Overexpression of 7a, a Protein Specifically Encoded by the Severe Acute Respiratory Syndrome Coronavirus, Induces Apoptosis via a Caspase-Dependent Pathway

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A novel coronavirus was identified as the etiological agent of severe acute respiratory syndrome (SARS) (8, 12, 16, 24). The SARS coronavirus (SARS-CoV) genome is ~30 kb in length and contains 14 potential open reading frames (ORFs). These contain the replicase gene 1a/1b and correspond to the four structural proteins (spike [S], envelope [E], membrane [M], and nucleocapsid [N]), as well as nine viral proteins, varying in length from 39 to 274 amino acids, with no homologue in other coronaviruses (19, 25, 29). Recently, we showed that two of these proteins, termed U274 and U122, are expressed in SARS-CoV-infected cells (11, 28). U274 (also known as ORF3, X1, or ORF3a [19, 25, 29]) is encoded by the first ORF of subgenomic RNA3, is expressed on the surface of infected cells, and undergoes endocytosis (28). U122 (also known as ORF8, X4, or ORF7a [19, 25, 29]) is encoded by the first ORF of subgenomic RNA7 and contains a signal peptide at the N terminus and a typical endoplasmic reticulum retrieval motif, KRKTE, at the C terminus (11). Since the nomenclature used by Thiel and colleagues (29) is more consistent with those used for other coronaviruses, we shall refer to U274 and U122 as 3a and 7a, respectively, in this paper.

Based on studies with other coronaviruses, it may be predicted that these group-specific proteins are dispensable for viral replication, at least in a cell culture system, but may be important for virus-host interactions and thus contribute to viral fitness (for reviews, see references 5 and 17). Many virus genomes encode gene products that can modulate apoptosis, which has been observed clinically. We showed that the overexpression of 7a, but not of 3a or the viral structural proteins, nucleocapsid, membrane, and envelope, induces apoptosis. 7a induces apoptosis via a caspase-dependent pathway and in cell lines derived from different organs, including lung, kidney, and liver.

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of this total cell suspension was subjected to Western blot analysis (11, 27, 28). For detection of endogenous poly(ADP-ribose) polymerase (PARP) protein, a polyclonal antibody (Cell Signaling Technology, Inc., Beverly, Mass.) that recognizes full-length PARP (116 kDa) and the cleaved form of PARP (83 kDa) was used.

The overexpression of HA-tagged 7a (7a-HA) induces apoptosis in 293T (human kidney epithelial) cells, as evidenced by an increase in caspase-3 protease activity, which is a hallmark of apoptosis, in cells that were transfected with a positive control (HA-BAX; column 1), a negative control (HA-GST; column 2), and the different SARS-CoV proteins (columns 3 to 7). All experiments were performed in duplicate; the average values with standard deviations are plotted. For cell viability assays, experiments were performed in triplicate, and the average percentages (± standard deviations) of live cells, compared to that for HA-GST, which is normalized to 100%, are shown in parentheses above each column. (B) Western blot analysis to determine the cleavage of endogenous full-length PARP, which is a substrate of activated caspase-3, from 116 to 83 kDa (upper panel). Expression levels of the HA-tagged proteins were determined with anti-HA antibody (middle panel), and the amounts of total cell lysates loaded were verified by measuring the level of endogenous actin (lower panel).

To determine the degree of cell death, cell viability was also determined by using the cell proliferation reagent WST-1 (Roche Molecular Biochemicals, Indianapolis, Ind.). In this case, 0.3 x 10^6 cells in a 24-well plate were transfected with 0.35 μg of DNA and then assayed after 16 h, and the percentages of live cells are shown in Fig. 1A in parentheses. Consistent with the caspase assay, overexpression of 7a leads to cell death, as evidenced by the decrease in cell viability to ~44% (Fig. 1A, column 3). Apoptosis was also observed with the expression of either the HA-tagged 7a or the untagged form of 7a and was mediated via caspases, since it was strongly blocked by the pan-

![Diagram A](image)

![Diagram B](image)

![Diagram C](image)

![Diagram D](image)
caspase inhibitor z-VAD-fmk (Fig. 1C and D, lanes 1 to 4). In contrast, no inhibition of apoptosis was observed in the presence of an irrelevant peptide, z-FA-fmk (Fig. 1C, D, lanes 5 to 7). As reported previously (11), about 50% of the precursor form of 7a (17.5 kDa) is cleaved after the signal peptide at the N terminus, yielding a product of 15 kDa (Fig. 1D, lanes 3 and 6). However, the HA-tagged form of 7a does not appear to undergo cleavage, since only a single band of 22 kDa, which corresponds to an unprocessed form of 7a (17.5 kDa) fused with three HA (YPYDVPDYA) motifs (~4 kDa) at the C terminus, is observed (Fig. 1D, lanes 2 and 5). It is yet unknown why the HA tag at the C terminus of 7a affects its processing, but our observations suggest that cleavage of the signal peptide is not essential for the induction of apoptosis.

The experiment was repeated with cell lines derived from different organs (Fig. 2): cervical (HeLa; human cervical carcinoma), lung (A549; human lung carcinoma), liver (HepG2; human hepatocellular carcinoma cells), and kidney (Vero E6 and COS-7; African green monkey kidney epithelial and fibroblast cells, respectively). Increases in caspase-3 protease activities and PARP cleavage were observed when 7a-HA (Fig. 2, lanes 3, 6, 9, 12, and 15) were overexpressed in all the cell lines tested, in comparison to cells transfected with a control plasmid, HA-GST (Fig. 2, lanes 1, 4, 7, 10, and 13). This is consistent with the findings that different organs infected by SARS-CoV showed extensive apoptosis (6, 32). The signal peptide at the N terminus of 7a is cleaved more efficiently in infected cells than in transfected cells (11), but this cleavage is not important for induction of apoptosis, since both mutants 7a-L, which contains mutations at the cleavage site (11), and mat7a, where the signal peptide has been deleted (11), induce the same degree of apoptosis as wild-type 7a (Fig. 3A). This result is also consistent with the data shown in Fig. 1C and D, where 7a and 7a-HA, where the signal peptide is not cleaved, induce similar degrees of apoptosis.

The levels of caspase-3 protease activity in lysates obtained from Vero E6 cells transfected with 7a cDNA (Fig. 3B, lanes 1 and 2) are significantly lower than that for SARS-CoV-infected Vero E6 cells (Fig. 3B, lane 4), even though the expression levels of 7a are comparable (Fig. 3B). These cells were infected at a multiplicity of infection of 0.1 and harvested 24 h postinfection as previously described (28). This implies that 7a is not the only apoptosis-inducing factor during SARS-CoV infection in Vero E6 cells. This is not surprising, since coronaviruses are known to cause cell-cell fusion during the late stages of infection, resulting in syncytium formation and cytopathic effects (17). In future studies, it would be crucial to determine the precise contribution of 7a to the cytopathic effects of SARS-CoV infection, for example, by studying infectious clones with...
the 7a gene deleted or by determining if there is a correlation between the expression of 7a and the degree of apoptosis in clinical samples.

Induction of apoptosis has been observed during infection by other coronaviruses, including mouse hepatitis virus (20), feline infectious peritonitis virus (13), transmissible gastroenteritis coronavirus (9), and human coronavirus strain 229E (7).

FIG. 3. Induction of apoptosis by overexpression of wild-type 7a and 7a mutants (7a-L and mat7a) in A549 and Vero E6 cells and by SARS-CoV infection of Vero E6 cells. (A) The CaspACE fluorometric assay system from Promega Corporation was used to measure the activation of caspase-3 protease in different cell lines that were transfected with HA-GST (negative control) (lanes 1 and 5), wild-type 7a (lanes 2 and 6), mutant 7a-L (lanes 3 and 7), and mutant mat7a (lanes 4 and 8) (first panel). 7a-L contains mutations at the signal peptide cleavage site and is cleaved less efficiently than the wild type, and mat7a does not contain the signal peptide (see reference 11). Western blot analyses were performed to determine the expression levels of the GST and 7a proteins (anti-7a; second panel) and endogenous actin as a loading control (antiactin; third panel). (B) Caspase-3 protease activities in Vero E6 cells transfected with 1.0 μg (lane 1) or 2.0 μg (lane 2) of 7a plasmid or mock-infected cells (lane 3) or SARS-CoV-infected cells (lane 4) were determined (first panel). Western blot analyses were performed to determine the expression levels of 7a (anti-7a; second panel), SARS-CoV N (anti-N [12a]; third panel), and endogenous tubulin as a loading control (monoclonal antitubulin [Sigma]; fourth panel) in 20 μg of cell lysates.
as the E protein of mouse hepatitis virus (1) or a 58-kDa protein encoded in ORF 1b of infectious bronchitis virus (18), was sufficient to induce apoptosis. Our data suggest that 7a of SARS-CoV is another example of a coronavirus protein that can induce apoptosis when overexpressed. Although 7a was detected in SARS-CoV-infected Vero E6 cells (11), it is still unknown at what level this protein is expressed in infected organs. Nevertheless, the ability of 7a to induce apoptosis in different cell types is consistent with the clinical observation of apoptosis in different organs infected by SARS-CoV and suggests that the expression of 7a during infection may be one of the underlying mechanisms for the pathogenesis of SARS-CoV infection.

REFERENCES


