Proceedings of the 2005 IEEE
Engineering in Medicine and Biology 27th Annual Conference
Shanghai, China, September 1-4, 2005

The 3a Protein of SARS-coronavirus Induces Apoptosis in Vero E6 Cells

Mary M.Y. Waye[1,5], Patrick T.W. Law[1,2], Chi-Hang Wong[2], Thomas C.C. Au[2], Chi-Pang Chuck[1], Siu-Kai Kong[1], Paul K.S. Chan[3], Ka-Fai To[4], Anthony W.I. Lo[4], Judy Y.W. Chan[1], Yick-Keung Suen[1], H.Y. Edwin Chan[1], Kwok-Pui Fung[1,5], Joseph J.Y. Sung[2,6], Y.M. Dennis Lo[2,7], Stephen K.W. Tsui[1,2,5]

[1]Department of Biochemistry, [2]Centre for Emerging Infectious Diseases, [3]Department of Microbiology, [4]Department of Anatomical and Cellular Pathology, [5]The Croucher Laboratory for Human Genomics, [6]Department of Medicine and Therapeutics and [7]Department of Chemical Pathology, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong SAR, China.

Summary
An outbreak of severe acute respiratory syndrome (SARS) occurred in China and the first case emerged in mid November 2002. The etiologic agent of this disease was found to be a previously unknown coronavirus, SARS-CoV. The detailed pathology of SARS-CoV infection and the host response to the viral infection are still not known. The *3a* gene encodes a non-structural viral protein which is predicted to be a transmembrane protein. In this study, we showed that the 3a protein was localized to the endoplasmic reticulum (ER) in 3a-transfected monkey kidney Vero E6 cells. *In vitro* experiments of chromatin condensation and DNA fragmentation suggest that the 3a protein may trigger apoptosis. Our data show that over-expression of a single SARS-CoV protein can induce apoptosis *in vitro*. Thus GFP-3a fusion protein could also be used as a biosensor for monitoring the cytopathic features of SARS infection, e.g. lymphopenia, in animal model systems, similar to nucleocapsid and 7a proteins.

Introduction
The severe acute respiratory syndrome (SARS) had affected more than 8,000 individuals and caused 774 deaths in 26 countries (http://www.who.int/csr/sars/en/). Different strain of the SARS-coronavirus (SARS-CoV) was sequenced since the outbreak of SARS, and their genomes were rapidly sequenced (Marra *et al*., 2003; Rota *et al*., 2003; Ruan *et al*., 2003; Tsui, Chim, and Lo, 2003; Yeh *et al*., 2004). The *3a* locus (also known as X1 or ORF3; CDS: 25,268 to 26,092 in Tor2 strain of SARS-CoV) encodes one of the ORFs with unknown function and is located between two structural genes encoding the spike and the envelope proteins of the SARS-CoV genome (Marra *et al*., 2003). Interestingly, the *3a* ORF is not found in other two human coronaviruses (OC43 and 229E). This suggests that the 3a protein is a newly emerged protein in coronavirus.

Methods, Results and Discussion
When the 3a sequence was searched against the SMART server (Letunic *et al*., 2004), a predicted signal sequence was found at aa 1 to 16. In addition, three transmembrane domains were predicted at aa 34-56, 77-99 and 103-125 (Fig. 1). Further, the carboxyl terminal region of the 3a protein shares 53% (aa 209-264) and 40% (aa 152-254) similarity respectively to the *Plasmodium* calcium pump and the *Shewanella*

outer membrane porin. Notably, the outer membrane porins are a family of bacterial proteins that may oligomerize to form transmembrane channels for the passive diffusion of small molecules across membranes.

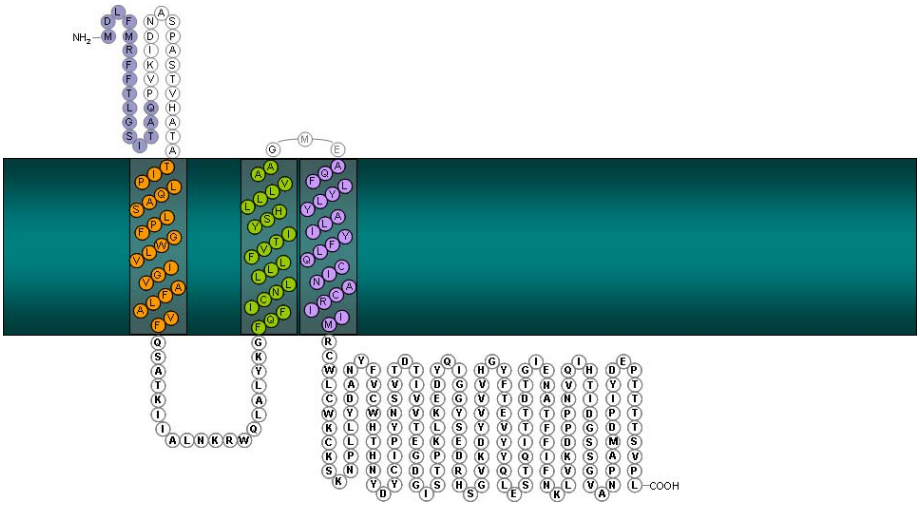

Fig. 1 Schematic diagram showing the topology of the 3a protein as predicted by the SMART server. A signal sequence (in grey colour) and 3 transmembrane regions (red, green and blue in colour) were located at residue 1 to16, 34 to 56, 77 to 99 and 103 to 125, respectively.

To determine the subcellular localization of the 3a protein, pEGFP-3a was co-expressed with the endoplasmic reticulum (ER)-specific construct DsRed2-ER in Vero E6 cells. At one day post-transfection, we observed a punctate fluorescent-signal pattern, similar to that of the ER, and co-localization of the fluorescent signals from the ER-specific protein was observed in pEGFP-3a-transfected Vero E6 cells (Fig. 2A-D). Similar immunofluorescent pattern was obtained in pcDNA4-3a-transfected Vero E6 cells (data not shown).

To validate the targeting of the 3a protein to the ER was driven by the putative signal sequence, deletion mutants pEGFP-3a-Δ16 and pEGFP-3a-Δ130 were prepared with the N-terminus 16 residues and 130 residues removed, respectively. The signal sequence was removed in the construct pEGFP-3a-Δ16 while both the signal sequence and the three predicted transmembrane domains were removed in the construct pEGFP-3a-Δ130. It was found that none of these deletion mutants demonstrated a characteristic ER location (Fig. 2E-J).

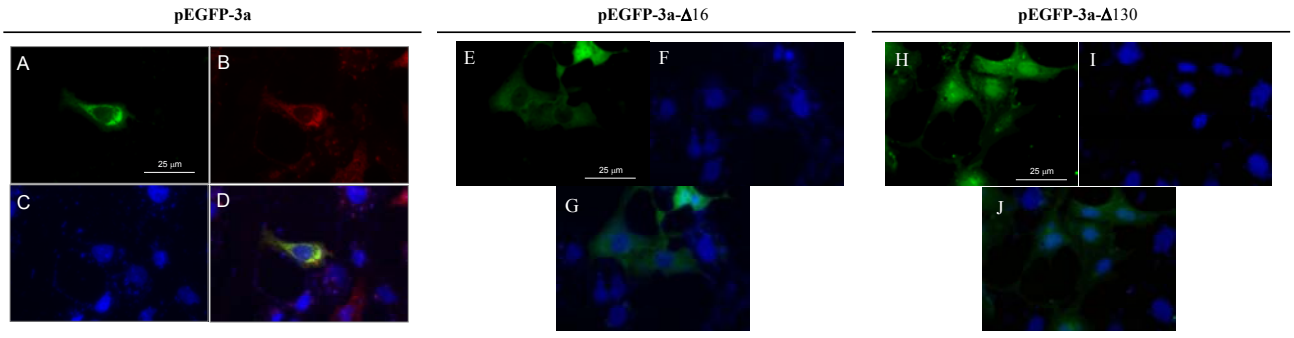

Fig. 2 Subcellular localization of the 3a protein in pEGFP-3a- and pEGFP-3a deletion mutants-transfected Vero E6 cells. (A-D) The GFP-3a protein was co-expressed with DsRed2-ER and the fluorescent signals were detected. (A) The fluorescent detection of the 3a protein, indicating the subcellular location of the ER. (B) The localization of DsRed2-ER tracker protein. (C) Hoechst 33342 staining showing the localization of the nucleus and (D) the overlay of fluorescent signals. Regions of overlapped are displayed in yellow. (E-J)

Fluorescent signals from the pEGFP-3a deletion mutants-transfected Vero E6 cells. The localization of the deletion mutants, Hoeschst 33342 counter staining and overlay of fluorescent signals are shown in E-G (pEGFP-3a-Δ16) and H-J (pEGFP-3a-Δ130), respectively.

SARS-CoV can induce cytopathic effect and apoptosis (Yan *et al*., 2004) in some cell culture models such as Vero E6 cells and the nucleocapsid protein is able to induce apoptosis in COS-1 monkey kidney cells in the absence of growth factors (Surjit *et al*., 2004). Recently, the ORF*7a* protein has been shown to induce apoptosis when overexpressed in Vero E6 cells (Tan *et al*., 2004). To investigate whether the 3a protein could induce apoptosis, Vero E6 cells were transfected with pEGFP-3a and morphological changes were examined using the inverted fluorescent microscope. On day 3 post-transfection, extensive chromatin condensation, a hallmark of apoptosis, was observed in GFP positive cells. These results imply that the 3a protein induces apoptosis in Vero E6 cells (Fig. 3).

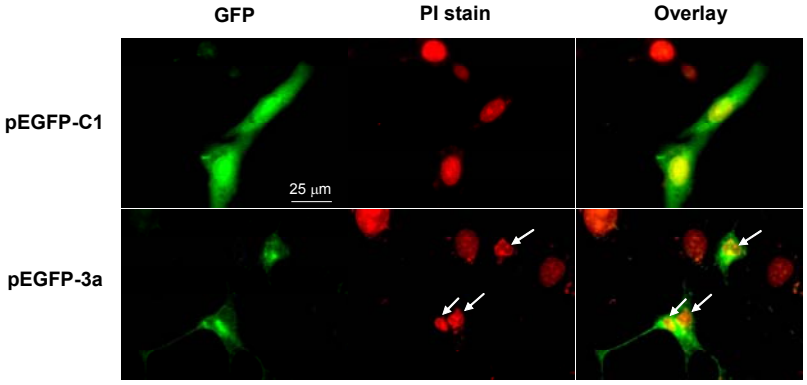

Fig. 3 Chromatin condensation in Vero E6 cells induced by the GFP-3a protein. Cells were transfected with pEGFP-C1 empty vector (upper panel) and pEGFP-3a (lower Panel). GFP–positive cells with chromatin condensation are indicated by arrows. The overlay of the GFP and propidium iodide (PI) fluorescent signals is shown.

To examine whether the 3a protein would induce DNA fragmentation, a common phenomenon of apoptosis, Vero E6 cells were transiently transfected with pcDNA4-3a. The expression level of the 3a protein and the possible internucleosomal DNA cleavage were monitored daily for 5 days (data not shown) and extensive low-molecular-weight apoptotic DNA fragments were observed from day 3 onwards (Fig. 4). There was no sign of DNA fragmentation in Vero E6 cells transfected with pcDNA4-HRPL29 (human ribosomal protein L29) (data not shown).

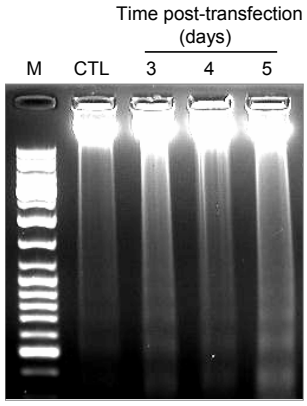

Fig. 4 DNA fragmentation in mammalian cells induced by the 3a protein. (A) The 3a protein induces apoptosis in Vero E6 cells. Lane M, 100 bp ladder molecular markers. Apoptotic laddering was observed in pcDNA4-3a-transfected Vero E6 cells from 3 days post-transfection onwards. No low-molecular-mass DNA fragments were observed following transfection of pcDNA4 empty vector (CTL).

Conclusion

The sequence analysis suggests that the 3a protein contains an N-terminal signal sequence and three transmembrane domains and our results indicate that the 3a protein localization is the ER. Apoptosis is an important defense mechanism that controls the viral infection (O'Brien, 1998; Roulston,Marcellus, and Branton, 1999). On the other hand, virus-induced apoptosis can limit the inflammatory response and somehow facilitate the dissemination of progeny undetected by the host immune system (O'Brien, 1998). Recently, the nucleocapsid protein and the non-structural protein ORF7a have been shown to induce apoptosis when overexpressed in COS-1 cells and Vero E6 cells, respectively (Surjit *et al*., 2004; Tan *et al*., 2004). Here we demonstrate for the first time that the non-structural protein 3a alone can induce apoptosis in SARS-CoV susceptible Vero E6 cells and our study shows that overexpression of the 3a protein can induce chromatin condensation and low-molecular-weight apoptotic DNA fragmentation from 3 days post-transfection.

Acknowledgments

This project team is supported by the Research Fund for the Control of Infectious Diseases (RFCID) from the Health, Welfare and Food Bureau of the Hong Kong SAR Government.

References

Letunic, I., Copley, R. R., Schmidt, S., Ciccarelli, F. D., Doerks, T., Schultz, J., Ponting, C. P., and Bork, P. (2004). SMART 4.0: towards genomic data integration. *Nucleic Acids Res* 32 Database issue, D142-4.

Marra, M. A., Jones, S. J., Astell, C. R. & 56 other authors. (2003). The Genome sequence of the SARS-associated coronavirus. *Science* 300(5624), 1399-404.

O'Brien, V. (1998). Viruses and apoptosis. *J Gen Virol* 79 (Pt 8), 1833-45.

Rota, P. A., Oberste, M. S., Monroe, S. S., and 32 other authors. (2003). Characterization of a novel coronavirus associated with severe acute respiratory syndrome. *Science* 300(5624), 1394-9.

Roulston, A., Marcellus, R. C., and Branton, P. E. (1999). Viruses and apoptosis. *Annu Rev Microbiol* 53, 577-628.

Ruan, Y. J., Wei, C. L., Ee, A. L. and 17 other authors. (2003). Comparative full-length genome sequence analysis of 14 SARS coronavirus isolates and common mutations associated with putative origins of infection. *Lancet* 361(9371), 1779-85.

Surjit, M., Liu, B., Jameel, S., Chow, V. T., and Lal, S. K. (2004). The SARS coronavirus nucleocapsid protein induces actin reorganization and apoptosis in COS-1 cells in the absence of growth factors. *Biochem J* 383(Pt 1), 13-8.

Tan, Y. J., Fielding, B. C., Goh, P. Y., Shen, S., Tan, T. H., Lim, S. G., and Hong, W. (2004). Overexpression of 7a, a protein specifically encoded by the severe acute respiratory syndrome coronavirus, induces apoptosis via a caspase-dependent pathway. *J Virol* 78(24), 14043-7.

Tsui, S. K.W, Chim, S. S., and Lo, Y. M. (2003). Coronavirus genomic-sequence variations and the epidemiology of the severe acute respiratory syndrome. *N Engl J Med* 349(2), 187-8.

Yan, H., Xiao, G., Zhang, J., Hu, Y., Yuan, F., Cole, D. K., Zheng, C., and Gao, G. F. (2004). SARS coronavirus induces apoptosis in Vero E6 cells. *J Med Virol* 73(3), 323-31.

Yeh, S. H., Wang, H. Y., Tsai, C. Y., Kao, C. L., Yang, J. Y., Liu, H. W., Su, I. J., Tsai, S. F., Chen, D. S., and Chen, P. J. (2004). Characterization of severe acute respiratory syndrome coronavirus genomes in Taiwan: molecular epidemiology and genome evolution. *Proc Natl Acad Sci U S A* 101(8), 2542-7.