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Molecular and genetic characterisation of the SARS coronavirus auxiliary protein X1 in *Drosophila*

Key Messages

1. We have generated monoclonal antibodies against the SARS coronavirus (SARS-CoV) X1/3a protein (3a), which are suitable for western blotting, immunocytochemistry, and immunohistochemistry.
2. We have established and characterised an in-vivo *3a* transgenic *Drosophila* model, and demonstrated its usefulness in studying SARS-CoV *3a* gene function.
3. We validated our in-vivo findings on *3a* gene function in mammalian Vero E6 cells.
4. Our findings raise the possibility of using ion channel blockers as a novel approach to suppress SARS-CoV–induced cell death.

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Introduction

SARS coronavirus (SARS-CoV) is the aetiological agent that caused a global outbreak of atypical pneumonia in 2003. To date, at least 14 open reading frames have been identified in the SARS-CoV genome; unravelling the molecular functions of these SARS-CoV gene products could provide a more complete understanding of SARS-CoV pathogenesis. The SARS-CoV *3a* locus encodes a 274 a.a. protein.[1] Expression of 3a is detected in patients' intestinal surface enterocytes and pneumocytes. The 3a protein preferentially localises to the Golgi apparatus and cell surface in both transfected and virus-infected cells. The 3a protein is predicted to possess a short extracellular N-terminal head, three transmembrane regions, and a long intracellular C-terminal tail. Several conserved domains are found in 3a, including: a cysteine-rich domain, a Yxxϕ domain, and a diacidic domain.[2] The Yxxϕ and diacidic domains are responsible for the intracellular protein trafficking of 3a, while the cysteine-rich domain is known to affect 3a self homo- and hetero-dimerisation, which is also crucial for its ion channel activity.[3] It has been demonstrated that SARS-CoV induces apoptosis in infected cells, and a number of SARS-CoV proteins were reported to be pro-apoptotic. Furthermore, evidently apoptosis can initiate viral cytopathic effects in SARS-CoV–infected cells. In this project, we investigated the pro-apoptotic property of 3a, both in vivo and in vitro.

Methods

This study was conducted from January 2005 to December 2006.

BALB/c mice were immunised intraperitoneally with KLH-conjugated 3a peptide in complete Freund's adjuvant (Sigma-Aldrich), and followed by monthly intraperitoneally boosts using the same antigen in incomplete Freund's adjuvant (Sigma-Aldrich). Mice showing high serum antibody titers to the antigen were given a final booster injection. The mice were sacrificed 3 days later, and the spleens harvested. Fusion of the spleen cells with a nonproducer myeloma line was carried out.

Fly strains were grown at 29°C on standard cornmeal medium supplemented with dry yeast. The *UAS-3a* transgene produces a full-length non-tagged 3a protein, whereas the *UAS-EGFP-3a* transgene produces a full-length 3a protein with EGFP fused to its N-terminus.[4] The *pUAST-3a-WT* construct was then used as a template to generate three *3a* mutant constructs: cysteine-rich (3a-CS; C127S, C130S, C133S), Yxxϕ (3a-YA; Y160A), and diacidic (3a-DE; E171A, D173A).

African green monkey kidney cell line Vero E6 was maintained at 37ºC in Dulbecco's modified Eagle's medium (Invitrogen), containing 10% heat-inactivated foetal bovine serum (Gibco-BRL), streptomycin (100 g/mL), and penicillin (100 U/mL). Cells were seeded onto 24-well plates 24 hours prior to transient transfection.[5]

Results

We performed detailed characterisation on three clones of monoclonal antibodies

(X61, X98 and X103) raised against the SARS-CoV 3a peptide antigen, mainly using clone X98 in this study. To investigate *3a* function in vivo, the wildtype *3a* gene (*3a-WT*) of the CUHK-W1 SARS-CoV isolate (AY278554) was used to generate *3a-WT* and *EGFP-3a-WT* transgenic flies. When misexpressed in *Drosophila*, the 3a-WT protein displayed a punctate cytoplasmic subcellular localisation and caused external eye malformation in adult flies.[4] As apoptosis has been reported in SARS-CoV–infected cells and the dysregulation of apoptotic cell death in *Drosophila* results in eye malformation, we investigated whether the *3a*-induced eye malformation phenotype was related to apoptotic cell death. We detected increased numbers of apoptotic cells in *3a-WT*–expressing flies as compared to controls. When *3a-WT* was coexpressed with anti-apoptotic genes, the eye malformation phenotype was mostly suppressed and the number of apoptotic cells was considerably reduced. Our data clearly demonstrate that the SARS-CoV *3a-WT* gene is pro-apoptotic.[4]

We then performed site-directed mutagenesis on the cysteine-rich region (3a-CS; C127S C130S C133S), YXXϕ (3a-YA; Y160A) and diacidic domains (3a-DE; E171A D173A), so as to investigate the significance of these regions on the pro-apoptotic function of 3a. Apoptotic cell staining was performed to assess the pro-apoptotic potential of the *3a* mutants. In contrast to *3a-WT*–expressing flies, relatively few apoptotic cells were detected in all three *3a* mutants (unpublished). Further expression of each of these *3a* mutants produced only a mild eye malformation phenotype compared to *3a-WT*, consistent with *3a* mutants being relatively less potent than their wildtype progenitor in inducing cell death. Our data clearly show that the cysteine-rich, Yxxϕ and diacidic domains are crucial for 3a's pro-apoptotic function.

As 3a has been shown to form ion channels on membranes,[3] we investigated the relationship between 3a's channel activity and its pro-apoptotic function. We fed *3a-WT*–expressing flies with barium chloride (barium [Ba] is a known ion channel blocker) and found that the numbers of apoptotic cells were considerably reduced when compared to the untreated control group (unpublished). Our findings therefore establish a link between the pro-apoptotic property and ion channel activity of 3a.

We then validated our data generated from the transgenic fly model in mammalian Vero E6 cells,[5] and to further investigate the molecular mechanisms of 3a functions (with an emphasis on dissecting its relationship to ion channel activity and pro-apoptotic function). Similar to the fly data, we showed that Ba significantly suppressed cell death induced by 3a-WT in Vero E6 cells (unpublished). To determine the involvement of 3a's channel activity in caspase-dependent apoptosis, we simultaneously treated 3a-WT–transfected Vero E6 cells with both Ba and caspase inhibitor; such drug co-treatment showed no further suppression of cell death (unpublished). These findings show that the ion channel activity of 3a-WT mainly regulates caspase-dependent apoptosis (unpublished).

Discussion

We generated and characterised several clones of 3a monoclonal antibodies; X98 and X61 are two that yielded high titers and specificity toward the 3a protein. Clone X98 was used primarily in our study; this clone was shown to be suitable for Western blotting, immnocytochemistry, and immunohistochemistry. We found that the 3a-WT protein displayed a puntate cytoplasmic localisation in *Drosophila* cells, comparable to the pattern observed in SARS-CoV–infected cells. We observed that the expression of 3a-WT protein caused external eye malformation.[4] When EGFP was expressed in the fly eye as a control, no morphological eye changes could be detected.[4] This suggests that at least to a certain extent, SARS-CoV protein can function in invertebrates as it does in mammalian cells. Together with its short life cycle and evolutionary gene pathway conservation (including the apoptotic cell death pathways), *Drosophila* therefore serves as a faithful complementary in-vivo experimental model to mammalians, for the investigation of SARS-CoV in relation to viral gene functions.

By means of genetic interaction studies, we demonstrated that the eye malformation phenotype caused by 3a-WT protein expression was related to the caspase-dependent cell death pathway. Furthermore, the extent of apoptosis was ameliorated when anti-apoptotic genes such as caspase inhibitor were coexpressed with 3a.[4] This prompted us to further investigate the protein domains in 3a that govern its pro-apoptotic property. By means of site-directed mutagenesis, we identified several regions essential for 3a-WT to execute its pro-apoptotic function (unpublished). We further focused our study on the cysteine-rich domain of 3a. It has been shown that 3a-WT protein possesses ion channel activity.[3] We first demonstrated that when *3a-WT*–expressing flies were fed the ion channel blocker Ba in vivo, apoptosis was largely suppressed (unpublished). Our data clearly show that 3a-WT–induced apoptosis is related to the disturbance of intracellular ion homeostasis. Accordingly, perturbation of intracellular ion flux in SARS-CoV–infected cells could therefore be one of its pathogenic mechanisms.

We used mammalian Vero E6 cells to validate our findings obtained from transgenic *Drosophila*,[5] to further investigate the relationship between 3a's ion channel activity and its pro-apoptotic property. After using multiple methods to confirm the pro-apoptotic role of 3a-WT in Vero E6 cells, we demonstrated involvement of caspase-8 and -9 activation in 3a-induced cell death (unpublished).[5] In addition, we showed that the cysteine-rich, Yxxϕ and diacidic domains are all responsible for caspase-dependent apoptosis (unpublished). We further demonstrated that ion channel blockers can effectively inhibit caspase-dependent cell death in Vero E6 cells. This again demonstrates the consistency of data obtained from both invertebrate and

mammalian models. Interestingly, we showed that 3a-WT induces both caspase-dependent and -independent cell death in Vero E6 cells (unpublished). In the future it might be important to further delineate details of non–caspase-dependent apoptosis of 3a, both in vitro and in vivo.

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