costa Mesa, CA), 100 μm zVAD-fmk (BioMol, Plymouth Meeting, PA) and 100 ng μl^{-1} of recombinant GST, GST-Reaper, GST-NSsCe, or GST-NSsSa to CSt extracts. Protein translation was assayed by SDS-PAGE analysis, or by TCA precipitation (80 μg of extract in 20% TCA). In vitro assays using RRL were conducted as previously described (Holley *et al.,* 2002).

Immunodepletion Assays

Affi-Prep protein A-beads (40 μl; Bio-Rad, Hercules, CA) were washed in PBS and incubated with 135 μl of preimmune or anti-scythe antisera overnight. The beads were washed twice in ELB and incubated 150 μl of CSt for 1 h at 4°C. Beads were then allowed to pellet by gravity, the supernatant was transferred to a fresh Microfuge tube, and the depletion process was repeated, using fresh beads, twice more. Depleted extracts were then assayed for their ability to translate in the presence of recombinant GST, GST-Reaper, or GST-NSsCe.

Cell Culture, Transfections, and Pulse Analysis

All cell culture reagents were obtained from Invitrogen (Carlsbad, CA) unless otherwise specified. BHR cells were maintained in G-MEM supplemented with 10% FBS, 10% tryptose phosphate broth, and 1 mg/ml geneticin. For pulse chase analysis, 200,000 cells were plated per well in six-well plates in medium lacking geneticin and transfected 24 h later using LipofectAMINE 2000 (Invitrogen), OptiMEM, and 2 μg of DNA, according to the manufacturer's instructions. Sixteen to 20 h after transfections, cells were washed once with prewarmed pulse medium (DMEM minus L-Met and L-Cys supplemented with 10% dialyzed fetal bovine serum and 1 mM sodium pyruvate) and then incubated for 15 min in pulse medium to deplete Met and Cys levels. Cells were then incubated for the times indicated in pulse medium containing 200 μCi ml^{-1} of ^{35}S-*trans* label. Radiolabeled proteins were harvested and separated by SDS-PAGE as previously described (Holley *et al.,* 2002).

DEVD and Nuclear Fragmentation Assay

Recombinant GST, GST-Reaper, GST-NSsCe, GST-NSsLac, and GST-NSsSa were added in 1:10 dilution to crude egg extracts. At the indicated times, 3 μl aliquots were withdrawn and incubated with 90 μl of assay buffer and 200 μm Ac-DEVD pNA colorimetric substrate (BioMol) as previously described (Holley *et al.*, 2002). Caspase-3 activity was monitored by the measurement of absorbance at 405 nm using a LabSystems MultiSkan MS microtiter 96-well plate reader (Helsinki, Finland). Nuclear fragmentation assay was performed as previously described (Evans *et al.*, 1997a).

Cytochrome c Release Assays

Recombinant GST, GST-Reaper, GST-NSsCe, or GST-NSsLac was added 1:10 dilution to CS extracts supplemented with 100 μM zVAD-fmk. At the indicated times, 20-μl aliquots were withdrawn and filtered through a 0.1-μm ultrafree-MC filter (Millipore, Bedford, MA). Aliquots were then analyzed for cytochrome c content by SDS-PAGE and immunoblotting as previously described (Evans *et al.*, 1997a).

Protein Refolding Assays

Protein refolding assays were performed as previously described (Freeman and Morimoto, 1996).

Scythe Binding and Release Assays

Scythe binding and release assays were performed as previously described (Thress *et al.*, 1999).

Viruses

Recombinant Sindbis virus either empty (dsTE12Q) or carrying the NSsSA or NSsCE genes under the regulation of an extra copy of the subgenomic promoter were generated as described (Levine *et al.*, 1996). Infectious viruses recovered from the supernatants of transfected BHK (baby hamster kidney) cells (American Type Culture Collection, Manassas, VA) were titered by standard plaque assay.

Mice Mortality Assays

Twelve-day-old CD-1 mice (Charles River Laboratories, Wilmington, MA) were inoculated by intracerebral injection of 5×10^3 plaque-forming units of each recombinant virus diluted in 30 μl HBSS.

TUNNEL Assays

Mice were inoculated as described above. At 2–3 d after infection brains were collected and fixed in 4% paraformaldehyde. The fixed tissue was processed and paraffin-embedded. Sections 5 μm in thickness were deparaffinized, rehydrated, and permeabilized using 0.1% Triton X-100 in 0.1% sodium citrate to perform TUNNEL assays as described by the manufacturer (Roche Diagnostics, Indianapolis, IN).

RESULTS

Sequence and Structural Similarities between California Serogroup Nonstructural NSs Protein and Drosophila Reaper

Although the extreme N-terminus of Reaper is required for IAP binding and stimulated degradation, this region of the protein is not critical for Reaper-induced mitochondrial cytochrome c release or translational inhibition (Evans *et al.*, 1997a; Holley *et al.*, 2002). Given that these activities of Reaper mapped to a region of the protein with no known orthologues, we wished to search known protein databases for proteins with sequence similarity to Reaper. We were particularly interested in identifying related proteins known to regulate protein synthesis. Accordingly, we queried public databases using a tBLASTn algorithm for proteins similar to Reaper amino acids 16–65. This search yielded a group of Non-Structural small-segment–encoded proteins (NSs) from some members of the *Orthobunyavirus* genus in the Bunyaviridae family of viruses. The *Orthobunyavirus* genus comprises 172 named viruses that are subdivided into 18 serogroups. The smallest of the three orthobunyavirus genomic RNA segments encodes two proteins, the nucleocapsid (N) protein and, in an overlapping distinct reading frame, a nonstructural protein termed NSs. We found that in the

California group, the major cause of viral pediatric encephalitis in North America (Gonzalez-Scarano *et al.*, 1992), NSs proteins are similar to Reaper at the C-terminal region in a 59-amino acid area we termed the Reaper-like region (RLR). The most similar of these to Reaper, the NSs from San Angelo virus (SA), has an RLR that is 50% similar to Reaper with 12 exact amino acid identities (25%; Figure 1A; also shown are NSs from slightly more distantly related viruses, La Crosse and California encephalitis).

The RLR overlaps the Trp/GH3 motif, a small homologous block shared by Reaper, Grim, and Sickle (Wing *et al.*, 1998, 2001, 2002; Christich *et al.*, 2002; Claveria *et al.*, 2002). This motif has been predicted to form an amphipathic α-helix (Claveria *et al.*, 2002; Wing *et al.*, 2002). Secondary structure analysis of the NSs proteins suggested that the RLR's Trp/GH3 motif also conforms to an amphipathic α-helix, with four hydrophilic amino acids in this highly hydrophobic region positioned at the same face of the helix (Figure 1B). Hydrophobicity analysis of the Trp/GH3 region corroborated that both proteins had similar hydrophobicity profiles and conserved amino acids responsible for the observed hydrophobicity patterns (Figure 1C). Collectively, these observations suggest that sequence similarities between NSs proteins and Reaper might extend to structural similarities of functional significance.

NSs Proteins Inhibit General Protein Translation

Little is known about NSs biological function, but these proteins are demonstrably important for viral pathogenesis. Moreover, a mutant Bunyamwera virus (Bunyamwera serogroup, *Orthobunyavirus* genus) deleted for its NSs (BUNdelNSs) lacks the characteristic ability to inhibit host cell protein synthesis during viral infection (Bridgen *et al.*, 2001). Although the failure of BUNdelNSs to impair translation implied that the NSs protein might be a translational regulator, the ability of NSs to inhibit translation directly has not been reported. Hence, we wished to determine whether the NSs proteins and *Drosophila* Reaper shared the ability to downregulate general protein synthesis. To test this, NSs protein from San Angelo virus (NSsSa) was produced in bacteria as a GST fusion protein. When equivalent levels of NSsSa, Reaper, or GST were added to translationally competent but transcriptionally inactive *Xenopus* eggs extracts, a profound inhibition of translation was observed in the presence of the NSs protein and *Drosophila* Reaper, but not GST alone (Figure 2, A and B). Interestingly, the dose-response curve for protein translation was very similar for *Drosophila* Reaper and viral NSs proteins (unpublished data). Additionally, to assess the translational effects of the NSs proteins in mammalian cells, we cloned the gene of NSsSa downstream of an EMCV IRES sequence, which allows protein expression even when bulk cap-dependent protein translation is attenuated (Moss *et al.*, 1990). This construct, along with a plasmid encoding GST, was transfected into BHR cells treated with the general caspase inhibitor zVAD-fmk to circumvent any translational inhibition that might result from NSs-mediated apoptotic induction (see further below). To assess de novo protein synthesis in these cells, we pulsed them briefly with [35S]methionine, prepared lysates, and isolated the newly synthesized GST protein on glutathione Sepharose beads. As shown in Figure 2C, the NSs protein was able to dampen GST synthesis markedly (note that synthesis of the endogenous protein migrating just below GST appears unaffected because most of the cells in the culture do not express the NSs/GST because of low transfection efficiency). Collectively, these observations extend
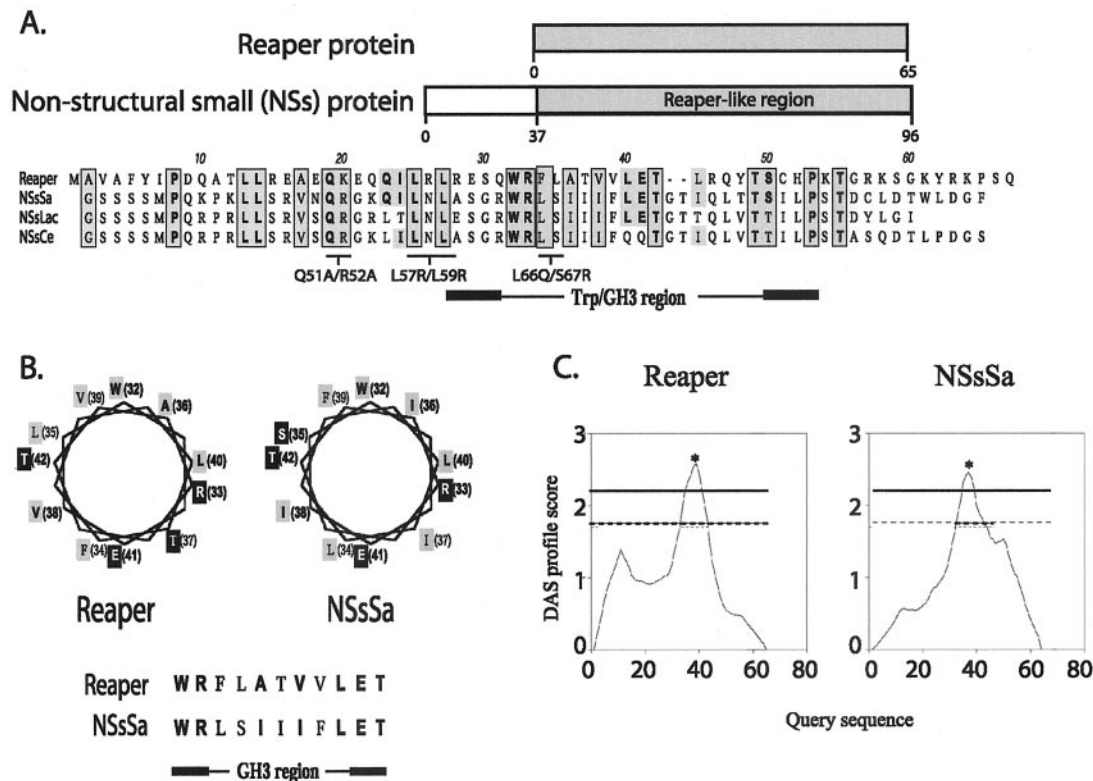
Figure 1. Sequence alignment of NSs and Reaper. (a) Public databases queried for Reaper homologues using a tBLASTn algorithm yielded a group of nonstructural proteins encoded by the small genome segment (NSs) expressed in the Bunyaviridae viral family. The Reaper-like region in the NSs protein is highlighted in the schematic diagram of the NSs. Areas of similarity between the NSs and Reaper are shaded. Amino acid identities are represented in bold. Areas of similarity and identity between the three NSs proteins and Reaper are boxed. Also indicated are areas of sequence similarity mutated and assayed for function (see Figure 3). (b) Helical wheel projection diagram of the Reaper and NSsSa GH3 region, which is predicted to conform to an amphipathic α-helix. Similar and identical residues are represented in bold. Hydrophobic residues are shaded in gray, and hydrophilic residues in black. (c) Hydrophobicity analysis of Reaper and the NSsSa RLR by DAS TM-segment prediction. Dashed horizontal line correspond to loose cutoff DAS score of 1.7, and the solid gray line corresponds to the strict cutoff DAS score of 2.2. Starred area of high hydrophobicity corresponds to amino acids 33–42 (in both Reaper and NSs RLR).

those made using BUNdelNSs virus and argue strongly that NSs proteins can suppress general protein translation.

Residues Conserved between Reaper and NSs Are Critical for Translational Inhibition

The shared ability of the NSs proteins and Reaper to inhibit protein synthesis suggested that the sequence similarities described above were functionally significant. Therefore, we speculated that a shared sequence motif might be responsible for the common ability of these proteins to inhibit translation. To address this possibility, we performed site directed mutagenesis of motifs shared by Reaper and NSs proteins. As shown in Figure 3A, NSsSa proteins altered at residues L57R/L59R or residues L66Q/S67R were still capable of inhibiting protein synthesis, suggesting that these conserved residues (equivalent to residues 25/27 and 34/35, respectively, in Reaper) were not required for the inhibitory activity. However an NSsSa mutant altered at residues 51 and 52 (Q51A/R52A) was incapable of inhibiting translation. Mutations of the corresponding residues in Reaper (residues 19–20) also abrogated its capacity to inhibit translation (unpublished data). These data suggest that there are areas of sequence similarity shared by these proteins that are critical for their capacity to inhibit protein synthesis, perhaps

responsible for mediating a common interaction with some component of the translation machinery.

NSs Proteins Can Trigger Caspase Activation in Cell-free Xenopus Egg Extracts

The ability of Reaper to promote caspase activation and cell death stems in part from its ability to inhibit IAP function. However, the Reaper 16–65 protein, lacking IAP inhibitory functions, is also able to induce apoptosis, suggesting that distinct determinants of cell killing lie within the C-terminal 2/3 of the protein (Chen *et al.*, 1996; Evans *et al.*, 1997a; Vucic *et al.*, 1997; McCarthy and Dixit, 1998; Wing *et al.*, 2001). Given the sequence homology between NSs proteins and Reaper in this region, we speculated that the viral NSs proteins could also affect apoptotic pathways. *Xenopus* egg extracts allow us to characterize the pro- or anti-apoptotic properties of proteins by assaying their ability to inhibit or accelerate caspase activation (Kluck *et al.*, 1997). As described above, we have previously reported that addition of Reaper to cell-free *Xenopus* egg extracts triggers several hallmarks of apoptosis, including caspase activation and nuclear fragmentation. To determine if the NSs proteins could modulate apoptotic caspase activation, we supplemented interphase extracts with GST fused NSsSa, NSsCe, NSsLac, and
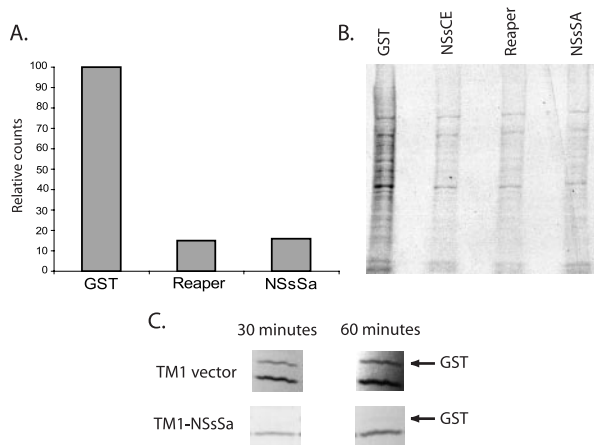
Figure 2. Repression of translation by NSs proteins. (a) Translationally competent, but transcriptionally inactive, *Xenopus* egg extracts were supplemented with 35S-Met/Cys and zVAD-fmk plus GST, GST-Reaper, or GST-NSsSa. Translation was allowed to proceed for 45 min, and products were subjected to TCA precipitations and quantified by scintillation counting. (b) Samples prepared as in panel a were resolved by SDS-PAGE. (c) BHR cells were transfected with GST and either NSs or vector control. Cells were radiolabeled in a pulse experiment, and the resulting radiolabeled GST protein was precipitated from cell lysates using GSH beads. GST expression was then analyzed by autoradiography. The synthesis of the endogenous protein that interacts with GSH beads (lower band) was also observed in mock-transfected cells (unpublished data). Its synthesis appears unaffected because most cells do not express NSs/GST because of low transfection efficiencies.

Reaper proteins. As with all of our previously reported apoptosis assays, these were performed in the presence of cycloheximide, so that the ability of these proteins to inhibit translation would not impact the assay. Surprisingly, we found that GST-Reaper and the NSs proteins (but not GST alone) were equivalently active in inducing caspase activation, as measured by cleavage of the artificial caspase 3 substrate, DEVD-pNA, and nuclear fragmentation (Figure 4, A and B). These data suggest that NSs proteins, like fly Reaper, have proapoptotic properties.

NSs Proteins Induce Mitochondrial Cytochrome c Release

Using *Xenopus* egg extracts, we have previously demonstrated that *Drosophila* Reaper can trigger mitochondrial cytochrome c release and that this release is absolutely required in this system for Reaper-induced caspase activation (Evans *et al.*, 1997a; Kluck *et al.*, 1997). To determine if the NSs proteins could also induce cytochrome c release, GST-NSsSa, GST-NSsCe, or GST-NSsLac proteins were incubated in *Xenopus* egg extracts supplemented with the caspase inhibitor zVAD-fmk. Caspase inhibitor was included in these incubations to preclude mitochondrial cytochrome c release induced *after* the activation of caspases. At various times after protein addition, aliquots of extract were withdrawn and filtered to remove mitochondria. The resulting supernatants were then immunoblotted for released cytochrome c. Like *Drosophila* Reaper, the NSs proteins accelerated cytochrome c release in a caspase-independent manner (Figure 4C). These data suggest that the observed caspase activation by the NSs proteins in egg extracts results from an acceleration of mitochondrial cytochrome c release.
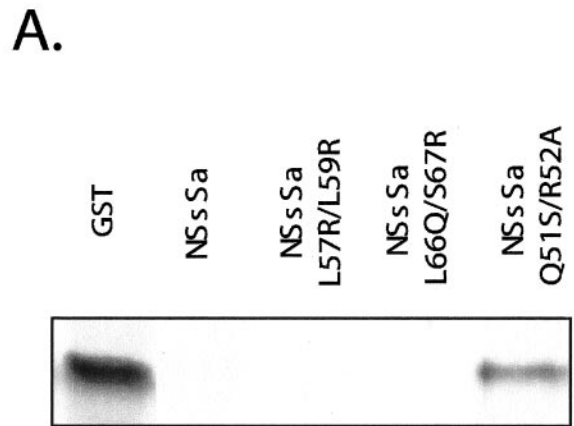

Figure 3. Sequence similarities are functionally significant. (a) GST, GST-NSsSa, GST-NSsSa L57R/L59R, GST-NSsSa L66Q/S67R, or GST-NSsSa Q51A/R52A was added to rabbit reticulocyte lysates programmed with nonspecific Cdc25 cDNA. The reticulocyte lysates were allowed to transcribe and translate for 45 min, and the product was resolved by SDS-PAGE as previously described (Holley *et al.*, 2002). Note that the GST-NSsSa Q51A/R52A mutant was still markedly defective in translational inhibition even when added at concentrations four-fold higher than the GST-NSsSa L57R/L59R or GST-NSsSa L66Q/S67R proteins. (b) GST, GST-NSsSa, or GST-NSsSa Q51A/R52A was added to crude *Xenopus* egg extracts and caspase activity was monitored by cleavage of the colorimetric peptide substrate DEVD-pNA.

Residues Conserved between Reaper and NSs Are Critical for Induction of Apoptosis

Given that residues shared between Reaper and NSs proteins were central for their ability to inhibit translation, we speculated that a shared sequence motif might also be responsible for the common ability of these proteins to induce apoptosis. To address this possibility, we tested the mutants described above for their capacity to activate caspases in *Xenopus* egg extracts. As shown in Figure 4D, NSsSa mutants L57R/L59R and L66Q/S67R, both which disrupt hydrophobic residues in or near the Trp/GH3 region, were unable to induce apoptosis (as opposed to the Q51A/R52A
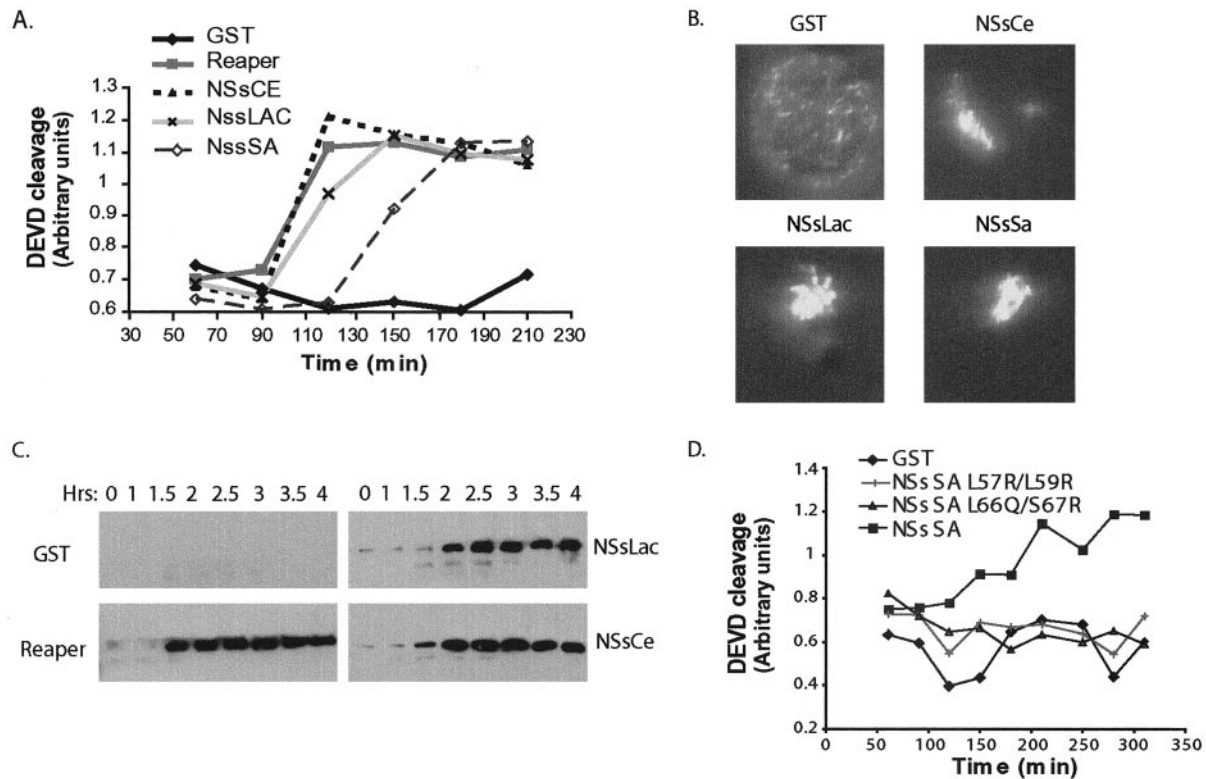
Figure 4. Induction of apoptosis by NSs proteins. (a) GST, GST-Reaper, GST-NSsCe, GST-NSsLac, and GST-NSsSa were added to crude *Xenopus* egg extracts and caspase activity was monitored by cleavage of the colorimetric peptide substrate DEVD-pNA. (b) Photomicrographs of representative nuclei formed in vitro by incubation of sperm chromatin in egg extracts after supplementation of extracts with either GST alone, GST-NSsCe, GST-NSsLac, or GST-NSsSa protein. Nuclear morphology was normal in the presence of GST, but the addition of GST-NSsCe, GST-NSsLac, or GST-NSsSa resulted in apoptosis and nuclear fragmentation. (c) Recombinant GST, GST-Reaper, GST-NSsLac, or GST-NSsCe proteins were added to crude extract in the presence of the caspase inhibitor zVAD-fmk. Samples were collected at the indicated times, and mitochondria were removed by passage of the extract through a 0.1-μm filter. The filtered extract was examined for cytochrome c release by anticytochrome c immunoblotting. (d) GST, GST-NSsSa, GST-NSsSa L57R/L59R, or GST-NSsSa L66Q/S67R was added to crude *Xenopus* egg extracts and caspase activity was monitored by cleavage of the colorimetric peptide substrate DEVD-pNA. Note that although the kinetics of caspase activation varies slightly from extract to extract, the relative activities of the wild-type and mutant proteins are constant.

mutants shown in Figure 3). These data suggest that there are regions of sequence similarity between Reaper and NS-sSa proteins, which are critical for apoptotic induction and for translation inhibition. It also strongly suggests that the observed sequence similarities are functionally significant.
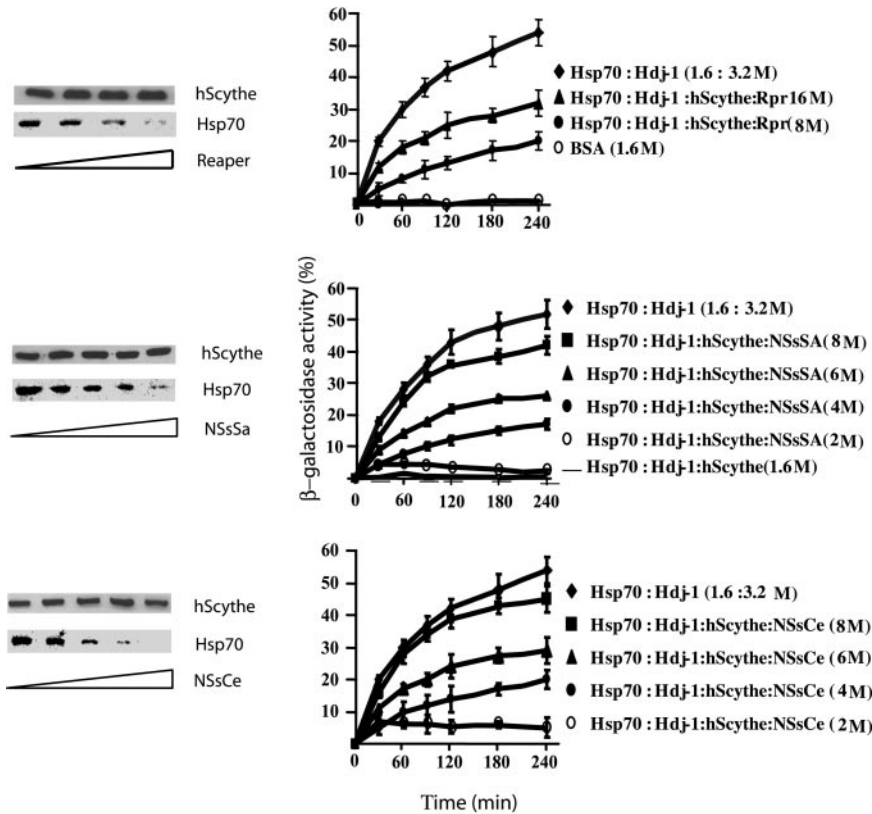
NSs Proteins Bind to Scythe and Modulate Its Function

We have previously demonstrated that Reaper can bind to and regulate the action of the Hsc/Hsp 70 modulator, Scythe, and that this activity of Reaper is central to its mitochondrial cytochrome c-releasing activity (Thress *et al.,* 2001). To determine if NSs and Reaper might promote cyto-chrome c release by a similar mechanism, we first wished to determine if the NSs proteins could, like Reaper, alleviate Scythe-mediated Hsp 70 suppression. To test this we followed the in vitro protein-folding assay for Hsp 70 chaper-ones described by Freeman and Morimoto (1996). Briefly, β-galactosidase was denatured in 6 M guanidine HCl and then incubated in refolding buffer with purified recombinant Hsp 70, ATP, and the cochaperone Hdj-1 in the presence or absence of Scythe, Reaper, NSsSa, or NSsCe proteins. After various times of incubation, samples were assayed for β-ga-lactosidase activity using the colorimetric substrate *o*-nitro-

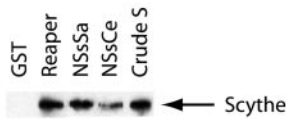phenyl-β-D-galactopyranoside. As shown in Figure 5A and reported previously, Scythe very effectively suppressed Hsp 70–mediated protein folding. Strikingly, the NSs proteins were as effective as Reaper at reversing this inhibition of protein folding (Figure 5A). Consistent with these data, we found that the NSs proteins bound well to Scythe (both *Xenopus*, Figure 5B, and Human Scythe/BAT3, unpublished data) and could, upon binding, displace Scythe-bound Hsp 70 (Figure 5A). Collectively, these observations strongly suggest that Reaper and NSs proteins can modulate Scythe in a similar manner.

Although the precise substrate of the Reaper/Scythe/Hsp 70 machine responsible for mitochondrial cytochrome c re-lease has been elusive, on an empirical level we know that Scythe immunoprecipitates contain a Reaper-releasable fac-tor that can trigger cytochrome c release from isolated mi-tochondria (Thress *et al.,* 1999). Therefore, we assayed the NSs proteins for the Reaper-like ability to induce the disso-ciation of a cytochrome c-releasing activity from Scythe pre-cipitates. Immunoprecipitates from *Xenopus* egg extracts formed using either preimmune or Scythe antisera linked to protein A Sepharose were washed extensively and then incubated with either recombinant GST protein, GST-
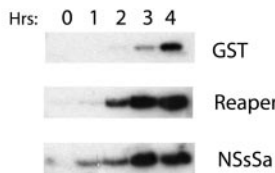
A.

Figure 5. NSs bind to Scythe and modulate its function. (a) His-*h*Scythe (2 μm) was incubated with Hsp70 (2 μm) and the complex was allowed to form. After complex formation, increasing concentrations of recombinant Reaper (2, 5, 8, and 10 μM in lanes 1–4, respectively), NSsCe or NSsSa (2, 4, 6, 8, and 10 μM in lanes 1–5, respectively) were added. Bound proteins were precipitated with Ni$^+$-agarose beads, washed, resolved by SDS-PAGE, and analyzed by immunoblotting using an Hsp70 mAb 5a5 or an anti-His antibody for *h*Scythe detection (left panel). Hsp70–dependent refolding of β-galactosidase was measured in the presence of full-length human Scythe and increasing concentrations of recombinant Reaper, NSsSa, or NSsCe (right-panel). (b) Recombinant GST, GST-Reaper, GST-NSsSA, or GST-NSsCe proteins attached to glutathione Sepharose beads were incubated in *Xenopus* egg extracts, retrieved by centrifugation, washed, and analyzed for the presence of bound Scythe by immunoblotting. (c) Anti-Scythe immunoprecipitates formed by incubation of protein A-linked anti-Scythe antibodies with *Xenopus* egg extracts were washed and then incubated with recombinant GST, Reaper, or NSsSa protein. The released activity was then incubated with purified isolated mitochondria. After removal of the mitochondria by filtration, released cytochrome c was detected by immunoblotting with anticytochrome c antibody.

Reaper, or GST-NSsSa to initiate release of presumptive cytochrome c-releasing factors. Bead-bound proteins (including Scythe, Reaper, and NSsSa) were removed by centrifugation and the collected supernatants were concentrated and added to purified mitochondria. As shown in Figure 5C and previously reported (Thress *et al.*, 1999), Reaper induced the release of proapoptotic activity from the immune resin and GST did not induce release of such factors. Interestingly, the NSsSa behaved like Reaper, promoting dissociation of a cytochrome c–releasing activity from anti-Scythe immunoprecipitates. These results point to a common mechanism whereby Reaper and NSs act in conjunction with Scythe to trigger mitochondrial cytochrome c release and consequent caspase activation.

Despite the apparent involvement of Scythe in both Reaper- and NSs-mediated apoptosis, it should be noted that their shared ability to regulate Scythe does not appear to account for their effects on protein translation. Specifically, removal of >95% of Scythe from *Xenopus* egg extracts by immunodepletion did not interfere with the ability of either Reaper or the NSs proteins to dampen translation (unpublished data). These data suggest that the 16–65 region of

Reaper and the NSs proteins must interact with factors other than Scythe to mediate translational repression.

NSs Proteins Can Induce Apoptosis in the Mouse Brain

Although the data above strongly suggest that NSs proteins can have proapoptotic activity, these experiments were all performed in vitro in the heterologous *Xenopus* system. We wished to extend these in vitro observations further by determining whether the NSs proteins could also induce apoptosis in vivo. Bunyaviruses are normally neuronotropic viruses; peripheral infection of young mammals with bunyaviruses leads to invasion of the CNS and increased cytopathy (Gonzalez-Scarano *et al.*, 1992; Nathanson, 1997). To induce expression of the NSs proteins in neuronal mouse tissue, we subcloned NSsSa and NSsCe genes into a Sindbis virus-based expression vector. Sindbis virus infection causes age-dependent encephalitis in mice, and its severity correlates with the degree of virus-induced apoptosis in neuronal cells (Griffin and Hardwick, 1997). Recombinant Sindbis virus (dsTE12Q) carrying genes coding for the NSs proteins were introduced by intracerebral injection into the brain of twelve-day-old mice, and animals were monitored for 10 d.
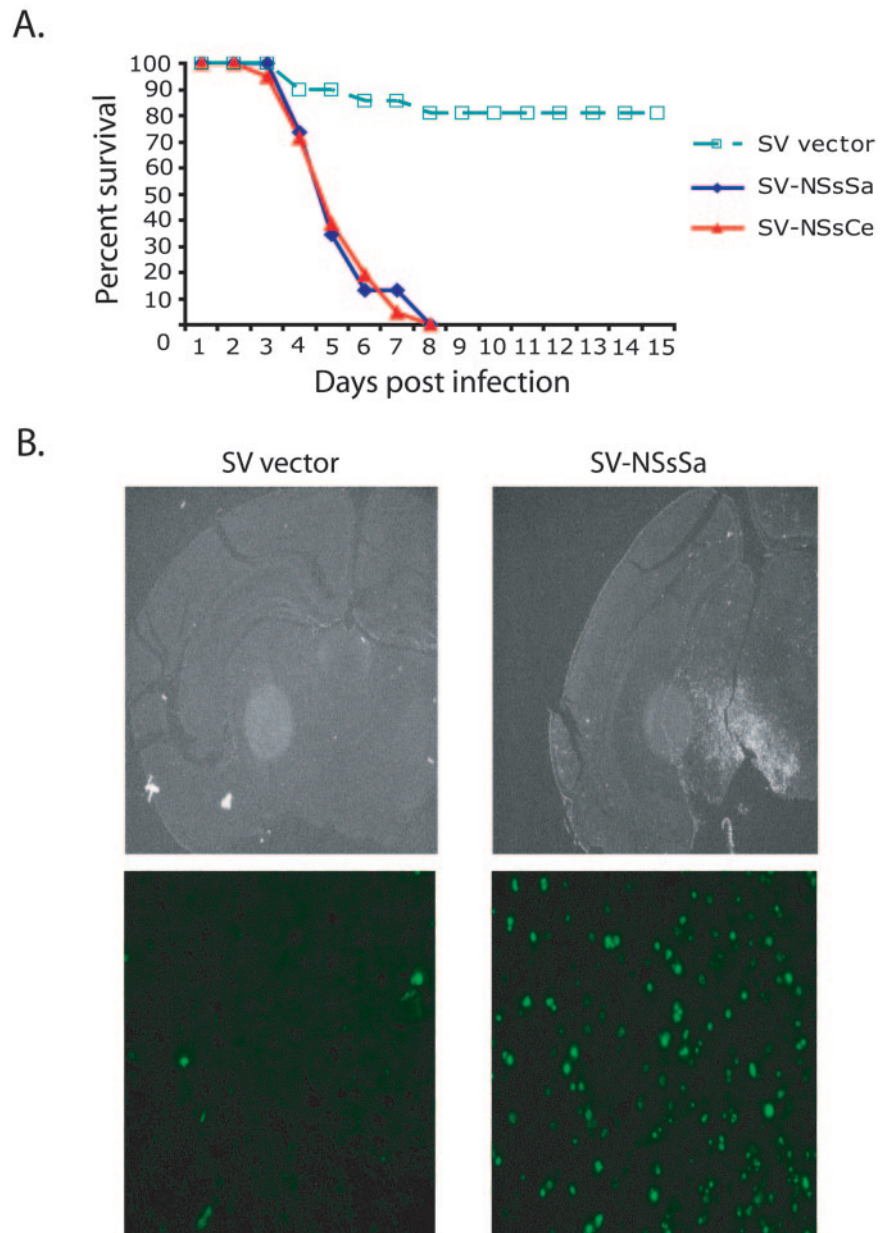
Figure 6. NSs induce programmed cell death in vivo. (a) Percent survival of 12-d-old CD-1 mice inoculated with recombinant Sindbis virus carrying no exogenous gene (SV vector) or carrying the NSsSa or NSsCe genes (SV- NSsSa or SV-NSsCe, respectively). A total of 50–60 mice were analyzed per treatment group. (b) TUNNEL staining of tissue sections from brains of 12-d-old CD-1 mice inoculated with SV vector or SV-NSsSa (top panel). Close-up of TUNNEL staining of brain tissue sections (bottom panel).

As shown in Figure 6A and previously reported (Levine *et al.*, 1996), mice this age are resistant to Sindbis-induced death, with >80% of animals surviving the infection. Remarkably, all of the animals inoculated with the recombinant virus expressing the NSs proteins died at approximately 6 days postinjection (Figure 6A).

To determine if the observed increase in mortality following NSs expression was due to the induction of neuronal programmed cell death, sagittal cross sections of brains from mice infected with the various Sindbis virus constructs were analyzed by TUNNEL staining. As shown in Figure 6B, brains from mice inoculated with control SV vector displayed low levels of TUNNEL-positive cells. In contrast, brain sections from SV-NSs–infected animals showed highly elevated levels of TUNNEL-positive cells, indicating a significant increase in virus-induced apoptosis. Taken together, these results indicate that expression of NSs proteins during SV infection resulted in exacerbated programmed cell death of infected neurons and efficient induction of fatal encephalitis in infected animals, demonstrating the ability of NSs proteins to function as prodeath factors in vivo.

DISCUSSION

Drosophila Reaper is a potent apoptotic inducer in cells of both vertebrate and invertebrate origin. Although Reaper is only 65 amino acids long, it can inhibit IAPs, promote IAP autoubiquitination, trigger mitochondrial cytochrome c release, and inhibit general protein translation (Evans *et al.*, 1997a; Goyal *et al.*, 2000; Holley *et al.*, 2002). A bona fide vertebrate Reaper homolog able to perform all of these functions has not yet been reported, though the extreme Reaper N-terminus shares limited similarity at the sequence level

with Smac/Diablo and Omi/HtrA2, which translates into the functional ability to bind IAPs and displace them from caspases (Martins *et al.,* 2002; Verhagen *et al.,* 2002; Verhagen and Vaux, 2002). We report here that a group of bunyaviral nonstructural NSs proteins share limited homology with the C-terminal region of fly Reaper. Although these sequence similarities do not necessarily indicate common evolutionary origins, the shared ability of the California NSs proteins and Reaper to inhibit generalized protein translation, induce mitochondrial cytochrome c release/caspase activation and modulate Scythe/Hsp 70 activities strongly suggest that the shared sequence motifs are functionally significant.

Homology and Functional Similarities

Structural predictions for the NSs RLR and Reaper suggest that the sequence similarities between these proteins might translate to structural similarities. For instance, both NSs proteins and Reaper share a similar pKi (NSsSa pKi is 10.10; Reaper pKi is 10.13), and their conserved GH3 motif is predicted to form an amphipathic α-helix. Interestingly, this region is also present in the *Drosophila* proteins Sickle and Grim (Claveria *et al.,* 2002; Wing *et al.,* 2002). Although the function of this region is not fully understood, it is important for Grim's apoptotic activity, and mutations in this region affect its capacity to localize to the mitochondria (Claveria *et al.,* 2002). Consistent with these observations, hydrophobic residues altered in or near this conserved α-helical area also affected NSsSa capacity to induce apoptosis. Moreover, recent data in our lab indicates that similar mutations in Reaper also disrupt its capacity to induce apoptosis (M. Olson and S. Kornbluth, unpublished observations). Interestingly, these mutations did not affect the capacity of the protein to inhibit translation. Instead, a conserved motif present outside of the Trp/GH3 area is apparently responsible for the inhibitory activity of these proteins. These data collectively show that sequence similarities in the RLR area are likely to account for the similar activities observed for Reaper and NSs proteins.

One interesting conclusion to be drawn from these studies concerns the evolutionary separation of Reaper's functions. One hallmark function of Reaper is the ability to inhibit IAP-caspase interactions. This activity of Reaper resides in its extreme N-terminus in a motif shared by the proteins Grim and Hid, as well as the mammalian proteins SMAC and Omi (Wang *et al.,* 1999; Goyal *et al.,* 2000; Christich *et al.,* 2002; Holley *et al.,* 2002; Martins *et al.,* 2002; Srinivasula *et al.,* 2002; Tenev *et al.,* 2002; Verhagen *et al.,* 2002; Verhagen and Vaux, 2002; Wing *et al.,* 2002; Yoo *et al.,* 2002). As far as we know, there is no evidence to suggest that either SMAC or Omi, which are unrelated to the C-terminal 2/3 of Reaper, can either induce mitochondrial cytochrome c release or inhibit protein synthesis. Based on the identification of regions of significant sequence homology shared between the C-terminal 2/3 of Reaper and NSs proteins and the absence of IAP inhibitory motifs in the NSs proteins, we would suggest that the N- and C-terminal functions of Reaper have evolved to be present in separate proteins. Moreover, this suggests that there may be as yet unreported mammalian proteins that share sequence similarity/function with the C-terminal 2/3 of Reaper. In this regard, it is interesting to note that we have found that coexpression of SMAC and amino acids 16–65 of Reaper in human cells (which are both very weak apoptotic inducers on their own) can reconstitute full Reaper-like cell killing activity (C. Holley and S. Kornbluth, unpublished observations).

Translational Inhibition by NSs Proteins and Reaper

In this report we show that NSs proteins, like Reaper, can act in isolation to inhibit protein synthesis. These observations support previous studies demonstrating that a mutant virus lacking NSs (BUNdelNSs) is impaired in its ability to inhibit host cell translation (Bridgen *et al.,* 2001). The ability of these Reaper-related proteins to inhibit protein translation also helps to validate this rather unexpected function of Reaper itself. As the nearly complete immunodepletion of Scythe did not compromise the translational inhibition, we suspect that there are other ligands for the NSs proteins/Reaper that mediate effects on the translational machinery. Further work will be required to elucidate the mechanism of this inhibition, but the availability of several proteins that act in a similar manner, and the identification of a common motif important for translation inhibition should facilitate isolation of the relevant targets.

The capacity of the viruses to inhibit translation raises the intriguing question as to how they can avoid inhibiting synthesis of their own RNA while affecting the host protein synthesis program. Interestingly, NSs proteins are encoded by the smallest of the three orthobunyavirus genomic RNA segments and are in an overlapping, but distinct reading frame with the nucleocapsid (N) protein (Bridgen *et al.,* 2001). This suggests that the synthesis of NSs proteins might be controlled by a cap-independent translational pathway such as that driven by internal ribosome entry sites. This is the case for other known classes of viral proteins able to inhibit translation (reviewed by Vagner *et al.,* 2001). If this is the case for the NSs proteins, they may inhibit cap-dependent host translation initiation while preserving their own expression (and presumably that of other bunyaviral proteins).

Apoptotic Induction by NSs Proteins

Viruses frequently inhibit cap-dependent translation of host proteins. For viruses whose own reading frames are translated from IRES sequences, this can serve as a means to enhance translation of their own IRES-driven viral proteins. We speculated that Reaper-mediated translational inhibition could contribute to apoptotic induction by preventing synthesis of short-lived apoptotic inhibitors, such as the IAPs (Holley *et al.,* 2002). It is not yet clear whether the translational inhibitory properties of the NSs proteins also contribute to their apoptotic activity. However, NSs proteins must possess apoptotic regulatory activity distinct from their ability to inhibit translation as they accelerated mitochondrial cytochrome c release and caspase activation in cycloheximide-treated *Xenopus* egg extracts.

Although the IAP inhibitory functions of fly Reaper are clearly significant for its full apoptotic potential, several reports have attributed distinct apoptotic activities to the C-terminal 2/3 of Reaper where the similarities to the RLR NSs lay (Evans *et al.,* 1997a; Vucic *et al.,* 1997; McCarthy and Dixit, 1998). Indeed, the 16–65 Reaper fragment, which lacks the RHG domain, can still induce apoptosis (Chen *et al.,* 1996; McCarthy and Dixit, 1998; Thress *et al.,* 1999). We have shown that NSs proteins, which lack an RHG domain, can induce apoptosis in both cell-free extracts and in vivo when introduced into mice. Furthermore, NSs proteins appear to act similarly to Reaper in modulating the BAG domain-containing protein Scythe. Reaper was the first protein documented to relieve inhibition of Hsc 70/Hsp 70 by BAG family members (Thress *et al.,* 2001). The NSs proteins constitute a second group of these ligands, potentially able to

modulate a number of cellular functions by controlling protein chaperones. Like Reaper, the NSs proteins appear to induce mitochondrial alterations (e.g., cytochrome c release) through dissociation of a Scythe-sequestered cytochrome c–releasing activity. Although the nature of this activity is not yet clear, the common ability of NSs and Reaper to invoke these activities further underscores their likely similar mechanism of action.

Viruses and Apoptosis

Under most circumstances, known viral regulators of cell death act to inhibit, rather than promote, apoptosis to preserve the host cells in which they replicate (reviewed by Hardwick, 2001; Benedict *et al.,* 2002). However, viruses have also been shown to elicit and use apoptosis as part of their infection strategy. For example, the Aleutian mink disease parvovirus (ADV) induces caspase activation and apoptosis. Direct inhibition of caspase 3 not only prevented viral-induced apoptosis, but also compromised ADV replication (Best *et al.,* 2002). Studies on coxsackie virus B2, hepatitis C virus, herpes simplex virus, influenza A virus, and HIV-1 have also found that particular viral proteins critical for the viral life cycle also directly induce apoptosis (Henke *et al.,* 2000; Anderson, 2001; Goh *et al.,* 2001; Henke *et al.,* 2001; Schultz-Cherry *et al.,* 2001; Muthumani *et al.,* 2002a, 2002b). It has been hypothesized that induction of apoptosis could facilitate the dissemination of viral particles. Furthermore, programmed cell death may also play an important role in the viral strategy to avoid the host immune response (Fazakerley, 2001; Fazakerley and Allsopp, 2001).

Bunyaviruses are cytopathic, causing hemorrhagic fevers and encephalitis in humans (Gonzalez-Scarano *et al.,* 1992; Elliott, 1997). Apoptotic cell death by members of the *Bunyaviridae* has been described for hantaviruses (Kang *et al.,* 1999) and of direct relevance for our study, for La Crosse virus (Pekosz *et al.,* 1996). La Crosse virus induces apoptosis in the brains of newborn mice and in some, but not all, neuronal cell lines. Moreover, BUN NSs protein has been shown to be critical for viral pathogenesis (Bridgen *et al.,* 2001; Weber *et al.,* 2002). Given our findings on NSs apoptotic activity, it is attractive to speculate that the NSs proteins are directly responsible, through the induction of apoptosis, for the observed bunyavirus-induced cytopathy. This makes them interesting targets of regulation in controlling human arbovirus encephalitis.

Neuronal Apoptosis and Reaper

Although Reaper was originally thought to be a universal regulator of apoptosis, analysis of flies defective only at the Reaper locus revealed that Reaper is required solely for developmental apoptosis of the central fly ganglia (Peterson *et al.,* 2002). Bunyaviruses are neurotropic viruses and the NSs proteins appear to be potent inducers of neuronal apoptosis (Gonzalez-Scarano *et al.,* 1992; Pekosz *et al.,* 1996; Figure 6). In several cytopathic neurotropic viruses, neurovirulence can be dependent upon the age of the host, with younger hosts being more susceptible to neurotropic virus infections (reviewed by Anderson, 2001; Fazakerley, 2001; Fazakerley and Allsopp, 2001; Gosztonyi and Koprowski, 2001; Liebert, 2001). This may be because these viruses can hijack the endogenous cell death pathways that are being actively used in wiring the developing nervous system. It is attractive to speculate that Reaper and bunyaviral NSs proteins share underlying properties that make them particularly suited to induce apoptosis in the neuronal milieu during development of the young nervous system. Factors

associating with both Reaper and the NSs proteins will be particularly attractive candidates for mediators of these biological functions.

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