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The Viral Nucleocapsid Protein of Transmissible Gastroenteritis Coronavirus (TGEV) Is Cleaved by Caspase-6 and -7 during TGEV-Induced Apoptosis

JEAN-FRANÇOIS ELÉOUËT,[1]* ELIZABETH A. SLEE,[2]† FRANÇOISE SAURINI,[1] NATHALIE CASTAGNÉ,[1] DIDIER PONCET,[1] CARMEN GARRIDO,[3] ERIC SOLARY,[3] AND SEAMUS J. MARTIN[2]†

Unité de Virologie et Immunologie Moléculaires, Institut National de la Recherche Agronomique, 78350 Jouy-en-Josas,[1] and U.F.R. Médecine et Pharmacie, INSERM U517, 21000 Dijon,[3] France, and Molecular Cell Biology Laboratory, National University of Ireland, Maynooth, County Kildare, Ireland[2]

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The transmissible gastroenteritis coronavirus (TGEV), like many other viruses, exerts much of its cytopathic effect through the induction of apoptosis of its host cell. Apoptosis is coordinated by a family of cysteine proteases, called caspases, that are activated during apoptosis and participate in dismantling the cell by cleaving key structural and regulatory proteins. We have explored the caspase activation events that are initiated upon infection of the human rectal tumor cell line HRT18 with TGEV. We show that TGEV infection results in the activation of caspase-3, -6, -7, -8, and -9 and cleavage of the caspase substrates eIF4GI, gelsolin, and α-fodrin. Surprisingly, the TGEV nucleoprotein (N) underwent proteolysis in parallel with the activation of caspases within the host cell. Cleavage of the N protein was inhibited by cell-permeative caspase inhibitors, suggesting that this viral structural protein is a target for host cell caspases. We show that the TGEV nucleoprotein is a substrate for both caspase-6 and -7, and using site-directed mutagenesis, we have mapped the cleavage site to $VVPD^{359}\downarrow$. These data demonstrate that viral proteins can be targeted for destruction by the host cell death machinery.

Apoptosis is a physiological and essential mechanism for controlling cell numbers in metazoan organisms (reviewed in reference 24). Viruses have evolved strategies to either inhibit or stimulate host cell apoptosis, depending on the particular virus-host interaction. Many viruses, such as herpesviruses, baculoviruses, and poxviruses, have developed strategies to inhibit or delay apoptosis, which usually results in increased virus production (23, 25, 31). Apoptosis of infected cells may also be advantageous by facilitating virus dissemination and limiting the host inflammatory response (31). In some situations, the death of virus-infected cells accounts for viral pathogenesis and related diseases. The capacity of host cells to rapidly undergo cell death in response to virus infection may be an important antiviral defense mechanism (23).

Transmissible gastroenteritis virus (TGEV) is a member of the *Coronaviridae* family, a group of enveloped viruses (33), and has a large, positive-stranded, capped and polyadenylated RNA genome of 28.6 kb (9). This enteropathogenic virus causes acute and fatal diarrhea in newborn piglets. TGEV replicates in enterocytes and provokes villous atrophy, is closely related to the human respiratory coronavirus HCoV-229E (9), and can also infect the respiratory tract. Moreover, some variant strains of TGEV, such as the porcine respiratory coronavirus (PRCoV), have lost their intestinal tropism (11, 18). The Purdue-115 strain (10) and the Miller strain (34) of TGEV have been shown to induce apoptosis in cell lines expressing the porcine aminopeptidase N (APN), which is a receptor for the virus (4). More recently, the murine coronavirus MHV was also found to trigger apoptosis upon infection of host cells (1).

Current evidence indicates that a family of proteases referred to as caspases (cysteine aspartate-specific proteases) play a central role in cell death by apoptosis. These proteases are synthesized as relatively inactive proenzymes that are activated by proteolytic cleavage at the onset of apoptosis (21, 32, 36, 41). The cleavage of procaspases generates two subunits, which assemble as a heterotetramer. Caspase activation involves a proteolytic cascade in which those with long prodomains, such as procaspase-8, -9, or -10, are activated first. In turn, these initiator caspases activate downstream proteases with short prodomains, such as procaspase-3, -6, and -7. The proteolytic cleavage of a limited number of essential cellular proteins by these effector caspases is thought to be responsible for the phenotypic changes that occur in cells undergoing apoptosis (21, 36, 41). The ability of the cell-permeative caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD.fmk) to inhibit TGEV-induced apoptosis in swine testis (ST) cells suggests that caspases are involved in the cytopathic effect of this virus (10).

The present study was undertaken to further explore the role of caspases in apoptosis triggered by TGEV. Since porcine-specific caspase antibodies were not available, we used the human rectal tumor adenocarcinoma cell line HRT18, which was modified to express the porcine APN (HRT18jap1). We observed that TGEV infection of HRT18jap1 cells resulted in the activation of caspase-3, -6, -7, -8, and -9, with the activation of caspase-8 preceding that of other caspases. As expected, TGEV-induced apoptosis was associated with caspase-mediated cleavage of various cellular proteins, such as eIF4GI, gelsolin, and α-fodrin. Surprisingly, the TGEV nucleocapsid protein (N protein)—a structural protein of the virus—also underwent caspase-mediated proteolysis within the host cell. Further studies revealed that the TGEV nucleocapsid protein

* Corresponding author. Mailing address: INRA, Unité de Virologie et Immunologie Moléculaires, 78352 Jouy-en-Josas Cedex, France. Phone: (33) 1 34 65 26 41. Fax: (33) 1 34 65 26 21. E-mail: eleouet@biotec.jouy.inra.fr.

† Present address: Division of Molecular and Cell Biology, The Smurfit Institute of Genetics, Trinity College, Dublin 2, Ireland.

could be cleaved by caspase-6 and -7 at a site within the C terminus, which we have mapped to Asp359. These results show that viral structural proteins are potential targets for the host cell death machinery.

MATERIALS AND METHODS

Materials. The broad-spectrum caspase inhibitor z-VAD.fmk was purchased from Bachem (Bubendorf, Switzerland). The caspase-8-selective inhibitor *N*-benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoro-methylketone (z-IETD.fmk) and the cathepsin B inhibitor *N*-benzyloxycarbonyl-Phe-Ala-fluoro-methylketone (z-FA.fmk) were purchased from Calbiochem (Meudon, France). Anti-caspase-3, anti-caspase-7, and antigelsolin monoclonal antibodies were purchased from Transduction Laboratories (Lexington, Ky.), anti-caspase-6 polyclonal antibody was purchased from Upstate Biotechnology (Lake Placid, N.Y.), anti-caspase-8 monoclonal antibody was from Pharmingen (San Diego, Calif.), and anti-α-fodrin antibody was obtained from Chemicon International Inc. (Temecula, Calif.). Anti-caspase-9 antibody was kindly provided by Doug Green (La Jolla Institute for Allergy and Immunology, San Diego, Calif.), and purified recombinant caspase-3, -6, -7, and -8 were a gift from Guy Salvesen (The Burnham Institute, La Jolla, Calif.). The anti-N antibodies 22.6, 5.1, and 19.1 have been described previously (17). Rabbit antiserum against EIF4GII (amino acids 1 to 480) was a gift of A. Gradi and N. Sonenberg (McGill University, Montréal, Canada).

Plasmid constructions. The N gene was derived from TGEV strain Purdue-115 and was PCR amplified using the plasmid pTG2.18 (29) as a template with the following primers: 5′GAGGAGCATATGGCCAACCAGGGACAACGT GTC3′ 5′GAGGAGCTCGAGGTTCGTTACCTCATCAATATTCTC3′. The amplified DNA was cloned by insertion between the *Nde*I and *Xho*I sites in pET-25b(+) (Novagen) downstream of the T7 promoter sequence. The C-terminal-deletion mutants and D355E, D359E, and D370E mutants were generated using the *Pfu* DNA polymerase with the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions, with the following primers: N-20+ (5′CCT GAT GCA TTA ATA TAG AAT TCT ACA GAT GTG TTT G3′) and N-20− (5′CAA ACA CAT CTG TAG AAT TCT ATA TTA ATG CAT CAG G3′) for the N(1–362) mutant, N-41+ (5′GAA CAG AGA AAA TGA ATT CCT CGT TCT AAA TC3′) and N-41− (5′GAT TTA GAA CGA GGA ATT CAT TTT CTC TGT TC3′) for the N(1–341) mutant, and N-63+ (5′GAT CCT AAG ACT TGA GAA TTC CTT CAG CAG3′) and N-63− (5′CTG CTG AAG GAA TTC TCA AGT CTT AGG ATC3′) for the N(1–319) mutant. To facilitate the screening of recombinant plasmids, an *Eco*RI restriction site was introduced downstream of the stop codons. The D355E, D359E, and D359A mutations were done using the following primers: D355E+ (5′AGGTCAGAGCAAGAGGTAGTACCTGATGCA3′), D355E− (5′TGCA TCAGGTACTACCTCTTGCTCTGACCT3′), D359E+ (5′GATGTGGTACC TGAGGCATTAATAGAA3′), D359E− (5′TTCTATTAATGCCTCAGGTACC ACATC3′), D359A+ (5′GATGTGGTACCTGCAGCATTAATAGAA3′), and D359A− (5′TTCTATTAATGCTGCAGGTACCACATC3′). The D355E mutation destroyed a *Kpn*I restriction site; the D359A and D359E mutations destroyed an *Nsi*I restriction site. Sequence analysis was carried out to confirm the amino acid changes. Plasmids encoding each of the caspases have been described previously (35).

Virus and cells. The American high-cell-passage Purdue-115 strain of TGEV was used as a virus source and propagated on ST cells as described previously (17). Cells were infected for 1 h with TGEV, with the end of infection designated 0 h postinfection (p.i.). The human rectal tumor cell line HRT18 stably expressing the porcine APN (HRT18jap1) has been described previously (5). ST and HRT18 cells were maintained as monolayer cultures in minimal essential Eagle's medium and RPMI medium, respectively, containing 10% fetal calf serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml).

DNA fragmentation assay. At different times p.i., 10^6 cells were collected, together with the floating cells in the supernatant, and low-molecular-weight DNA was extracted as described previously (10). DNA preparations were then electrophoresed through 2% agarose gels and stained with ethidium bromide.

Fluorescence microscopy. Cells (10^6) (including floating cells) were collected and fixed in 70% ethanol for 1 h, washed in phosphate-buffered saline, incubated for 15 min at 37°C with 100 μM RNase A, and stained by propidium iodide as described previously (10). Cells were centrifuged onto microscope slides for 5 min at 100 × *g* using a Cytospin II centrifuge (Shandon) and were then mounted with Glycergel (Dako). Stained cell preparations were then observed by UV microscopy.

Cell fractionation and subcellular localization of cytochrome *c*. Mitochondrial and cytosolic (S100) fractions for cytochrome *c* release studies were prepared and analyzed by Western blotting as described previously (38).

SDS-PAGE and Western blot analysis. For caspase activation and N cleavage studies, 10^6 cells were mock or TGEV infected using a multiplicity of infection (MOI) of 5. Floating and adherent cells were lysed together in 100 μl of standard Laemmli buffer. From each sample, 10 μl was subjected to standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and was transferred onto 0.45-μm reinforced nitrocellulose membranes (Optitran BA-S85; Schleicher & Schuell, Inc.). The membranes were blocked in PBS containing 5% nonfat dry milk powder for 15 min before incubation with the appropriate antibodies described in "Materials," above. Bound antibodies were detected using appropriate peroxidase-coupled secondary antibodies (Amersham), followed by detection using the Supersignal chemiluminescence system (Pierce), as previously described (22, 35). Western blotting using the EIF4GI antibody was done as described previously (28).

Cell-free reactions. [^{35}S]methionine-labeled N protein and caspase-7 were in vitro transcribed and translated using the TNT kit (Promega). Reactions were done using 1 μg of plasmid in a 50-μl transcription/translation reaction mixture containing 2 μl of translation grade [^{35}S]methionine (1,000 μCi/ml; ICN). Cell extracts were generated from Jurkat T lymphoblastoid cells as previously described (35). Depletion of caspase-3 from cell extracts was done as described previously (35), by incubation with 50 μl of either anti-caspase-3 antibody or a control (anti-RelA; Santa Cruz Biotechnology) rabbit polyclonal. For cell-free reactions, 10 μl of cell extract (~5 mg/ml) and 1 μl of transcription/translation reaction products were combined. In vitro apoptosis was induced by the addition of bovine heart cytochrome *c* to extracts at a final concentration of 50 μg/ml and the addition of dATP to a final concentration of 1 mM. [^{35}S]methionine-labeled N was then incubated in cell extracts at 37°C in the presence or absence of cytochrome *c* and dATP for periods of up to 2 h. Reaction products were removed at times indicated below and frozen at −70°C for the subsequent SDS-PAGE and fluorographic determination of substrate cleavage profiles or caspase activation.

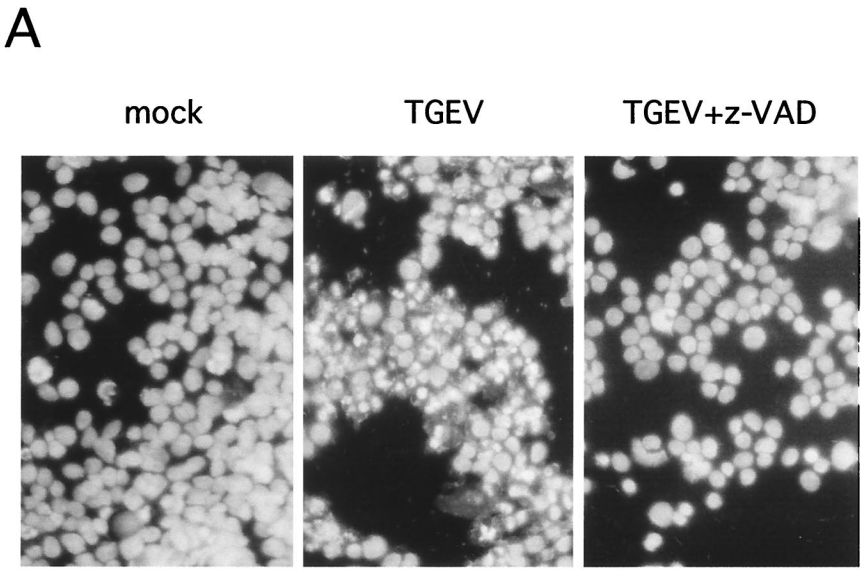
In vitro cleavage by caspases of truncated or mutated N. Truncations or point mutations of N were transcribed and translated in vitro in the presence of [^{35}S]methionine as described above. One to two microliters of the transcription/translation reaction products was incubated for 2 h at 37°C with or without purified recombinant caspases, prepared as described previously (39), in a total reaction volume of 10 μl. Reactions were carried out in protease reaction buffer {20 mM piperazine-*N*,*N*′-bis(2 ethanesulfonic acid)-KOH (pH 7.2), 100 mM NaCl, 1 mM EDTA, 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, 10% sucrose, 10 mM dithiothreitol}. Breakdown products were analyzed by SDS-PAGE followed by fluorography.

RESULTS

TGEV triggers caspase-dependent apoptosis in human HRT18 cells expressing the TGEV receptor. It has been previously shown that HRT18jap1 cells are sensitive to TGEV infection, as demonstrated by the synthesis of viral antigens and cytopathic effects, although these cells do not produce significant amounts of infectious TGEV virions (6). As observed with the porcine and canine cell lines previously tested (10), HRT18jap1 cells infected by TGEV (MOI, 5) showed typical features of apoptosis at 18 h p.i. (Fig. 1A). As expected, the synthetic caspase inhibitor z-VAD.fmk or z-IETD.fmk inhibited TGEV-induced DNA fragmentation in HRT18jap1 cells (Fig. 1B). As previously observed with ST cells (10), treatment of HRT18jap1 cells with z-VAD.fmk prior to infection also inhibited nuclear condensation and cell shrinkage (Fig. 1A).

TGEV triggers the processing of procaspase-3, -6, -7, -8, and -9 in HRT18jap1 cells. To explore the caspase activation events initiated during TGEV-induced apoptosis, we prepared lysates from TGEV-infected cells at different times p.i. Proteins from these lysates were then probed with a panel of caspase-specific antibodies. Figure 2A shows that upon infection of the cells with TGEV, caspase-3, -6, -7, -8, and -9 were processed, as assessed by the disappearance of the proforms of these proteases and—depending on the antibody used for immunoblotting—the appearance of breakdown products corresponding to the sizes of their mature forms.

Caspase-8 processing was consistently detected prior to that of the other proteases, suggesting that caspase-8 was the most proximally activated caspase in this context. Caspase-6 processing, as assessed by the disappearance of the zymogen form of the protease (the antibody used did not detect the mature protease), was also detected early in response to TGEV infection (8 to 10 h p.i.), followed by the processing of caspase-3, -7, and -9. Maturation of caspase-3 was detected between 12 and 14 h p.i. and correlated with the onset of cleavage of eIF4GI,



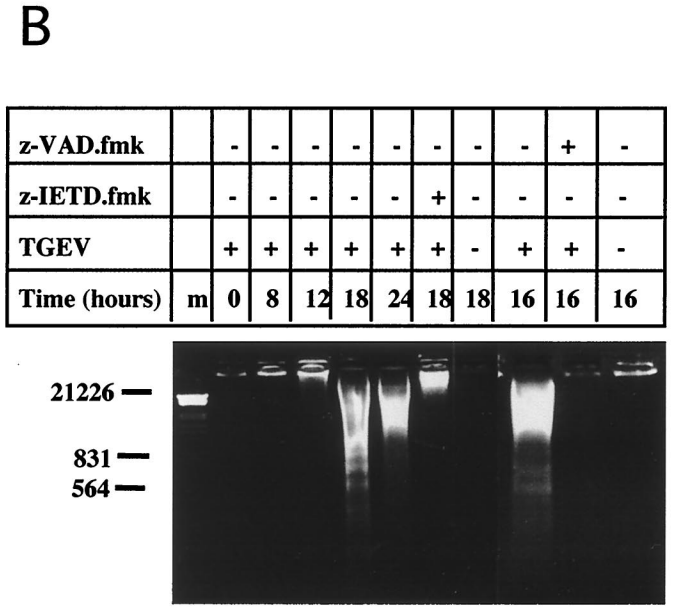


FIG. 1. TGEV-induced apoptosis in HRT18 cells is caspase dependent. Confluent cell monolayers were infected with TGEV at an MOI of 5 and incubated for the indicated times at 38.5°C. (A) Fluorescence microscopy of nuclei from HRT18 jap1 cells mock infected, TGEV infected, and TGEV infected in the presence of z-VAD.fmk (100 μM), as indicated. Nuclei were stained with propidium iodide 24 h p.i. (magnification, ×500). (B) Time course of internucleosomal DNA cleavage in HRT18jap1 cells. Low-molecular-weight DNA was extracted at the indicated times p.i. from TGEV- or mock-infected cells either left untreated or treated with 100 μM z-IETD.fmk and 100 μM z-VAD.fmk, as indicated. DNA marker band sizes (lane m) are indicated in base pairs.

a well-characterized caspase-3 substrate (Fig. 2B). Two other caspase-3 substrates, gelsolin and α-fodrin, appeared to be cleaved prior to appreciable processing of caspase-3 (by 6 and 9 h p.i., respectively) (Fig. 2B). This suggests that processing of these substrates may be partly mediated by other caspases, such as caspase-6 or -8, or that biochemically undetectable caspase-3 is already present at these time points.

TGEV infection induces the redistribution of cytochrome *c* from mitochondria to the cytosol. Cytochrome *c* is known to translocate from the mitochondria to the cytosol during apoptosis (15, 44), resulting in the activation of caspase-9 through the formation of a complex including cytochrome *c*, dATP, Apaf-1, and procaspase-9 (20). Because caspase-9 was activated upon TGEV infection, we explored whether cytochrome *c* redistribution occurred during this form of apoptosis. Figure 3 shows that cytochrome *c* was released from mitochondria between 6 and 12 h p.i., preceding caspase-9 activation (Fig. 2A).

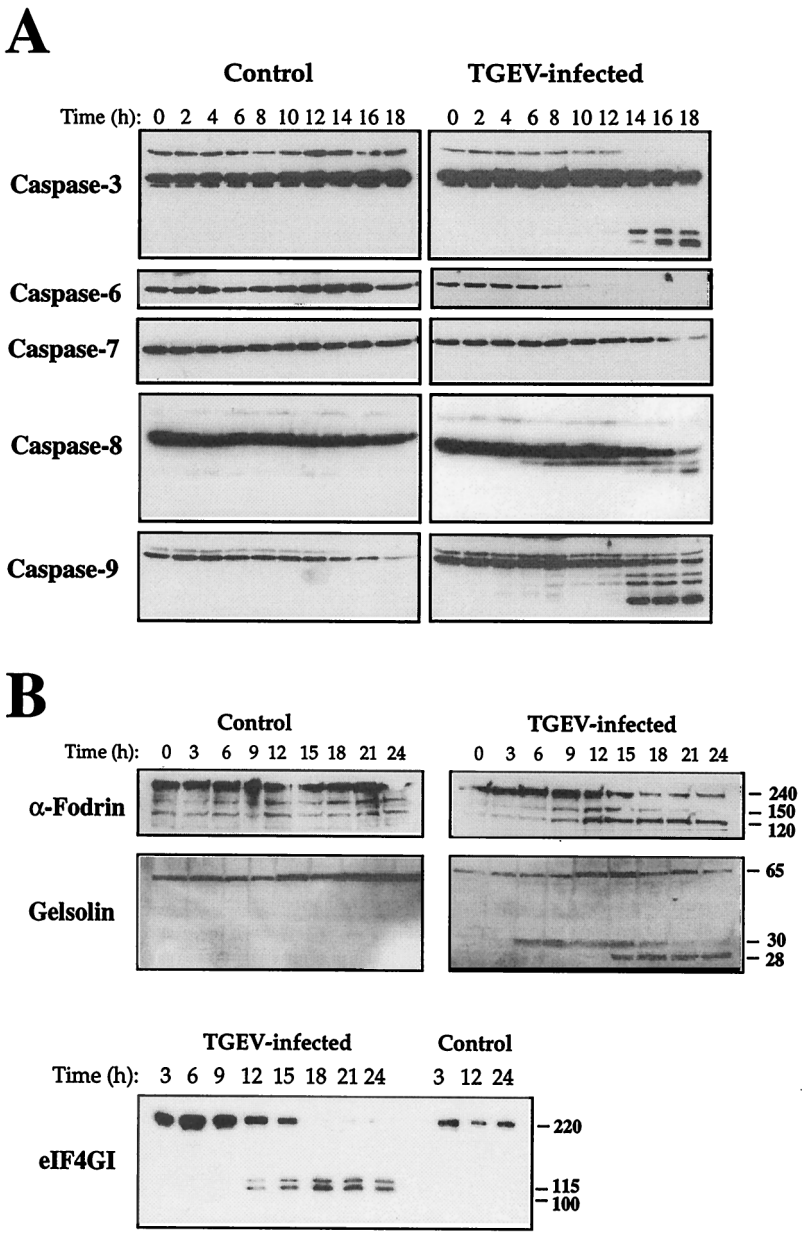


FIG. 2. Processing of multiple caspases and proteolysis of caspase substrates during TGEV-induced apoptosis of HRT18 cells. (A) Time course analysis of caspase-3, -6, -7, -8, and -9 processing; (B) kinetics of cleavage of the caspase substrates α-fodrin, gelsolin, and eIF4GI during TGEV-induced apoptosis. At the indicated times p.i., cell lysates (10^5 cell equivalents per lane) from mock- or TGEV-infected cells were separated by SDS-PAGE and transferred to nitrocellulose membranes by Western blotting, followed by probing for the indicated proteins. Numbers at the right are molecular masses, in kilodaltons.

The TGEV nucleocapsid protein is cleaved during infection in a caspase-dependent manner. The nucleocapsid protein of coronaviruses is believed to be the most abundant viral protein present at all stages of infection (reviewed in reference 19). To confirm productive viral infection of the cells used in this study, we examined the accumulation of the TGEV nucleocapsid protein within the host cells. As shown in Fig. 4A, significant synthesis of N protein was observed 4 h p.i. in ST cells and 6 h p.i. in HRT18jap1 cells. At later time points, we observed the appearance of a faster-migrating band that cross-reacted with the anti-TGEV nucleocapsid protein antibody. This N′ band appeared around 8 h p.i. in ST cells and slightly later in HRT18jap1 cells. The apparent molecular masses of the faster-

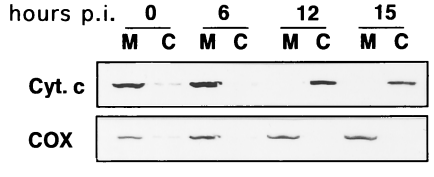


FIG. 3. Redistribution of mitochondrial cytochrome *c* accompanies TGEV-induced apoptosis in the human HRT18jap1 cell line. At the indicated times, cell lysates were analyzed by Western blotting with an anti-human cytochrome *c* antibody (Cyt. c) and, as a control for mitochondrial localization, an anti-cytochrome oxidase (COX) antibody. M, mitochondrial fraction; C, cytosolic fraction.

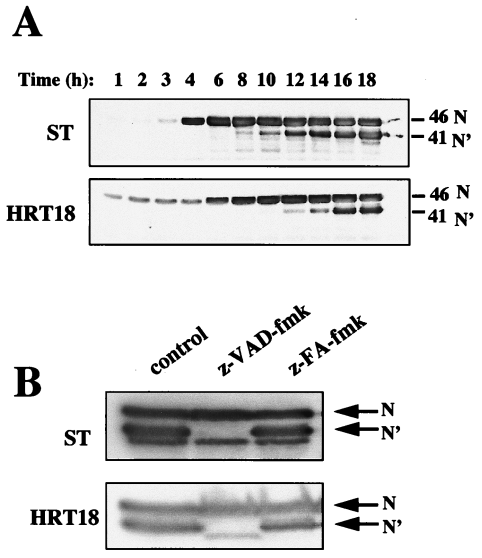


FIG. 4. The TGEV nucleocapsid protein is degraded during infection in a caspase-dependent manner. (A) Kinetics of N synthesis and subsequent degradation (N′) in ST and HRT18jap1 cells. Cell lysates were prepared at the indicated times p.i. and subsequently analyzed by Western blotting using anti-N antibodies. Numbers at the right are molecular masses, in kilodaltons. (B) Appearance of N′ is inhibited by the cell-permeative caspase inhibitor z-VAD.fmk but not by the cathepsin B cell-permeative inhibitor z-FA.fmk. At 0 h p.i., cells were treated either with 100 μM z-VAD.fmk or with 100 μM z-FA.fmk, as indicated. Cells were lysed at 16 h p.i., followed by analysis of N by Western blotting.

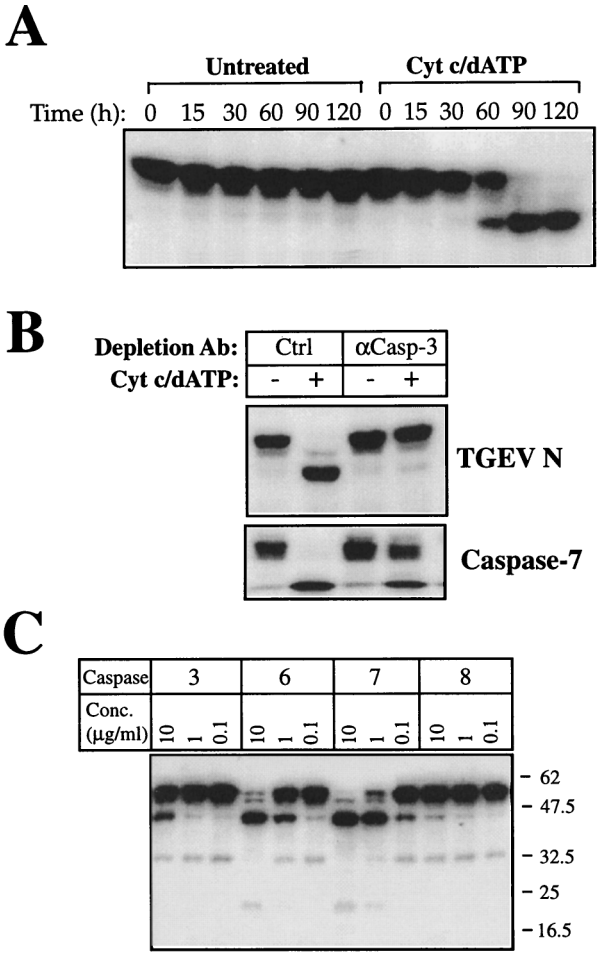


FIG. 5. Cleavage of the TGEV N protein in Jurkat cell extracts requires caspase-3. (A) ^{35}S-labeled N, prepared by coupled in vitro transcription/translation, was incubated for the indicated times in Jurkat cell extracts in the presence or absence of cytochrome *c* (Cyt c) (50 μg/ml) and dATP (1 mM) as indicated, followed by analysis by SDS-PAGE and fluorography. (B) Immunodepletion of caspase-3 from Jurkat extracts abolished proteolytic cleavage of the TGEV N protein and partially inhibited caspase-7 activation. Ab, antibody; Ctrl, control; αCasp-3, anti-caspase-3. (C) ^{35}S-labeled TGEV N was incubated for 2 h with the indicated concentrations of purified recombinant caspase-3, -6, -7, or -8, as described in Materials and Methods, and reaction products were analyzed by SDS-PAGE and fluorography. Numbers at the right are molecular masses, in kilodaltons.

migrating form of N (41 kDa) were identical in the two cell lines.

To determine whether the N′ band could be the product of a caspase-mediated TGEV nucleocapsid protein attack during apoptosis of the host cell, we used the cell-permeative caspase inhibitors z-VAD.fmk and z-IETD.fmk. As shown in Fig. 4B, the appearance of the faster-migrating form of N was abolished by z-VAD.fmk in ST and HRT18jap1 cells. The peptide z-IETD.fmk also inhibited the appearance of the N′ band in HRT18jap1 cells (data not shown). In contrast, the cathepsin B inhibitor z-FA.fmk did not inhibit the appearance of the N′ fragment. These data strongly suggested that N′ was a proteolytic fragment of N generated by caspase-mediated cleavage. Thus, cleavage of N seemed to be a direct consequence of the induction of apoptosis in TGEV-infected cells, with the viral nucleocapsid protein coming under direct attack by the host cell death machinery. The observation that N was cleaved by caspases during viral infection suggested that this could be a strategy adopted by the host cells for limiting virus production. As a preliminary approach to testing this possibility, we treated TGEV-infected cells with the caspase inhibitor z-VAD.fmk to block caspase-mediated cleavage of N. However, this approach did not significantly enhance viral yields by ST cells, nor did it restore virus production by HRT18jap1 cells (reference 10 and data not shown).

Cleavage of TGEV nucleocapsid protein in cell extracts. To explore further the possibility that the TGEV nucleocapsid protein is a caspase substrate, we used a cell model of apoptosis that recapitulates the Apaf-1–caspase-9-driven caspase cascade (35). Members of our group have previously shown that the addition of cytochrome *c* and dATP to Jurkat T lymphoblastoid cell extracts is sufficient to trigger the caspase-9-dependent activation of caspase-2, -3, -6, -7, -8, and -10 (35). In this system, caspase-9 activates caspase-3 and -7, and caspase-3 then in turn activates caspase-2 and -6. Finally, caspase-6 drives the activation of caspase-8 and -10 (35).

Using this system, we explored whether N was cleaved during apoptosis triggered by the addition of cytochrome *c* and dATP to Jurkat cell extracts. Figure 5A shows that ^{35}S-labeled N, prepared by in vitro transcription and translation, was cleaved in cell extracts where cytochrome *c* and dATP were added but not in control extracts. Immunodepletion of caspase-3 from the extracts—which also abolishes activation of caspase-2, -6, -8, and -10, which are downstream of caspase-3 in this system (35), and partly abolishes the activation of caspase-7—blocked the cytochrome *c*- and dATP-induced cleavage of N (Fig. 5B). This observation implicated caspase-3, or a caspase activated downstream of caspase-3 in this system, in the cleavage of N.

TGEV N is cleaved by caspase-6 and -7 in vitro. To explore the nature of the caspase(s) that is capable of cleaving the N

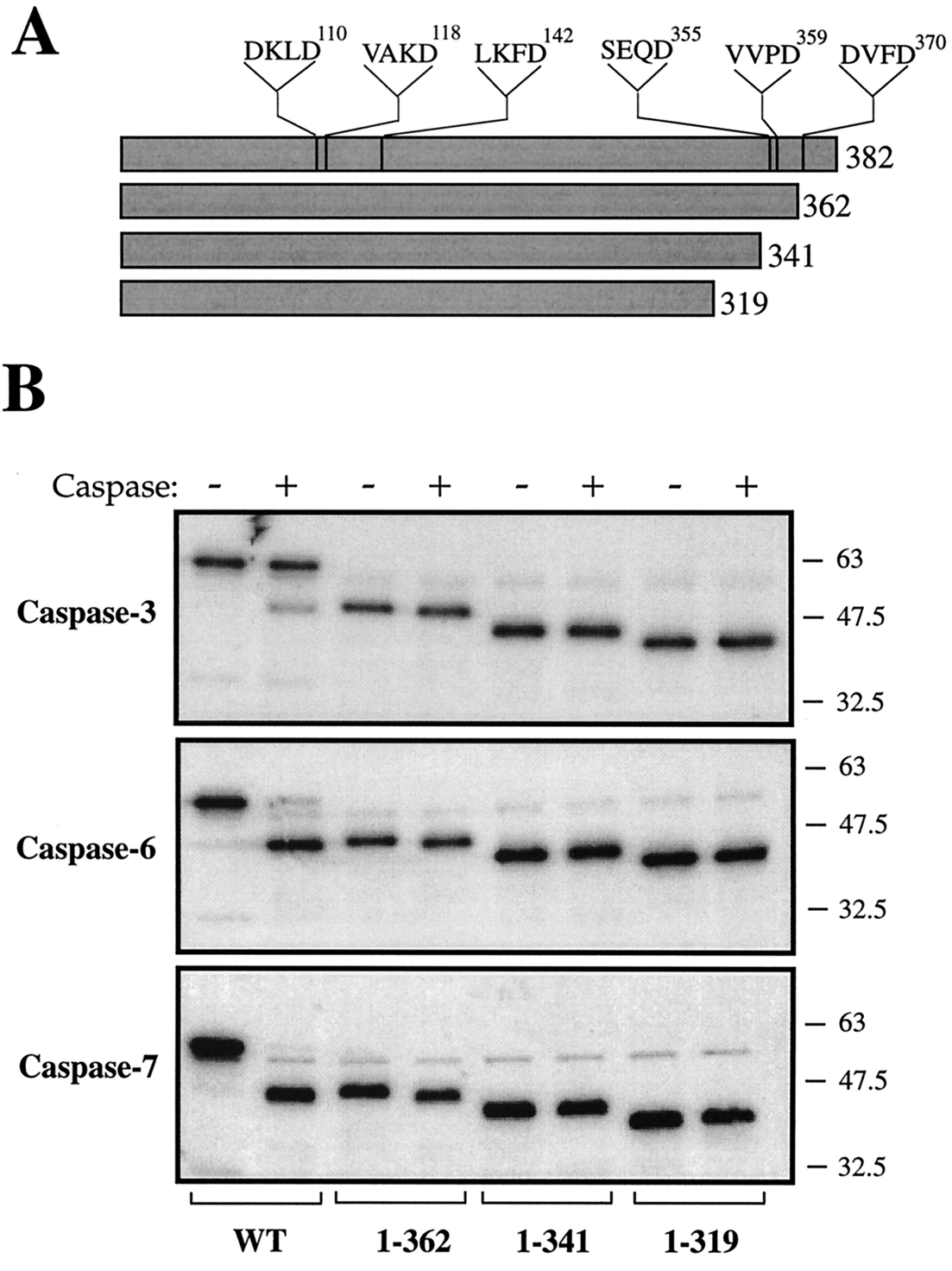


FIG. 6. The TGEV N protein is cleaved by caspase-3, -6, and -7 between residues 342 and 363. (A) Schematic representation of wild-type TGEV N and the potential caspase cleavage sites within the molecule, along with the different C-terminal-deletion mutants generated by the introduction of stop codons using site-directed mutagenesis. The lengths of the wild-type and truncated proteins and the amino acid positions of the potential caspase cleavage sites are indicated. (B) ^{35}S-labeled wild-type N (WT) or the indicated N truncations were incubated for 2 h either alone or with purified recombinant caspase-3, -6, or -7 at final concentrations of 10 μg/ml. Reaction products were analyzed by SDS-PAGE and fluorography. Numbers at the right are molecular masses, in kilodaltons.

protein, we exposed N to recombinant caspase-3, -6, -7, and -8 over a range of concentrations. Figure 5C shows that caspase-6 and -7 were capable of cleaving N very efficiently, whereas caspase-3 cleaved poorly and caspase-8 failed to cleave at any of the concentrations tested.

Site-directed mutagenesis identifies the VVPD^{359} sequence as the caspase cleavage site within the N protein. A number of potential caspase cleavage motifs are present in the N and C termini of the N protein (Fig. 6A). We attempted to microsequence cleaved N but were unsuccessful, suggesting that the N

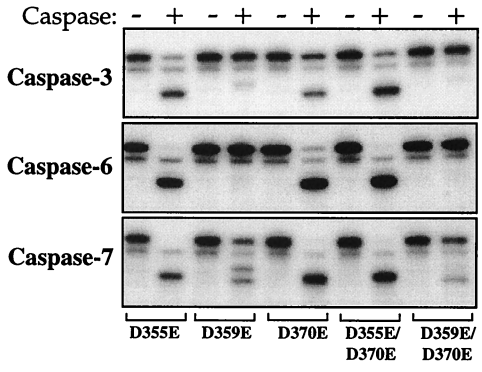


FIG. 7. Mapping and identification of the caspase cleavage sites within the TGEV N protein. The indicated ^{35}S-labeled N point mutants were incubated for 2 h either alone or with purified recombinant caspase-3, -6, or -7 at final concentrations of 10 μg/ml. Reaction products were analyzed by SDS-PAGE and fluorography.

terminus of the protein was blocked and that cleavage occurred at the C terminus (data not shown). We therefore constructed three N deletion mutants lacking different portions of the C terminus by introducing stop codons at the positions corresponding to amino acids E363, R342, and G320. These mutants produced proteins that terminated at amino acids 362, 341, and 319, respectively (Fig. 6A). The mutant N proteins were incubated with recombinant caspase-3, -6, and -7 to assess whether cleavage still occurred. As shown in Fig. 6B, cleavage was detected with the wild-type protein N(1–382), and a small downshift was observed with the N(1–362) deletion mutant but not with the N(1–341) and N(1–319) mutants. This indicated that the caspase cleavage site(s) was located between R342 and E363, implicating Asp residues, D355 and D359, which are present in this region.

We then constructed a panel of point mutants containing amino acid substitutions for Asp355, Asp359, or Asp370 and assessed the cleavage of these mutants by caspase-3, -6, and -7. Figure 7 shows that in all cases, the replacement of Asp359 substantially abolished the cleavage of N by the three caspases tested, thereby implicating the VVPD^{359} motif as the major cleavage site. The mutation of Asp355 had no effect on the cleavage of N by caspase-6 or -7. The mutation of Asp370 had no inhibitory effect with any of the caspases. Although the mutation of Asp359 to Glu substantially inhibited caspase-7-mediated N proteolysis, some cleavage was consistently detected with this mutant. This suggests that caspase-7 also cleaves at another (non-Asp) site within N or that this protease can cleave after Glu to some degree.

DISCUSSION

Several viruses have been shown to encode proteins which inhibit or activate the apoptotic process by interacting with various cellular components (for review, see references 23, 25, and 31). Viral proteins that inhibit cell death could facilitate virus replication, host cell transformation, or tumor progression (16, 40). Virus-induced apoptosis of host cells could either facilitate virus dissemination (7) or be part of the host defense response invoked to counteract viral infection (3). The present study adds a new dimension to the last possibility by suggesting that infected cells could attack the virus from within through caspase-mediated proteolysis of an essential structural protein. During review of this report, Zhirnov et al. also reported N-terminal cleavage of the influenza virus nucleocapsid protein by caspases (45), indicating that many viruses might be targeted by host cell caspases.

TGEV infection is shown to provoke apoptosis of HRT18 cells that have been modified to express the porcine APN. Cell death has been demonstrated to be triggered through caspase-dependent and caspase-independent pathways (13, 42). TGEV induces cell death through a caspase-dependent mechanism that involves the processing of two initiator enzymes (caspase-8 and -9), as well as three downstream effector caspases (caspase-3, -6, and -7). Processing of caspase-8 was detected before that of the other caspases, and treatment of cells with a caspase-8-selective inhibitor (z-IETD.fmk) inhibited apoptosis-associated DNA cleavage, suggesting that caspase-8 might be the primary initiator caspase in this context. Caspase-8 has been recently shown to be activated during the apoptosis of erythroid cells infected with the human parvovirus B19 (37) as well as during the death of cells infected with the Sendai virus (2). Although caspase-8 is the first activated caspase in CD95 ligand- and tumor necrosis factor alpha-mediated cell death, these receptors were shown not to be required for caspase-8 activation in cells infected by the latter virus (2).

The most striking finding of the present study is that a structural protein of the virus—the TGEV nucleocapsid protein—is degraded during the apoptosis of infected cells through caspase-mediated cleavage. This cleavage was replicated in Jurkat cell extracts under conditions designed to trigger caspase activation. The immunodepletion of caspase-3 from the cell extracts abolished proteolytic cleavage of the N protein, indicating that caspase-3, or a downstream protease activated by caspase-3, was required for N protein cleavage. Exploring this further, we found that TGEV N was efficiently cleaved in vitro by recombinant caspase-6 and -7 and rather inefficiently cleaved by caspase-3. Because caspase-6 was activated in TGEV-infected cells as early as 8 h p.i., this caspase is likely to be responsible for N cleavage in vivo, since cleavage was typically detected at 10 h p.i. in TGEV-infected HRT18jap1 cells. The number of proteins identified as substrates for caspase-6 and caspase-7 remains limited (reviewed in reference 8). Using site-directed mutagenesis, we identified the site of caspase-mediated cleavage in the N protein as VVPD^{359}. Interestingly, both caspase-6 and caspase-7 cleave human protein MDM2 at a DVPD site (12). This sequence is similar to the VVPD sequence of the TGEV nucleocapsid protein that is cleaved by caspase-6 and caspase-7.

The appearance of a shorter form of the N protein late in infection has been observed previously with a different strain of TGEV (FS772/70) during infection of porcine LLC-PK1 cells (14). Degradation of the nucleocapsid protein from 47 to 42 kDa was more marked in the LLC-PK1 cells than in other cell lines, and this was correlated with a 10^2-fold reduction in virus production. In addition, other groups have reported for the nucleocapsid protein one or more intracellular polypeptides with lower molecular masses than expected (~2 to 5 kDa less) in cells infected with murine (MHV), feline (FIPV), bovine (BCV), and avian (IBV and TCV) coronaviruses (see reference 19). The VVPD^{359} sequence that is cleaved by caspases during TGEV infection is located 23 amino acid residues upstream of the carboxy-terminal end of the N protein. This VVPD sequence is also present in the C terminus of the MHV (residues 448 to 451) N protein and in the respiratory variant of TGEV called PRCoV (residues 449 to 452). A perfect cleavage site (IETD) for group III caspases, including caspase-6, is also present at the C terminus (residues 385 to 388) of the human coronavirus HCoV-229E N protein. These observations suggest that cleavage of viral nucleocapsid pro-

tein by host cell caspases could be a general mechanism by which infected cells eliminate coronaviruses.

Caspase-mediated cleavage of the N protein might preserve its RNA binding domain, which is located in the central part of the protein (19). Accordingly, the N′ form of the MHV N protein was shown to conserve its RNA binding properties (30). The function of the acidic carboxy-terminal domain of coronavirus N protein remains unknown (19). There is a general agreement that only the full-length N protein is incorporated into coronavirus particles (19). This suggests that the cleaved form of N is unlikely to be used to encapsidate RNA to form new virions. Thus, production of virions might depend on the ability of the virus to replicate rapidly, before the activation of caspases.

Other TGEV proteins contain potential cleavage sites for caspases; e.g., the product of open reading frame 3a (ORF3a)—the function of which is unknown—contains a DELD sequence. This sequence has been identified as the cleavage site for caspase-3 in D4-DGI, a regulator of the Rho family of GTPases that is cleaved by caspase-3 in vitro (22, 26). Three (I/L/V)ExD tetrapeptides are present in the ORF1a product beginning at residues 132 (IEGD), 1010 (VEED), and 1350 (LEPD), and one (VEPD) is present beginning at residue 4806 of the predicted product of ORF1ab, in the polymerase locus. These sequences correspond to the consensus VExD of group III caspases (27, 43), including caspase-6 and caspase-8, that are activated early during TGEV infection. Whether these potential cleavage sites are targeted by caspases during host cell apoptosis remains to be determined.

In conclusion, we have shown that TGEV triggers caspase activation events during infection. A recent study performed with MHV indicated that the E structural protein could be responsible for this activation, whereas other MHV structural proteins, including the M protein, the N protein, and the hemagglutinin-esterase protein, were not involved in virus-induced cell death (1). Some of the caspases activated by TGEV, most likely caspase-6 and/or caspase-7, cleave the viral nucleocapsid protein. This event appears to have limited influence, if any, on viral yields. Ongoing studies might determine whether caspase-mediated N protein cleavage plays a role in viral pathogenicity. Understanding of the mechanisms by which TGEV interacts with host cell death machinery will lead to a better understanding of viral pathogenicity and might also shed light on the cell death machinery itself.

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