

Severe acute respiratory syndrome coronavirus 3a protein activates the mitochondrial death pathway through p38 MAP kinase activation

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The molecular mechanisms governing severe acute respiratory syndrome coronavirus-induced pathology are not fully understood. Virus infection and some individual viral proteins, including the 3a protein, induce apoptosis. However, the cellular targets leading to 3a protein-mediated apoptosis have not been fully characterized. This study showed that the 3a protein modulates the mitochondrial death pathway in two possible ways. Activation of caspase-8 through extrinsic signal(s) caused Bid activation. In the intrinsic pathway, there was activation of caspase-9 and cytochrome *c* release from the mitochondria. This was the result of increased Bax oligomerization and higher levels of p53 in 3a protein-expressing cells, which depended on the activation of p38 MAP kinase (MAPK) in these cells. For p38 activation and apoptosis induction, the 3a cytoplasmic domain was sufficient. In direct Annexin V staining assays, the 3a protein-expressing cells showed increased apoptosis that was attenuated with the p38 MAPK inhibitor SB203580. A block in nuclear translocation of the STAT3 transcription factor in cells expressing the 3a protein was also observed. These results have been used to present a model of 3a-mediated apoptosis.

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INTRODUCTION

The aetiological agent for severe acute respiratory syndrome (SARS) was identified as a novel coronavirus (SARS-CoV) (Peiris *et al.*, 2003). SARS-CoV has a polyadenylated, positive-sense RNA genome of approximately 30 kb (Marra *et al.*, 2003). In addition to the prototypic coronavirus genes, the SARS-CoV genome also contains nine unique open reading frames (ORFs) (Marra *et al.*, 2003). Of these, *orf3a* is the largest and encodes a protein of 274 aa, variously called ORF3A (Ito *et al.*, 2005), X1 (Rota *et al.*, 2003) or U274 (Tan *et al.*, 2004b). The 3a protein has been predicted to contain an N-terminal signal peptide followed by three transmembrane domains and a C-terminal cytoplasmic domain of approximately 150 aa (Zeng *et al.*, 2004).

The 3a protein is associated with virus particles produced following infection of Vero E6 or Caco-2 cells (Ito *et al.*, 2005; Shen *et al.*, 2005) and can assemble into virus-like particles when co-expressed with the membrane and envelope proteins in insect cells (Shen *et al.*, 2005). *In vitro* studies have also shown the 3a protein to interact with the viral envelope, membrane and spike proteins (Tan *et al.*, 2004b; Tan, 2005) and the cellular protein caveolin-1 (Padhan *et al.*, 2007). A deletion of *orf3a* was shown to reduce virus titres, but not to eliminate virus replication

(Yount *et al.*, 2005), and convalescent sera from SARS patients have antibodies to the 3a protein (Tan *et al.*, 2004a), suggesting that it is expressed during virus infection of the host. The 3a protein was shown to upregulate the expression of fibrinogen in A549 lung epithelial cells (Tan *et al.*, 2005) and to possess an ion-channel activity selective for monovalent cations (Lu *et al.*, 2006).

Ectopic expression of the 3a protein has been shown to induce apoptosis in Vero E6 cells through activation of caspase-8, chromatin condensation and DNA fragmentation (Law *et al.*, 2005). In a *Drosophila melanogaster* expression system, it was also found to modulate cellular cytochrome *c* levels and caspase activity (Wong *et al.*, 2005). It has recently been shown to induce G₁-phase cell cycle arrest via the cyclin D3/pRb pathway in HEK293, COS-7 and Vero cells (Yuan *et al.*, 2007).

Apoptosis is a cell-death programme that is initiated in response to various extrinsic and intrinsic signals such as cellular stress, including virus infection, and plays an important role in the pathogenesis of many viruses (Wang *et al.*, 2007). The extrinsic pathway initiates at the cell surface following occupation of death receptors by their cognate ligands; the best examples of this are the Fas/Fas ligand and tumour necrosis factor receptor (TNFR)/TNF

pathways (Tartaglia *et al.*, 1993). The intrinsic pathway is controlled by multiple sensor proteins, such as those of the Bcl-2 family, and initiates at the mitochondria, leading to their depolarization and leakage of molecules such as cytochrome *c* (Green & Kroemer, 2004). Following the initial signal and its transduction, the effector caspases are activated, causing the activation of endonucleases and degradation of cellular proteins (Zou *et al.*, 1999). Whilst different caspases are activated downstream of the extrinsic and intrinsic pathways – caspase-8 for the former and caspase-9 for the latter – the signal is eventually integrated through the activation of caspase-3, the central effector for both pathways (Mayer & Oberbauer, 2003).

The Bcl-2 family includes both pro-apoptotic (Bad, Bak and Bax) and anti-apoptotic (Bcl-2 and Bcl-X_L) proteins that are critical modulators of the intrinsic death pathway (Adams & Cory, 1998). The multi-domain Bax protein exists as a monomer in the cytoplasm of viable cells, but upon stimulation by various death insults it undergoes conformational changes, dimerizes and translocates to the mitochondria, where it inserts into the outer membrane, causing the release of cytochrome *c* (Wolter *et al.*, 1997). The BH3-only pro-apoptotic proteins Bid, Bim, Noxa, Puma and as-yet-unidentified proteins or lipids mediate induction of the active conformation of Bax by death stimuli (Kuwana *et al.*, 2002; Oda *et al.*, 2000). This is either through association with anti-apoptotic Bcl-2 family members or by stimulating other apoptosis-promoting factors (Chen *et al.*, 2005). Many apoptotic stimuli utilize Bax to kill cells (Zhang *et al.*, 2000). Its overexpression alone is also sufficient to induce apoptosis in many cell types (Xiang *et al.*, 2000). The tumour suppressor and transcription factor p53 can also enhance the pro-apoptotic activity of Bax, either by inducing its expression or by sequestering Bcl-2 or Bcl-X_L in the cytoplasm (Miyashita & Reed, 1995).

We show here that, in addition to caspase-8 activation (Law *et al.*, 2005), the SARS-CoV 3a protein also activates caspase-9 and other elements of the intrinsic pathway, including cytochrome *c* release, Bax oligomerization, p53 upregulation and p38 MAP kinase (MAPK) activation. We also show p38 MAPK activation to be important for the pro-apoptotic effects of the 3a protein.

METHODS

Materials and plasmids. All common reagents were from Sigma Chemical Co. unless otherwise stated. Huh7 cells were cultured at 37 °C in 10% CO₂ in complete Dulbecco's modified Eagle medium and 10% fetal bovine serum. Antibodies generated against a recombinant 3a protein have been described previously (Padhan *et al.*, 2007). Antibodies to caspase-3, cleaved caspase-3, caspase-9, cleaved caspase-9 and cleaved PARP were part of the Apoptosis Sampler kit (Cell Signaling Technology). All other antibodies were obtained from either Santa Cruz Biotechnology or Cell Signaling Technology. The phospho-specific antibodies included: pErk (T202/Y204), pJNK (T183/Y185), pp38 (T180/Y182), pMKK3/6 (S189/207) and pStat3 (Y705) (Cell Signaling Technology). The cloning of *orf3a*

and construction of the pSGI-3a-HA, pSG-3a-cyto, 3a-ECFP and 3a-DsRed expression vectors has been described previously (Padhan *et al.*, 2007). The pEGFP-cytC plasmid was a gift from Dr Israrul Haq Ansari (University of Nebraska, Lincoln, USA). The STAT3 reporter pLucTKS3 and the control pLucTK plasmids were obtained from Dr Gulam Waris (University of Colorado Medical School, USA). The STAT3-EGFP plasmid was a kind gift of the Darnell laboratory (Rockefeller University, NY, USA).

Transfection and Western blotting. Typically, 5 µg plasmid DNA was used for each 60 mm dish of Huh7 cells. Transfections were carried out using Lipofectin reagent (Invitrogen) according to the manufacturer's instructions. At the indicated times post-transfection (p.t.), cells were scraped, lysed in 1 × cell lysis buffer [20 mM Tris/HCl (pH 8), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM sodium orthovanadate and protease inhibitor cocktail (Roche)] and clarified by centrifugation at 16 000 g for 20 min at 4 °C. The protein concentration of the lysate was estimated using Bradford reagent (Bio-Rad). Western blotting was carried out as described elsewhere (Padhan *et al.*, 2007).

Cross-linking experiments. Transfected Huh7 cells were washed and harvested in PBS at 48 h p.t. The p38 inhibitor SB203580 (Sigma) was added 12 h before harvest at a concentration of 20 µM in fresh complete medium. Washed cells were resuspended in PBS containing protease inhibitor cocktail (Roche) and lysed by repeated freeze–thaw cycles in liquid N₂ and 37 °C. The unclarified lysate was then cross-linked with 10 mM disuccinimidyl suberate (DSS; Sigma) at 30 °C for 30 min and the reaction stopped with 50 mM Tris/HCl (pH 8.0). The lysate was then prepared in 2 × in SDS loading buffer, boiled and separated by 10% SDS-PAGE.

Immunofluorescence and subcellular localization. Cells grown on coverslips to 40–50% confluence were transfected and then imaged as described previously (Padhan *et al.*, 2007). For subcellular localization of cytochrome *c*, Huh7 cells were co-transfected with pEGFP-cytC and pDsRed-mito (Clontech) either with or without plasmid 3a-ECFP. The percentage co-localization was calculated using LaserPix software (Bio-Rad). For STAT3 imaging, Huh7 cells were co-transfected with the STAT3-EGFP vector together with either the 3a-DsRed or the pDsRed-N1 plasmid. After 36 h, cells were serum-starved overnight and then treated with 100 ng epidermal growth factor (EGF) ml⁻¹ for 3 h, washed with PBS and imaged.

Flow cytometry. Huh7 cells were transfected as above and, 36 h later, the cells were switched to fresh medium containing 20 µM SB203580 for 12 h. Subsequently, 1.5 × 10⁶–1.8 × 10⁶ cells were harvested in 0.02% EDTA, washed twice with PBS and stained with 3 µl phycoerythrin (PE)-conjugated Annexin V (BD Pharmingen) in 40 µl binding buffer [10 mM HEPES (pH 7.4), 140 mM NaCl, 2.5 mM CaCl₂]. The cells were then fixed with 100 µl fixative (Intrastain kit; Dako) for 15 min at room temperature and washed with PBS. The pellet was resuspended in 100 µl permeabilization buffer (Intrastain kit; Dako) at room temperature for 15 min. After washing with PBS, the cells were resuspended in 1:100-diluted rabbit anti-3a and incubated for 30 min at room temperature, followed by two washes in PBS. The cells were then counterstained with 1:500-diluted Alexa 488-conjugated anti-rabbit antibody (Molecular Probes) at room temperature for 45 min. After two washes in PBS containing 1% BSA, the cells were resuspended in 400 µl of the same buffer for acquisition. For each sample, 30 000 events were acquired using a CyAn-ADP flow cytometer (Dako) and the data analysed using Summit software (version 4.3). The cells were gated for 3a expression, and Annexin V staining was determined in 3a-positive and -negative cells, the latter being treated as controls. Apoptosis (±SD) was calculated over four different experiments and the results

were calculated as percentage values. *P* values were calculated using Student's *t*-test with 95 % confidence intervals.

Luciferase assay. Transfected cells were collected at 36 h p.t., serum-starved overnight and then treated with 100 ng EGF ml⁻¹ for 3 h, washed with PBS and harvested. The preparation of cytosolic extracts and luciferase assays were carried out using a Luciferase assay kit (Promega) according to the supplier's protocol and readings were taken on a luminometer (Sirius).

RESULTS

SARS-CoV 3a protein activates the intrinsic pathway of apoptosis

The 3a protein has been shown previously to induce apoptosis in Vero E6 cells. In this system, there was increased caspase-8 activation, but no change in Bad or Bcl-2 levels, suggesting that the death-receptor (extrinsic) apoptosis pathway was activated (Law *et al.*, 2005). We chose to study the apoptotic activity of the 3a protein in the Huh7 human hepatocellular carcinoma cell line, as liver cells have been shown to be permissive for SARS-CoV infection and replication (Kaye, 2006). Furthermore, these cells are negative for caveolin, unlike Vero or A549 cells (Damm *et al.*, 2005). We recently showed that the SARS-CoV 3a protein interacts with caveolin (Padhan *et al.*, 2007), a cellular protein with multiple effects, including effects on apoptosis (Timme *et al.*, 2000). Huh7 cells transfected with the pSGI-3a-HA or control plasmid were harvested at different times and checked for various cell-death markers by Western blotting. There was increased activation (cleavage) of caspase-3, the central effector caspase, and its downstream substrates caspase-7 and PARP, in 3a protein-expressing cells (Fig. 1a). Caspase 3 can be activated by either caspase-8 from the extrinsic

pathway or caspase-9 from the intrinsic pathway. Higher levels of cleaved caspase-9 were detected in 3a protein-expressing cells, these effects being more prominent following serum starvation (Fig. 1b). As the 3a protein has been shown previously to activate caspase-8 (Law *et al.*, 2005), we also checked for cleavage of Bid. Truncated Bid (tBid) has effects on the mitochondria and is important for cross-talk between the extrinsic and intrinsic pathways (Luo *et al.*, 1998). Higher levels of tBid were observed in 3a protein-expressing cells (Fig. 1c).

Another hallmark of the intrinsic pathway of apoptosis is the release of cytochrome *c* from mitochondria. In Huh7 cells co-transfected with pEGFP-cytC and pDsRed-mito, the cytochrome *c* signal was found quantitatively in the mitochondria in the absence of 3a protein (Fig. 2, upper panels). However, in cells that co-expressed 3a, cytochrome *c* was found to be extra-mitochondrial (Fig. 2, middle panels). The 3a protein itself did not localize to the mitochondria (Fig. 2, lower panels) and was found predominantly at the plasma membrane and in the Golgi region, as shown previously (Padhan *et al.*, 2007). On image quantification across multiple cells that expressed the 3a protein, only 7.1 ± 4.0 % of the cytochrome *c* signal was found to co-localize with the mitochondrial marker. Thus, caspase-9 activation and cytochrome *c* release in cells expressing the 3a protein indicated that this viral protein also activates the intrinsic pathway of apoptosis.

Bax oligomerization and p53 upregulation are promoted by the 3a protein

Members of the Bcl-2 family of proteins play a major role in regulating cytochrome *c* release from the mitochondria. There were no significant differences in the levels of Bak, Bcl-X_L, Bcl-2 and Mcl-1, as well as the outer-mitochondrial

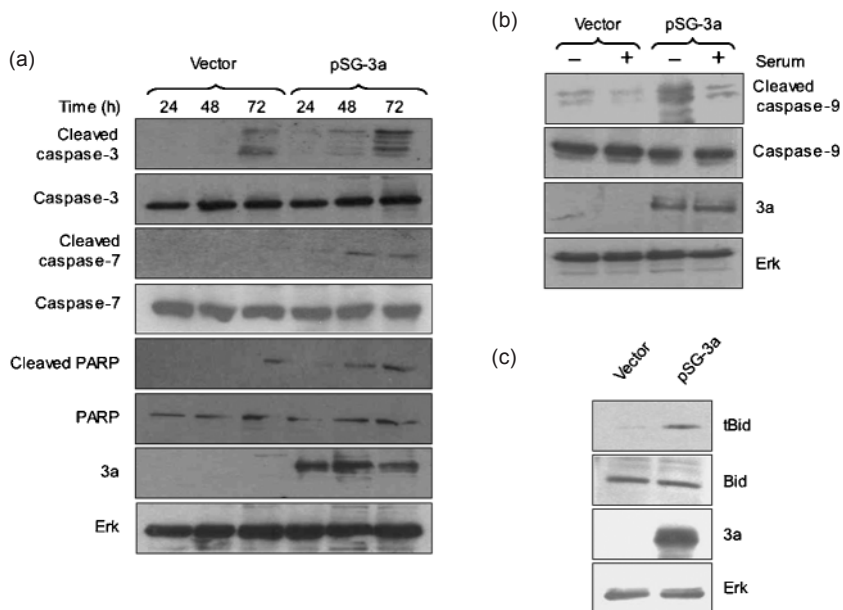


Fig. 1. Apoptosis markers in 3a protein-expressing cells. (a) Huh7 cells were transfected with pSGI-3a or the control vector pSGI. At various times, cell lysates were analysed by Western blotting for the indicated apoptosis marker proteins. (b) Transfected Huh7 cells were either shifted to serum-free medium at 36 h p.t. for 12 h or kept in serum-containing medium. Cell lysates were analysed by Western blotting. (c) Transfected Huh7 cells at 48 h p.t. were analysed by Western blotting.

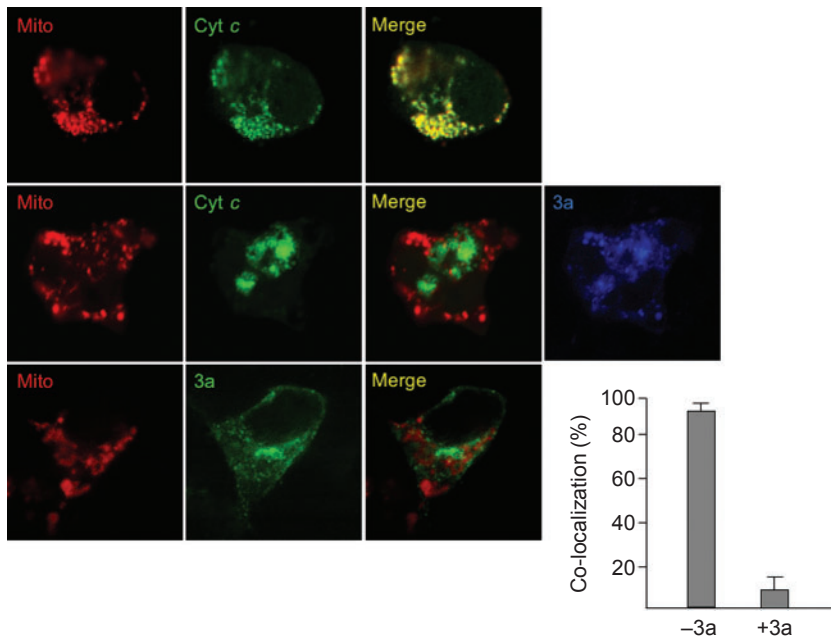


Fig. 2. Cytochrome *c* is released from mitochondria in 3a protein-expressing cells. Huh7 cells were co-transfected with plasmids pEGFP-cytC, pDsRed-mito and either 3a-ECFP or pECFP (vector control). After 48 h, cells were fixed and analysed by confocal microscopy for the distribution of DsRed-mito (mitochondrial marker), cytochrome *c* and 3a protein. Individual pseudocoloured and merged images are shown. The graph on the right shows the percentage co-localization of the mitochondrial marker and cytochrome *c* in cells lacking or expressing the 3a protein. The quantification is based on 20 cells of each kind.

membrane porin VDAC, in 3a protein-expressing versus control cells (Fig. 3a). However, cells expressing the 3a protein showed increased levels of oligomeric Bax (Fig. 3b). These levels also varied with time, peaking at 48 h following pSGI-3a-HA transfection of Huh7 cells. Oligomeric Bax forms a channel in the outer-mitochondrial membrane through which cytochrome *c* and other apoptogenic factors move into the cytosol.

The tumour suppressor protein p53 is pro-apoptotic and works through diverse mechanisms. One of these is based on the activation of *bax* transcription, as the p53-mediated activation of Bax at cytoplasmic sites (Baptiste & Prives,

2004). We found an approximately twofold increase in the levels of p53 in cells expressing the 3a protein at various times p.t. (Fig. 3c). This is likely to be an underestimation, as not all of the transiently transfected cells expressed the 3a protein. In multiple experiments, the transfection efficiency was in the range of 40–50 % of Huh7 cells.

The 3a protein causes p38-mediated upregulation of p53

Many viral proteins regulate cell survival or death by modulating the activity of the MAP kinase family of proteins. In cells expressing the 3a protein, we found no

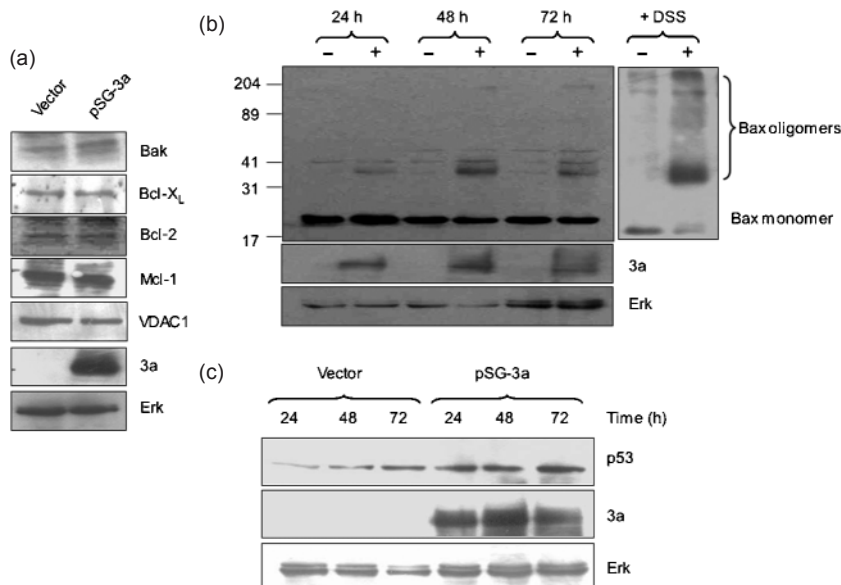


Fig. 3. The 3a protein promotes Bax oligomerization and upregulates p53. (a) Huh7 cells at 48 h p.t. were analysed by Western blotting for the indicated proteins. (b) Huh7 cells at various times p.t. were analysed for Bax by Western blotting. The 48 h sample was also cross-linked with DSS, as described in Methods, prior to SDS-PAGE and Western blotting. (c) Huh7 cells at various times p.t. were analysed for p53 levels by Western blotting.

change in pERK levels and only a small increase in pJNK levels, but significant upregulation of p38 phosphorylation (Fig. 4a). To confirm whether p38 mediated the 3a protein effects, we employed SB203580, a specific inhibitor of p38 activity. As expected, SB203580 did not show any effect on p38 phosphorylation. However, it abolished the increase in p53 levels seen in 3a protein-expressing cells (Fig. 4b), placing p53 downstream of p38. Treatment with SB203580 also reduced Bax oligomerization in cells expressing the 3a protein (Fig. 4c). A small decrease in the levels of cleaved PARP was also observed following SB203580 treatment (Fig. 4b). Complete inhibition of PARP cleavage was not expected, as the 3a protein can also induce apoptosis through caspase-8; this would also contribute to PARP cleavage, but would be insensitive to SB203580.

The activation of p38 is regulated by various stress signals and is mediated by the upstream MAPK kinase isoforms 3 and 6 (MKK3/6). Increased levels of phosphorylated (activated) MKK3/6 were also observed in 3a protein-expressing cells (Fig. 4d). Taken together, these results

support a stress pathway-mediated p38 activation that is directly responsible for p53 upregulation and Bax oligomerization in 3a protein-expressing cells.

The cytoplasmic domain of the 3a protein, but not its caveolin-binding activity, is required for apoptotic activity

The 3a protein of SARS-CoV is predicted to contain three transmembrane regions (aa 34–56, 77–99 and 103–125) and a C-terminal cytoplasmic domain of 149 aa. Expression of the 3a cytoplasmic domain in Huh7 cells led to increases in PARP cleavage and p38 phosphorylation at levels similar to those observed with the full-length protein (Fig. 5a). On densitometric quantification, cells transfected with pSG-3a or pSG-3a-Cyto showed a mean 2.3- and 2.1-fold increase in cleaved PARP, respectively, compared with vector-transfected cells. This indicated that the 3a cytoplasmic domain was sufficient for its pro-apoptotic effects.

We showed recently that the 3a protein interacts with caveolin, a protein that regulates many cellular processes, including apoptosis (Padhan *et al.*, 2007). As the analysis thus far was in caveolin-null Huh7 cells, we also tested the pro-apoptotic effects of the 3a protein in caveolin-expressing monkey kidney COS cells. Immunofluorescent staining of caveolin and 3a protein was carried out in cells transfected with pSG-3a. This confirmed that Huh7 cells did not express caveolin whilst COS cells did, and that both cells showed a similar intracellular distribution of ectopically expressed 3a protein (Fig. 5b). Western blotting of COS cell lysates showed that, in this cellular background, the 3a protein also induced PARP cleavage and p38 MAPK activation (Fig. 5b). Other elements of the 3a protein-mediated apoptotic pathway such as Bax oligomerization, p53 upregulation and caspase-9 activation were also observed in COS cells (Fig. 5c). These results suggested that the pro-apoptotic nature of the 3a protein is not influenced by its ability to bind caveolin.

Activation of p38 MAPK is important for 3a protein-mediated apoptosis

The results reported thus far indirectly suggested a pro-apoptotic role for the 3a protein. To demonstrate directly an effect of the 3a protein on cell death, we measured the ability of 3a protein-expressing and control cells to bind Annexin V. An early event in apoptosis is the loss of membrane phospholipid asymmetry, resulting in the exposure of phosphatidylserine at the surface of the cell. Annexin V binds with high affinity to phosphatidylserine and this binding is used as a marker of apoptosis (Koopman *et al.*, 1994). Using flow cytometry, we measured Annexin V binding to Huh7 cells that expressed the 3a protein and compared this with control cells that did not express the protein. There was a significant increase in Annexin V binding to cells that expressed the 3a protein

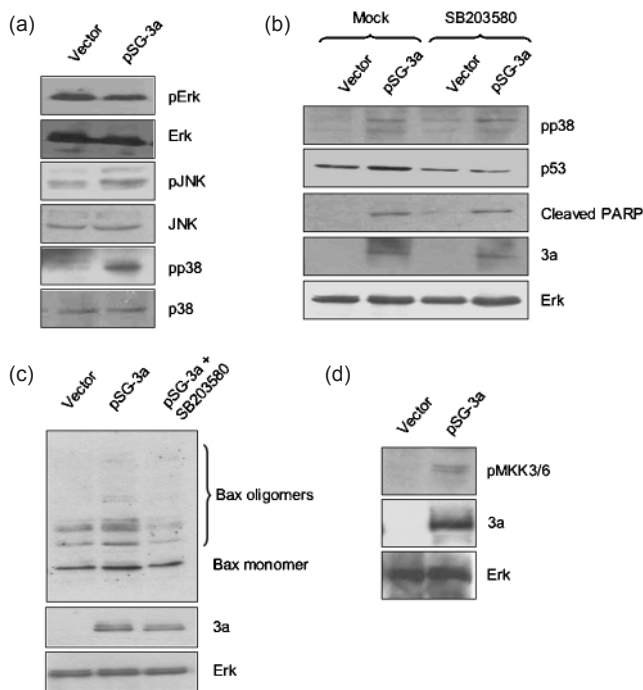


Fig. 4. The 3a protein-mediated p53 upregulation and Bax dimerization are p38-dependent. (a) Huh7 cells at 48 h p.t. were analysed for various MAPKs by Western blotting. (b) Transfected Huh7 cells at 36 h p.t. were shifted to fresh medium without (mock) or with 20 μ M SB203580. After 12 h, cell lysates were prepared and analysed by Western blotting for the indicated proteins. (c) Lysates prepared from transfected Huh7 cells either treated with SB203580 or not were subjected to cross-linking with DSS, as described in Methods. The lysates were then Western blotted for Bax. (d) Lysates from Huh7 cells at 48 h p.t. were analysed for pMKK3/6 by Western blotting.

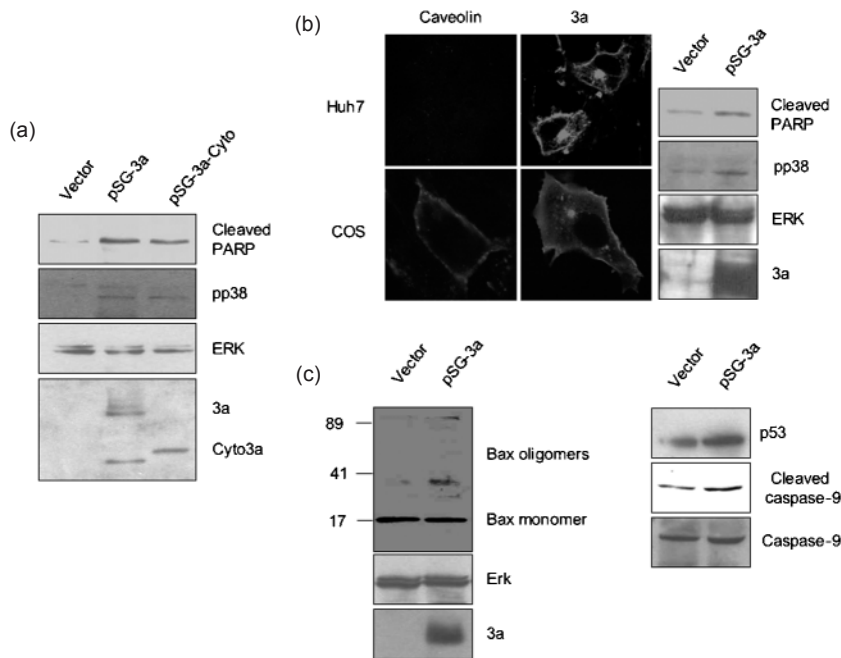


Fig. 5. (a) Huh7 cells were transfected with expression plasmids for full-length or the cytoplasmic domain of 3a protein. Lysates prepared at 48 h p.t. were analysed by Western blotting. (b) Left panels: Huh7 and COS cells transfected with pSG-3a were fixed, permeabilized and stained with antibodies against 3a protein or caveolin-1, followed by confocal microscopy. Right panels: lysates prepared from cells at 48 h p.t. were analysed by Western blotting against the indicated proteins. (c) Lysates of transfected COS cells at 48 h p.t. were cross-linked with DSS and Western blotted for Bax. Cell lysates without cross-linking were analysed for p53 and caspase-9 levels.

compared with control cells (Fig. 6a). However, treatment of the 3a protein-expressing cells with the p38 MAPK inhibitor SB203580 led to reduced Annexin V binding (Fig. 6a). In four separate experiments, we observed a mean of approximately 40% of cells expressing the 3a protein undergoing apoptosis, compared with <0.5% of control cells. Treatment with SB203580 reduced this to about half, with a mean of 19% of 3a-expressing cells demonstrating apoptosis (Fig. 6b). This indicated that p38 MAPK activation is important for the apoptosis of 3a protein-expressing cells.

The 3a protein inhibits nuclear translocation of STAT3

STAT3 is a key factor that negatively regulates p53 transcription by binding to its promoter (Niu *et al.*, 2005). We checked the subcellular localization of transfected STAT3-EGFP either alone or in cells expressing the 3a protein. In control cells following EGF stimulation, most of the STAT3 was found in the nuclear compartment [Fig. 7a(i)]. However, in 3a protein-expressing cells under similar conditions of EGF stimulation, STAT3 was retained in the cytoplasm, showing a punctate distribution and colocalization with the 3a protein [Fig. 7a(ii–iv)]. We tested whether the 3a protein binds STAT3 to retain it in the cytoplasm. In a co-immunoprecipitation assay, no direct interaction was detected between these two proteins (data not shown). To confirm further the inhibition of STAT3 nuclear translocation, we used a reporter gene transfection assay in which plasmid pLucTKS3 containing a minimal thymidine kinase promoter and seven STAT3 response elements was used to drive luciferase expression.

Transiently transfected Huh7 cells showed a marked reduction in luciferase activity when pLucTKS3 was co-transfected with the pSG-3a expression plasmid compared with the vector control (Fig. 7b). Furthermore, by Western blotting, no significant differences were observed in the levels of phospho-STAT3 and total STAT3 in control versus 3a protein-expressing cells (Fig. 7c). Taken together, these results showed that, in cells that express the SARS-CoV 3a protein, there is reduced nuclear localization and consequently decreased transcription factor activity of STAT3.

DISCUSSION

The host uses apoptosis to limit virus replication and remove virus-infected cells (Fujimoto *et al.*, 2000). Apoptosis can also assist in the dissemination of viral particles and is the cause of tissue damage observed following many viral infections (Lyles, 2000). In SARS patients, apoptosis of cells of the lung epithelium has been observed, suggesting SARS-CoV-induced apoptosis to have a deleterious pathogenic role, leading to severe tissue damage (Yan *et al.*, 2004). There are indications that apoptosis and the antiviral immune response may both contribute to the pathology in SARS patients (Yang *et al.*, 2005).

The SARS-CoV 3a, 3b, nucleocapsid and 7a proteins have been shown to induce apoptosis (Law *et al.*, 2005; Yuan *et al.*, 2007). In Vero E6 cells that transiently express the 3a protein, nuclear fragmentation, chromatin condensation, DNA laddering and an increased terminal deoxynucleotidyl transferase dUTP nick end-labelling signal have been

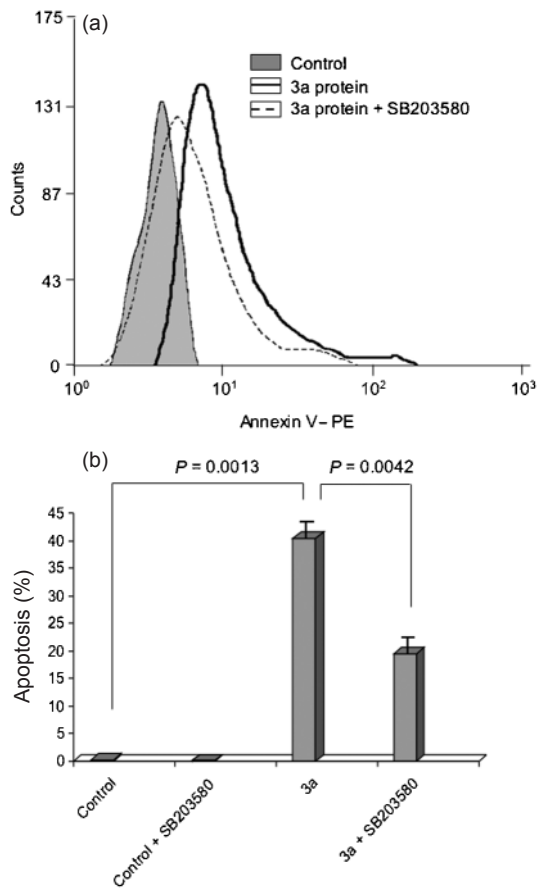


Fig. 6. The apoptotic effect of the 3a protein is mediated by p38. Transfected Huh7 cells were treated with SB203580 or not and the cells stained for Annexin V and the 3a protein as described in Methods. (a) Single-parameter overlay plot for cells gated on 3a expression. (b) Percentage apoptosis (\pm SD) calculated over four different experiments. The *P* values for the indicated sets were calculated using Student's *t*-test and values of $P \leq 0.05$ were considered significant.

observed (Law *et al.*, 2005). Furthermore, there was increased caspase-8 activation, but no change in Bcl-2, Bad or PCNA levels (Law *et al.*, 2005). As caspase-8 is downstream of the cell-surface death receptors (Green & Kroemer, 2004) and Bcl-2 family protein levels regulate the mitochondrial death signal (Zou *et al.*, 1999), this suggested that the 3a protein activated the extrinsic, but not the intrinsic pathway of apoptosis. We studied Huh7 cells that transiently expressed the 3a protein to understand its role in apoptosis. There was increased activation of caspase-3 and -9 as well as cleavage of the caspase-3 substrate PARP in cells expressing the 3a protein. We also observed increased levels of tBid in cells expressing the 3a protein. The Bid protein is cleaved by caspase-8 to generate tBid, which can form pores in the mitochondrial outer membrane and is a key factor that links the extrinsic and intrinsic pathways of apoptosis (Luo *et al.*, 1998).

As caspase-9 is activated downstream of the mitochondrial pathway of apoptosis (Zou *et al.*, 1999), we looked for cytochrome *c* release, a key feature of this pathway. This was observed in cells expressing the 3a protein, confirming a role for this protein in activating the intrinsic pathway as well. The Bcl-2 family proteins are important regulators of the intrinsic pathway (Adams & Cory, 1998). Whilst there was no effect of the 3a protein on the levels of Bcl-2, Bcl-X_L, Mcl-1 and Bak, increased oligomerization of Bax was observed in cells expressing the 3a protein. In viable cells, Bax mainly exists as a monomer in the cytoplasm, but upon apoptotic stimulation it undergoes conformational changes and translocates to the mitochondria, where it inserts into the outer membrane as oligomers; this results in the release of cytochrome *c* and initiation of apoptosis (Wolter *et al.*, 1997).

What might be the cue for Bax oligomerization? Whilst there are many cellular signals that regulate Bax levels, the tumour suppressor protein p53 is unique in that it can activate nuclear transcription of *bax* and it also promotes cytoplasmic oligomerization of Bax monomers (Baptiste & Prives, 2004). Increased levels of p53 were found in 3a protein-expressing cells. The regulation of p53 is complex and includes transcriptional as well as post-translational events such as phosphorylation and acetylation (Meek, 1998). Phosphorylation at Ser-15 and Ser-37 disrupts the interaction between p53 and MDM2, leading to increased stability of p53 (Shieh *et al.*, 1997). p38 MAPK activates transcription as well as phosphorylation of p53 at Ser-15 (Bulavin *et al.*, 1999; She *et al.*, 2000), and p38 activation was observed in cells that expressed the 3a protein. The MAPKs are signal transducers that respond to extracellular stimulation by cytokines, growth factors, virus infection and stress, and in turn regulate cell differentiation, proliferation, survival and apoptosis (Chang & Karin, 2001). p38 MAPK is involved in the cell-death cascade and is strongly activated by stress and inflammatory cytokines following phosphorylation of its Thr-180 and Tyr-182 residues by the upstream kinase MKK3/6 (Noh *et al.*, 2000; Yamagishi *et al.*, 2001), which was also activated in 3a protein-expressing cells. Whilst the upstream events that induce a conformational change in Bax are still unclear, recent studies suggest that caspase activation, Bid association and p38 MAPK initiate changes in Bax conformation, followed by its mitochondrial translocation (Desagher *et al.*, 1999; Ghatan *et al.*, 2000). In SARS-CoV-infected cells, there is increased activation of p38 MAPK and phosphorylation of its downstream targets (Mizutani *et al.*, 2004). Here, we showed that the SARS-CoV 3a protein activates p38 MAPK. The use of SB203580, an inhibitor of p38 activity, showed that it was upstream of p53 and Bax in the 3a protein-mediated apoptotic pathway. Direct flow cytometric assays also showed increased levels of death in cells expressing the 3a protein and inhibition of this following SB203580 treatment. Thus, p38 MAPK activation is an upstream effector of apoptosis directed by the SARS-CoV 3a protein.

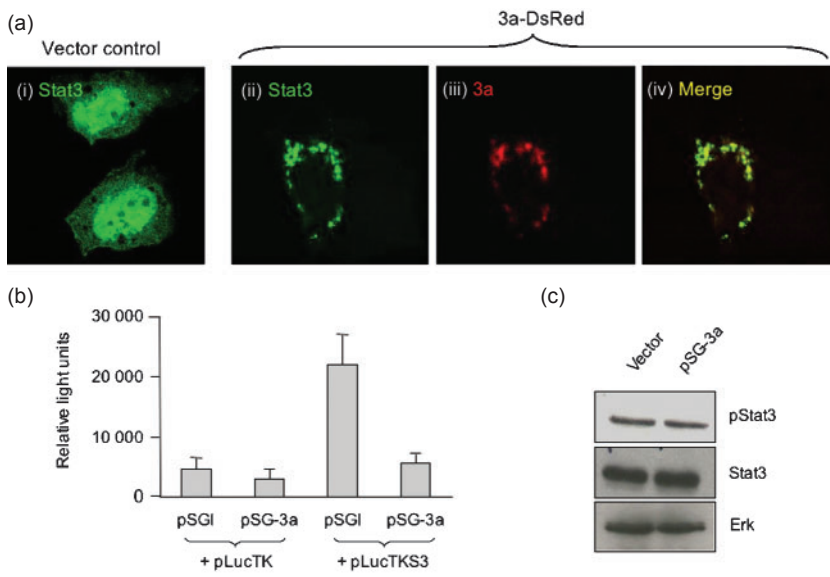


Fig. 7. The 3a protein blocks the nuclear translocation of STAT3. (a) Huh7 cells were co-transfected with the STAT3-EGFP and 3a-DsRed expression constructs. Transfected cells were stimulated with EGF, fixed and analysed by confocal microscopy for STAT3 and 3a protein localization. The pseudo-coloured and merged images are shown. (b) Huh7 cells were co-transfected with the indicated plasmids. After 48 h, cell lysates were prepared, normalized for protein content and assayed for luciferase activity as described in Methods. The graph shows the results from three separate experiments. (c) Lysates prepared from cells at 48 h p.t. with the indicated vectors were separated by SDS-PAGE and analysed by Western blotting.

The STAT proteins are transcription factors that mediate virtually all cytokine-driven signalling (Darnell, 1997). These proteins are latent in the cytoplasm and become activated on tyrosine phosphorylation by cytokine or growth factor receptor-associated kinases, dimerize, translocate to the nucleus and activate target genes (Bromberg, 2002). Whilst the STAT proteins generally regulate cell-cycle progression and apoptosis, STAT3 promotes cell-cycle progression and cellular transformation, and prevents apoptosis. It mediates a survival function by inducing the expression of anti-apoptotic genes such as Bcl-2 and Bcl-X_L (Levy & Lee, 2002). STAT3 can also promote cell survival by inhibiting the transcription of pro-apoptotic genes, including p53 (Niu *et al.*, 2005). Whilst we found no change in the levels of either pSTAT3 or STAT3 following expression of the 3a protein, there was a marked reduction in the nuclear translocation of STAT3 and its transcription factor activity in these cells.

The 3b, ORF6 and nucleocapsid proteins of SARS-CoV function as interferon antagonists by inhibiting the expression of beta interferon (Kopecky-Bromberg *et al.*, 2007). Of these, the ORF6 protein inhibits nuclear translocation of STAT1, a transcription factor that is required for the activation of interferon-responsive promoters. Interestingly, this protein also showed no effect on STAT1 phosphorylation. Our findings showed that the SARS-CoV 3a protein does not affect STAT3 phosphorylation, but inhibits its nuclear entry. Analogous to effects of ORF6 on STAT1, this could be one way of suppressing the inhibitory effects of STAT3 on p53, leading to increased p53 levels and its downstream effects on Bax and promotion of apoptosis.

Earlier work has demonstrated apoptosis of SARS-CoV-infected cells, and a role for the 3a protein was proposed based on its activation of caspase-8 (Law *et al.*, 2005). Although caspase-8 is activated downstream of the

extrinsic death pathway, the mechanism(s) remained unexplored. Here, we showed cleavage of Bid, a substrate of caspase-8, and a link between the extrinsic and intrinsic death pathways. Our results also showed that the 3a protein promotes the intrinsic death pathway, which involves the release of mitochondrial cytochrome *c* and the downstream activation of caspase-9. In cells expressing the 3a protein, the stress pathway is activated, which includes activation of p38 MAP kinase, a concomitant increase in p53 levels, increased Bax oligomerization and cytochrome *c* release. Mitochondrial depolarization and cytochrome *c* release would result from the effects of tBid as well as Bax oligomers, and both pathways appear to function downstream of the 3a protein. This is also reflected in the reversal of only about half of cell death by SB203580 in cells expressing the 3a protein. Whilst the intrinsic pathway, through caspase-9, Bax and p53, is dependent upon p38 MAPK, the extrinsic pathway operating through caspase-8 would be independent of it (Fig. 8). We also observed reduced nuclear translocation of STAT3 in 3a protein-expressing cells; this is likely to inhibit the expression of survival genes and to relieve STAT3-mediated inhibition of p53 expression. Taken together, we propose that the SARS-CoV 3a protein utilizes multiple pathways (Fig. 8) to promote apoptosis.

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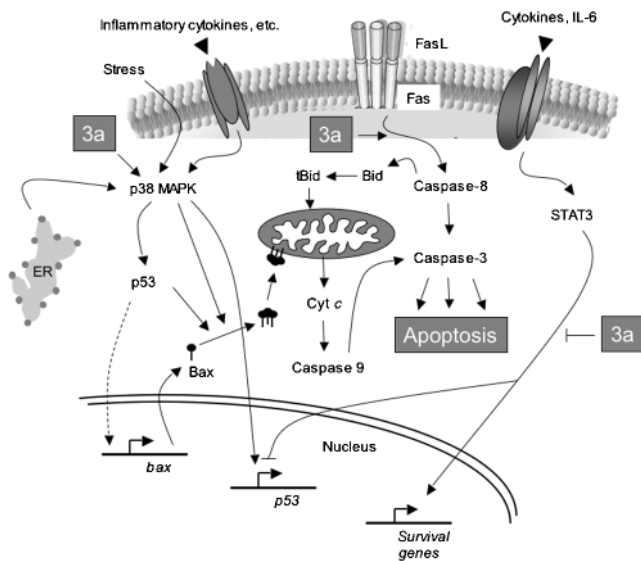


Fig. 8. Proposed model of 3a protein-mediated apoptosis. The 3a protein has been shown previously to activate caspase-8, caspase-3 and apoptosis (Law *et al.*, 2005). It also promotes the activation of Bid downstream of caspase-8. Truncated Bid (tBid) can form pores in the mitochondria, causing the release of apoptogenic factors. In a separate pathway, the 3a protein also activates p38 kinase, leading to upregulation of p53, which can increase nuclear transcription of the *bax* gene and promote cytoplasmic oligomerization of the Bax protein, the latter being observed in cells expressing the 3a protein. Bax multimers insert in the mitochondrial outer membrane, causing loss of potential and release of cytochrome *c* and other apoptogenic factors from the inter-membrane space. Cytochrome *c* promotes formation of the apoptosome and activation of caspase-9. This also activates caspase-3. Following the binding of cytokines and growth factors to cell-surface receptors, various survival pathways are activated. One of these involves the phosphorylation, dimerization and nuclear translocation of STAT3, a transcription factor that promotes the expression of various survival genes and repression of the p53 gene. The 3a protein blocks the nuclear translocation of STAT3, possibly adding to its pro-apoptotic effect.

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