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ScienceDirect
Archives of Biochemistry and Biophysics 459 (2007) 197–207
ABB
www.elsevier.com/locate/yabbi

The SARS-Coronavirus Membrane protein induces apoptosis through modulating the Akt survival pathway

Chak-Ming Chan [a,b], Cheuk-Wing Ma [a,c], Wood-Yee Chan [d], Ho Yin Edwin Chan [a,b,c,*]

[a] *Laboratory of Drosophila Research, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong SAR, China*
[b] *Department of Biochemistry, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong SAR, China*
[c] *Molecular Biotechnology Programme, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong SAR, China*
[d] *Department of Anatomy, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong SAR, China*

Received 11 October 2006, and in revised form 20 December 2006
Available online 31 January 2007

Abstract

A number of viral gene products are capable of triggering apoptotic cell death through interfering with cellular signaling cascades, including the Akt kinase pathway. In this study, the pro-apoptotic role of the SARS-CoV Membrane (M) structural protein is described. We found that the SARS-CoV M protein induced apoptosis in both HEK293T cells and transgenic *Drosophila*. We further showed that M protein-induced apoptosis involved mitochondrial release of cytochrome *c* protein, and could be suppressed by caspase inhibitors. Over-expression of M caused a dominant rough-eye phenotype in adult *Drosophila*. By performing a forward genetic modifier screen, we identified *phosphoinositide-dependent kinase-1* (*PDK-1*) as a dominant suppressor of M-induced apoptotic cell death. Both PDK-1 and Akt kinases play essential roles in the cell survival signaling pathway. Altogether, our data show that SARS-CoV M protein induces apoptosis through the modulation of the cellular Akt pro-survival pathway and mitochondrial cytochrome *c* release.

Keywords: Cytochrome *c*; *Drosophila*; HEK293T; PDK-1; Severe Acute Respiratory Syndrome

In 2003, the Severe Acute Respiratory Syndrome-Coronavirus (SARS-CoV) emerged and caused an outbreak of atypical pneumonia worldwide. The SARS-CoV genome contains 13–15 open reading frames (ORFs) [1–3] which encode the ORF1a–1b (replicases and protease enzymes), Spike (S), Envelope (E), Membrane (M), Nucleocapsid (N), and a number of less well-characterized regulatory proteins [1,2]. Previous studies revealed that SARS-CoV infection triggers a number of cellular responses in infected cells including modulation of signal transduction cascades such as the p38 MAPK and Akt cell survival pathways [4–12], and induction of apoptosis [11,13,14]. Apoptosis is a main pathologic feature induced by SARS-CoV infection [4,11,13–15], and a number of SARS-CoV proteins have been shown to be pro-apoptotic [12,16–22]. Apoptosis induction has further been demonstrated to initiate viral cytopathic effect (CPE)[1] in the infected cells [13] but appears not to be a mechanism for dissemination of new virions [15]. Over-expression of individual viral proteins is now known to be capable of interfering with various cell signaling cascades. For example, the N protein can modulate the MAPK pathway and down-regulate 14-3-3 protein level [12,23]. It is proposed that alteration of the status of various cellular signaling cascades, such as the Akt pathway, through the concerted effort of individual viral

* Corresponding author. Fax: +852 2603 7732.
E-mail address: hyechan@cuhk.edu.hk (H.Y.E. Chan).

[1] *Abbreviations used:* AO, acridine orange; CPE, cytopathic effect; DIAP1, *Drosophila* inhibitor of apoptosis 1; E, Envelope; EGFP, enhanced green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSK3β, glycogen synthase kinase3β; IAP, inhibitor of apoptosis; M, Membrane; MAPK, mitogen-activated protein kinase; N, Nucleocapsid; ORF, opening reading frame; PDK-1, phosphoinositide-dependent kinase-1; PKCζ, protein kinase Cζ; S, Spike; SARS-CoV, Severe Acute Respiratory Syndrome-Coronavirus; Ser, serine; Thr, threonine.

0003-9861/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2007.01.012

proteins would eventually deliver detrimental consequences to SARS-CoV infected cells [24].

The SARS-CoV *Membrane* locus is located in the 3′ region of the viral genome which encodes a protein of 221 amino acids [1,2]. The SARS-CoV M protein is one of the major proteins among all viral gene products [25]. The M protein possesses a triple membrane-spanning region, an extracellular N-terminal and a long cytosolic C-terminal domains [26]. Physical interactions between M and other viral proteins, including N [27–29], S [30], 3a [31] and 7a [32], have been elucidated; and such protein–protein interaction events are predicted to be essential for the biological functions of M in the viral life cycle. Postulated roles of M include promoting membrane fusion, regulating viral replication, and packing genomic RNA into viral particles [26,33]. Apart from the well-documented roles in viral infection and propagation, novel functions of various SARS-CoV structural proteins such as N and S have recently been assigned [12,19,20,22]. In the present study, we aimed at investigating previously unidentified roles of the SARS-CoV M protein. We over-expressed the SARS-CoV M protein in cells and transgenic *Drosophila*, and observed that M was capable of inducing a mitochondrial-mediated caspase-dependent apoptosis. We also showed that M over-expression down-regulated Akt protein phosphorylation.

Materials and methods

Construction of mammalian expression vector

Full length ORF of the SARS-CoV *Membrane* locus was PCR amplified from viral cDNA template (CUHK-Su10 SARS-CoV isolate; GenBank Accession No. AY282752) and subcloned into *pcDNA3.1(+)* vector using *Eco*RI and *Xba*I enzymes to generate the *pcDNA3.1(+)-Membrane* construct.

Mammalian cell culture and transient transfection

Human embryonic kidney cell line HEK293T was maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Gibco-BRL), streptomycin (100 g/ml), and penicillin (100 U/ml). Cells were seeded onto 6- or 24-well plates 24 h prior to transfection. Four (6-well plate) or 0.8 (24-well plate) μg of plasmid DNA were used for transient transfection with Lipofectamine 2000 reagent (Invitrogen). Cells were collected for the subsequent analyses 48 h post-transfection.

Semi-quantitative RT-PCR analysis

Total RNA was prepared as previously described [34]. Primers used were GAPDH-F: 5′ ACC ACA GTC CAT GCC ATC AC 3′; GAPDH-R: 5′ TCC ACC ACC CTG TTG CTG TA 3′; M-F: 5′ ATT ACC GTT GAG GAG CTT AAA CAA 3′; and M-R: 5′ CAA TGA CAA GTT CAC TTT CC 3′.

Immunofluorescence staining of HEK293T cells

Cells were seeded onto poly-D-lysine-coated coverslips at a density of 2×10^4 cells/coverslip. After transfection, cells were fixed with 3.7% formaldehyde for 15 min and then permeabilized by 1% Triton X-100 for 5 min. After blocking with 1% goat serum for 30 min, cells were incubated with rabbit anti-SARS-virus-PUPM antibody (C-term) (1:100; Abgent) alone or in combination with mouse anti-native cytochrome *c* clone 6H2.B2 (1:100; Pharmingen) at 4 °C overnight, and followed by secondary antibody incubation with goat anti-rabbit IgG (H+L)−FITC (1:250; Zymed) alone or in combination with goat anti-mouse IgG (H+L)−TRITC (1:250; Zymed) at room temperature for 1 h. Golgi body was stained by BODIPY® TR ceramide complexed to BSA (5 μM, Molecular Probes) and cell nuclei were labeled with Hoechst 33342 (trihydrochloride trihydrate) (5 μM, Molecular Probes) at room temperature for 10 min. Cell permeable synthetic caspase inhibitors (Merck) Z-DQMD-fmk (caspase-3 inhibitor V), z-IETD-fmk (caspase-8 inhibitor II) and z-LEHD-fmk (caspase-9 inhibitor I) were dissolved in DMSO. HEK293 cells were treated with caspase inhibitors (50 μM in 1% DMSO) 24 h post-transfection, and were further incubated for another 24 h. Fluorescence images were captured using an Olympus BX51 upright fluorescence microscope or a Leica NT confocal microscope.

Drosophila genetics

Fly strains were grown at 29 °C on standard cornmeal medium supplemented with dry yeast. *PDK-1*^{1} and *PDK-1*^{2} lines were kind gifts of Jongkyeong Chung [35]; *gmr-GAL4, UAS-DIAP1, UAS-P35, P[GMR-Akt1.Exel]2*, Exelixis, and DrosDel lines were obtained from Bloomington *Drosophila* Stock Center; and the *dc3*^{EP2305} and *PDK-1*^{EP837} lines were obtained from Szeged *Drosophila* Stock Centre.

Generation of transgenic fly lines

Full length ORF of the SARS-CoV *Membrane* gene was PCR amplified from viral cDNA template and subcloned into *pUAST* vector [36] using *Eco*RI and *Xba*I enzymes to generate *pUAST-Membrane* plasmid. Standard microinjection technique was employed to generate transgenic lines. A total of 10 *UAS-Membrane* transgenic fly lines were generated. When crossed to *gmr*-GAL4, all lines showed dominant rough-eye phenotype. A transgenic fly line J3 carrying an *UAS-Membrane* insert on the third chromosome was used in this study.

Scanning electron microscopy of adult fly eyes

In brief, fly heads were fixed in 2.5% glutaraldehyde (EM grade, Electron Microscopy Sciences) in phosphate buffer (pH 7.4) for 4 h, then post-fixed with 1% osmium tetroxide (Electron Microscopy Sciences), dehydrated to 100% ethanol and critical-point dried with liquid CO_2. Gold-palladium-coated specimens were examined with a Jeol JSM-6301FE microscope operated at 5 kV [37].

Acridine orange and immunofluorescence staining of larval eye discs

Acridine orange staining of third-instar larval eye discs was performed as previously described [38]. Quantification of acridine orange-positive cell was performed by Image-Pro Plus 5.1 (Media Cybernetics). Immunofluorescence staining was performed as previously described [16]. Rabbit anti-SARS-virus-PUPM antibody (C-term) (1:100; Abgent) and goat anti-rabbit IgG (H+L)–FITC secondary antibody (1:250; Zymed) were used. Propidium iodide (10 μg/ml, Molecular Probes) was used to label cell nuclei. Images were captured using an Olympus BX51 upright fluorescence microscope or a Leica NT confocal microscope.

Western blot analysis

For fly protein sample preparation, 16 adult fly heads of appropriate genotypes were homogenized in 75 μl of 6× SDS sample buffer. SDS–PAGE separation and Western blotting were performed as previously described [16]. Primary antibodies used include phospho-*Drosophila* Akt (Ser505) antibody (1:1000; Cell Signaling), total Akt antibody (1:1000; Cell Signaling) and anti-β-tubulin E7 (1:2000; Developmental Studies Hybridoma Bank, under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA