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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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Besides genes that are homologous to proteins found in other coronaviruses, the severe acute respiratory syndrome coronavirus genome also contains nine other potential open reading frames. Previously, we have characterized the expression and cellular localization of two of these "accessory" viral proteins, 3a (previously termed U274) and 7a (previously termed U122). In this study, we further examined whether they can induce 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.

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 (for a review, see references 2–4, 10, 14, 26, and 30). Induction of apoptosis in infected cells can contribute directly to viral pathogenesis, while inhibition of apoptosis can prevent premature death of the infected cells, allowing the virus to replicate to a high titer or allowing the establishment of a persistent infection. In the case of SARS-CoV, studies on the clinical features of the disease have revealed some common abnormalities, such as elevated lactate dehydrogenase, lymphopenia, thrombocytopenia, hypocalcemia, and liver enzyme abnormalities (for a review, see references 15, 21, 22, and 23). Besides lymphopenia, which is caused by the depletion of T lymphocytes by apoptosis, one study also reported apoptosis in the hepatocytes of three SARS patients who had liver impairment (6). Apoptosis was also observed in Vero E6 cells infected by SARS-CoV (31). Overall, these findings indicate that apoptosis plays an important role during SARS-CoV infection. In order to determine if 3a and 7a play any role in the induction of apoptosis during infection, we examine the ability of these two SARS-CoV-specific proteins to induce apoptosis in cell lines derived from different organs.

We first transfected 293T cells with cDNA constructs for expressing the various SARS-CoV genes, using a lipofectamine reagent (Invitrogen, Carlsbad, Calif.). These constructs were obtained from SARS-CoV 2003VA2774, an isolate from a SARS patient in Singapore (11, 27, 28). Two micrograms of DNA was used to transfect 10^6 cells, and the cells were left for approximately 16 h. When HA-tagged glutathione *S*-transferase protein (HA-GST) was expressed, it was observed that the expression level of this protein was extremely high; therefore, 0.25 μg of the plasmid for expressing HA-GST together with 1.75 μg of empty vector were used instead. The cells were harvested and washed with phosphate-buffered saline and then resuspended in ~100 μl of hypotonic cell lysis buffer. The suspension of cells was divided into two aliquots, one to be used for measuring caspase-3 protease activity and one for Western blot analysis.

One aliquot was subjected to five rounds of freeze-thaw cycles and then centrifuged to remove the cell debris. The total protein concentration in the lysate was determined by using Coomassie Plus reagent from Pierce (Rockford, Ill.). The amount of caspase-3 protease activity in 10 μg of total protein was then determined by using the CaspACE fluorometric assay system from Promega Corporation (Madison, Wis.). Laemmli's sodium dodecyl sulfate buffer was added to the other aliquot, which was then heated at 100°C for 10 min, and then

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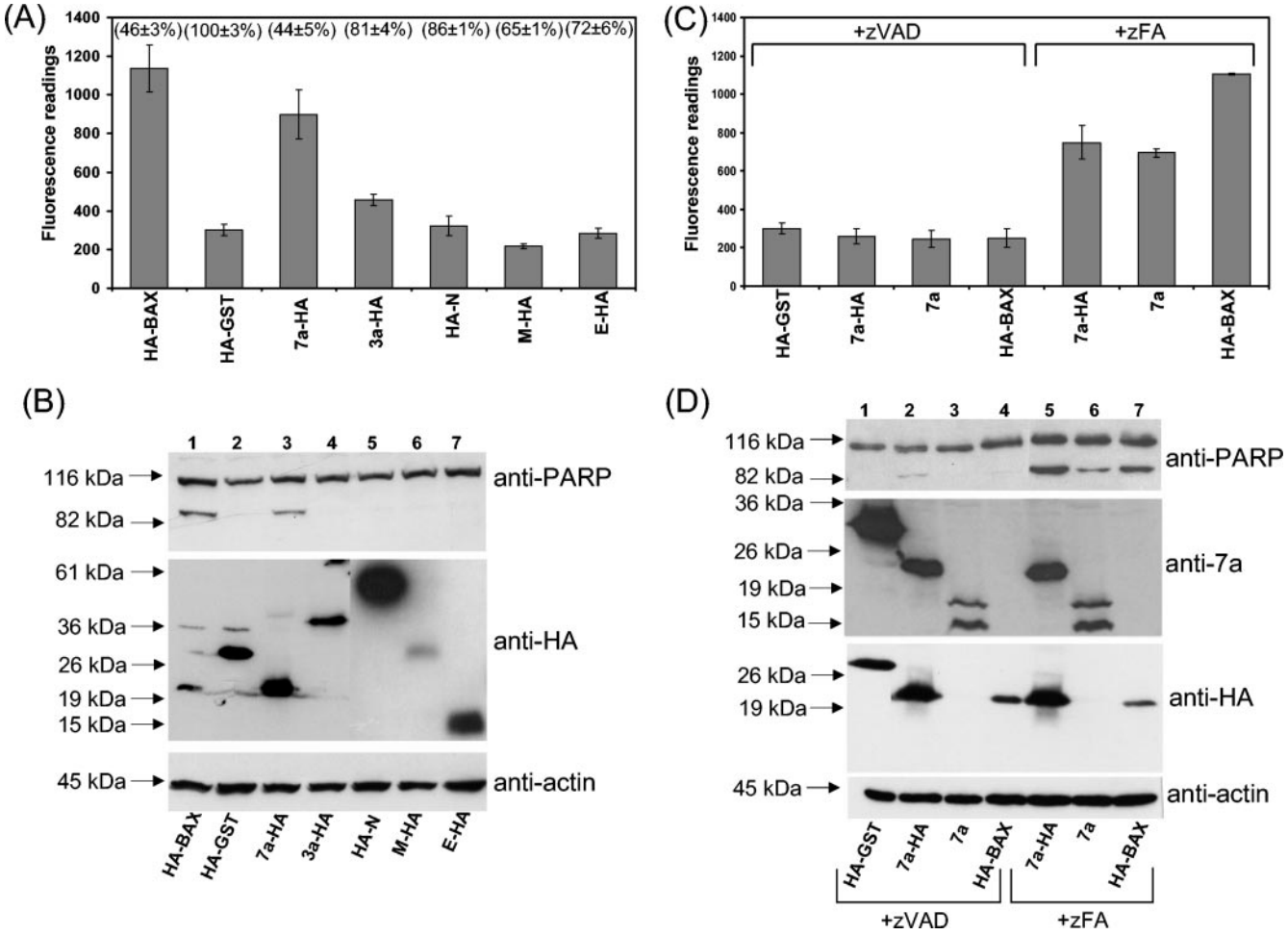

FIG. 1. Expression of the viral proteins in 293T cells and the effects on apoptosis. (A) The CaspACE fluorometric assay system from Promega Corporation was used to measure the activation of caspase-3 protease activity, which is a hallmark of apoptosis, in cells that were transfected with a positive control (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). (C) The CaspACE fluorometric assay system from Promega Corporation was used to measure the activation of caspase-3 in cells that were transfected with 7a-HA, 7a, or HA-BAX in the presence of a pan-caspase inhibitor, zVAD-fmk (columns 2 to 4) or an irrelevant peptide, zFA-fmk (columns 5 to 7). (D) Western blot analysis were performed to determine the cleavage of endogenous PARP (upper panel), expression levels of HA-GST, 7a-HA, 7a, and HA-BAX (anti-7a or anti-HA; middle panels), and endogenous actin as a loading control (antiactin; lower panel). Since the anti-7a antibody was obtained by using a GST-fusion protein, it recognizes both the 7a and GST proteins.

20 μl 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, a hallmark of apoptosis, which is comparable to that caused by the overexpression of BAX, a proapoptotic member of the Bcl-2 family (Fig. 1A, columns 1 and 3). Cleavage of endogenous PARP was also observed when either 7a-HA or HA-BAX was overexpressed (Fig. 1B, lanes 1 and 3). On the other hand, 3a-HA did not significantly induce apoptosis; neither did the structural proteins, M-HA, E-HA, and HA-N (Fig. 1A and B, lanes 4 to 7). 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, 0.3×10^6 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-

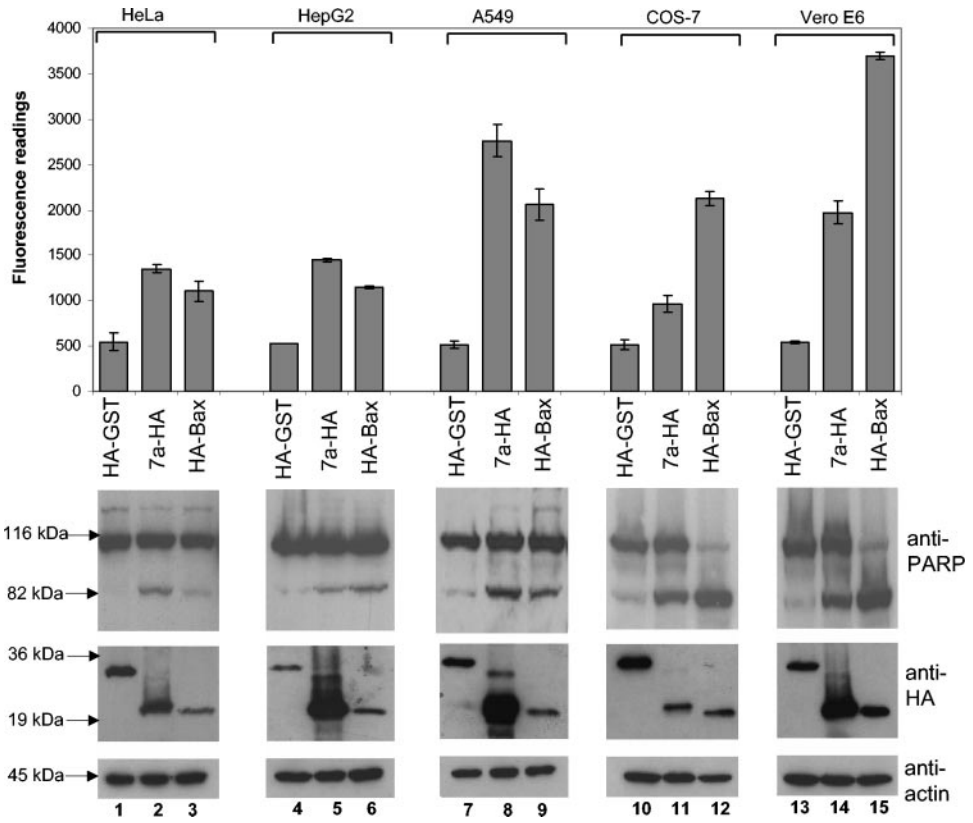

FIG. 2. Induction of apoptosis by 7a in cell lines derived from different organs. The cell lines used were HeLa (cervical carcinoma) (lanes 1 to 3), HepG2 (liver carcinoma) (lanes 4 to 6), A549 (lung carcinoma) (lanes 7 to 9), and COS-7 and Vero E6 (kidney) (lanes 10 to 12 and 13 to 15, respectively). All cell lines were purchased from the American Type Culture Collection (Manassas, Va.). 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, 7a-HA, and HA-BAX (first panel). Western blot analysis were performed to determine the cleavage of endogenous PARP (second panel), expression levels of HA-GST, 7a-HA, and HA-BAX (anti-HA) (third panel), and endogenous actin as a loading control (antiactin) (fourth panel).

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 cells), lung (A549; human lung carcinoma cells), 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 2, 5, 8, 11, and 14) or HA-BAX (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

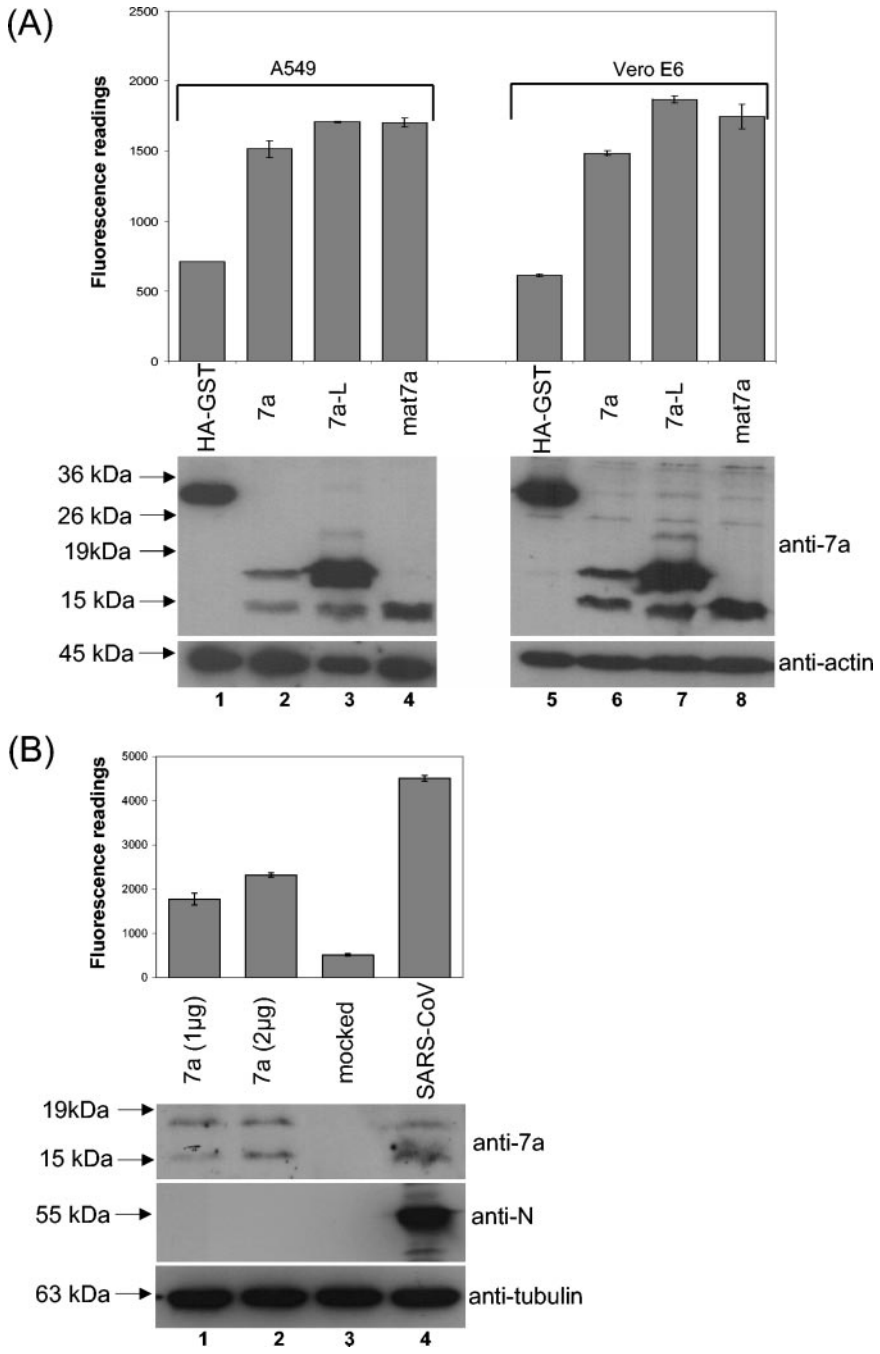

FIG. 3. Induction of apoptosis by overexpression of wild-type 7a and 7a mutants (7a-L and mat7a) in A549 and Vero E6 cells and by SARS-CoV infection of Vero E6 cells. (A) The CaspACE fluorometric assay system from Promega Corporation was used to measure the activation of caspase-3 protease in different 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.

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). In some cases, the overexpression of a single viral protein, such

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.

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