JKL Chan 陳家駱
P Cheung 張寶燕
M He 何明亮
Z Wu 鄔振國

Impact of SARS-coronavirus-encoded proteins on cellular signalling pathways and cytokine/chemokine gene expression

Key Messages

1. Although GFP-tagged ORF8 and ORF3 potently activate the JNK and p38 MAPK pathways, expression of non-tagged SARS-CoV-encoded ORF8 or ORF3 has no obvious effect.
2 Although both GFP-tagged ORF8 and ORF3 induce cell death, expression of non-tagged ORF8 or ORF3 has no obvious effect on cell survival.
3 Addition of an epitope tag to a protein of interest, a common way to study novel proteins in the absence of suitable antibodies, may generate unexpected artefacts. Caution should be taken with any results derived from epitope-tagged proteins.
4 When studying a novel protein, it is essential to prepare suitable antibodies to facilitate detection and purification (eg by immunoprecipitation) of the native or endogenous proteins.

Introduction

Severe acute respiratory syndrome coronavirus (SARS-CoV) was responsible for the global SARS pandemic in 2003.[1,2] Although all CoVs have similar microscopic appearance, gene products, and genomic organisation, SARS-CoV is unique in that it is associated with high mortality rates in humans. Spike, membrane, envelope, and nucleocapsid proteins as well as replicase are commonly conserved among all CoVs. The genome of SARS-CoV also encodes nine other novel open-reading frames (ORFs) with unknown functions (Fig 1).[1,2]

Various viruses exert their pathogenic effects through interaction of their viral proteins with distinct cellular targets. We hypothesised that the severe inflammation and high mortality caused by SARS-CoV are contributed in part by these novel ORFs. Therefore, we aimed to evaluate the functions of these novel ORFs by overexpressing them in human cell lines.

Mitogen-activated protein kinases (MAPK) are important cellular signalling molecules involved in cell growth, differentiation, and apoptosis under both normal and pathological conditions. Three major classes of MAPKs, namely extracellular signal-regulated kinases, cJun N-terminal kinases (JNKs), and p38 MAPKs, have been extensively characterised in the past 15 years. Many viral proteins are known to activate these MAPKs to exert their cytotoxic effects and trigger host inflammatory responses. For example, the Tax protein of human T-cell leukaemia virus type 1 and the latent membrane protein 1 (LMP1) of the Epstein-Barr virus potently activate the JNK pathway. We hypothesised that the novel ORFs of SARS-CoV may trigger inflammation and promote apoptosis of host cells through activation of MAPKs, especially the JNK and p38 MAPKs, which are known to be activated by pro-inflammatory and apoptotic stimuli.

Methods

This study was conducted from September 2004 to December 2006. To amplify the novel SARS ORFs from the SARS-CoV genome and to insert them into two sets of expression vectors (Flag-tagged pcDNA3 and eGFP-tagged pEGFP-C1), two sets of polymerase chain reaction primers were respectively designed: one containing the Nhe I (or Xba I for ORFs3 and 14)/Not I sites and another containing the Bgl II/Sal I sites. All constructs were verified by restriction enzyme digestion and sequencing.

All DNA constructs were transiently transfected into HEK293 cells using Lipofectamine Plus reagents (Invitrogen) following the manufacturer's instructions. Fluorescent images of live HEK293 cells containing green fluorescence protein (GFP) fusion proteins were acquired by an Olympus IX70 fluorescent microscope linked to a charge-coupled device digital camera (Spot RT, Diagnostic Instruments Inc, MI, USA).

HEK293 cells were co-transfected with HA-JNK2 together with individual viral ORFs. After 24 hours of transfection, cells were harvested, lysed, and

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Department of Biochemistry, Hong Kong University of Science and Technology
JKL Chan, P Cheung, Z Wu
Centre for Emerging Infectious Diseases, The Chinese University of Hong Kong
M He

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Principal applicant and corresponding author:
Dr Zhenguo Wu
Department of Biochemistry, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong SAR, China
Tel: (852) 2358 8704
Fax: (852) 2358 1552
Email: bczgwu@ust.hk

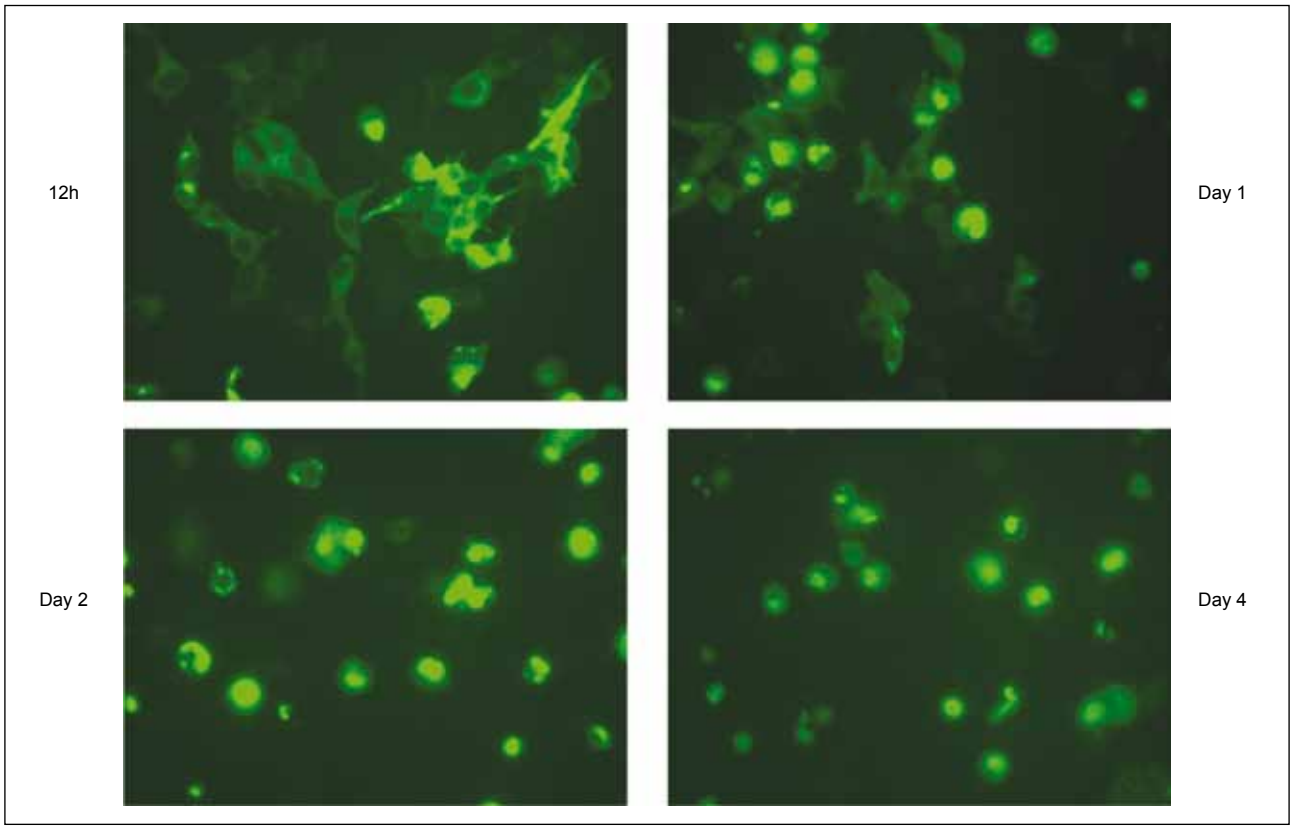

Fig 1. Expression of GFP-ORF8 in HEK293 cells results in significant cell death
HEK293 cells are transiently transfected with a construct encoding GFP-ORF8. At different time points after transfection, fluorescent images are taken using an Olympus IX70 fluorescent microscope linked to a charge-coupled device digital camera

the soluble whole cell lysates were prepared. After normalisation by Western blotting, an equal amount of HA-JNK was immunoprecipitated from cell lysates using anti-HA antibodies. The immunoprecipitates were then subjected to kinase assays using either GST-cJun(1-79) or GST-ATF2 (1-92) as substrates.

A segment of ORF8 gene encoding aa17-94 was subcloned into pET32M expression vector to generate the recombinant His-thioredoxin-ORF8 fusion protein in bacteria. The proteins were injected into rabbits to generate polyclonal antibodies according to standard procedures.

Results

Subcellular localisation of novel ORFs from SARS-CoV

As antibodies against the novel ORFs from SARS-CoV were not available, an enhanced GFP inframe was first fused to the N-terminus of all nine ORFs. After transfecting individual fusion constructs into HEK293 cells, the subcellular localisation patterns of these nine ORFs were classified using live cell imaging into three categories: GFP-ORFs3, 7, 8, 9, and 14 (which were mainly cytoplasmic); GFP-ORFs10, 11, and 13 (which were evenly distributed in both the cytoplasm and nucleus); and GFP-ORF4 (which was mainly nuclear).

Effect of GFP-ORFs on cell survival

After transfecting GFP-ORFs into HEK293 cells, the behaviour of the GFP-positive cells was monitored by fluorescent microscopy every 12 to 24 hours for up to 4 days. Several GFP-ORFs, especially GFP-ORF8, caused obvious cell death. After 24 hours of transfection into HEK293 cells, GFP-ORF8-positive cells started to round up and then detached from culture plates (Fig 1). By 48 hours, most of the GFP-positive cells had died. This was consistent with other reports.[3,4] Similarly, GFP-ORF3, GFP-ORF9, and ORF14 also promoted cell death.

GFP-tagged ORF8 activates both JNK and p38 MAPK

Based on the finding that GFP-ORF8 promoted cell death, we tested whether GFP-ORF8 could activate JNK and p38 MAPK (both of which are often activated by pro-apoptotic stimuli). The HEK293 cells were co-transfected with GFP-ORF8 together with either HA-JNK2 or HA-p38α. EBV-encoded LMP1 was used as a positive control. In immune-complex kinase assays, GFP-ORF8 potently activated both JNK and p38 as well as LMP1 (Fig 2).

There is a 15-aa leader peptide at the N-terminus of ORF8, which might correct targeting of the viral protein to the endoplasmic reticulum and Golgi networks.[5] To make sure that the effects seen above were not due to inappropriate

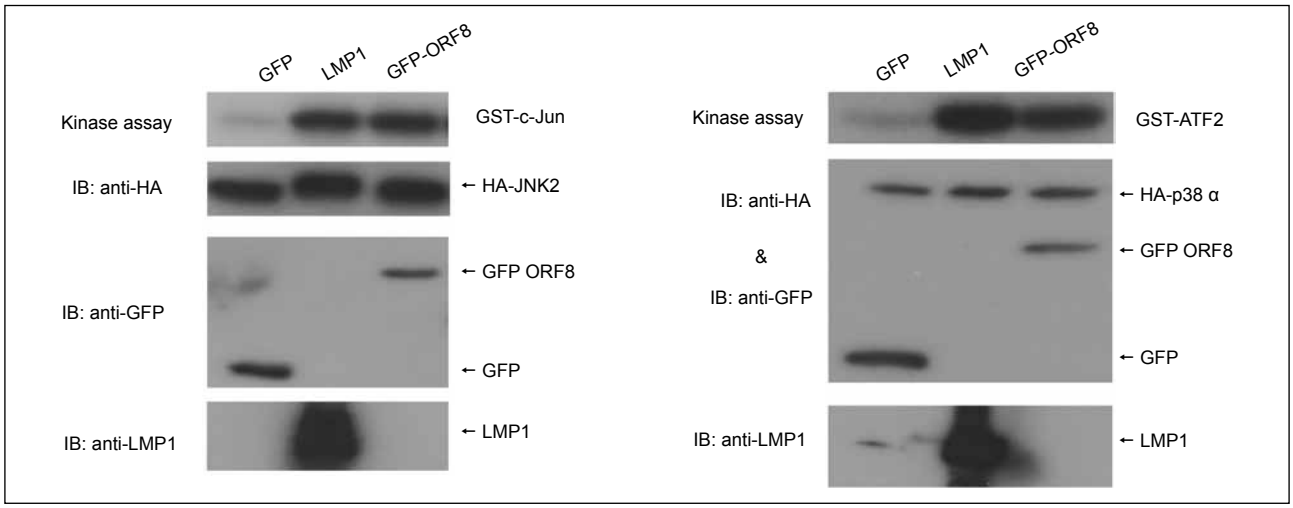

Fig 2. Activation of JNK and p38 MAPK by GFP-ORF8
HEK293 cells are co-transfected with GFP-ORF8 together with either HA-JNK2 or HA-p38α HA-tagged kinases are immunoprecipitated from cell lysates and subjected to kinase assays

positioning of the GFP tag in the fusion protein, another expression construct (ORF8-GFP) was generated with GFP-fused inframe to the C-terminus of ORF8. When the construct encoding ORF8-GFP was introduced into HEK293 cells, ORF8-GFP was as efficient as GFP-ORF8 (ie the N-terminally tagged ORF8) in promoting cell death. Furthermore, when co-transfected together with HA-JNK2 or HA-p38α into HEK293 cells, ORF8-GFP potently activated both JNK and p38 as well as GFP-ORF8.

Non-tagged ORF8 did not cause cell death and activate JNK and p38

To further exclude the possibility that the effects of ORF8 fusion proteins seen above were caused by inappropriate fusion of the GFP tag, an expression construct encoding the native ORF8 was generated, without any additional tag at either end of ORF8. To ensure that the ORF8 protein was actually expressed from the construct, polyclonal antibodies against ORF8 were generated using recombinant ORF8 protein spanning aa 17-84. The antibodies we used were able to detect ORF8 either as a GFP-ORF8 fusion protein or a non-tagged protein (Fig 3). When transfected into HEK293 cells, the non-tagged ORF8 did not cause obvious cell death. In addition, although GFP-ORF8 potently activated JNK, the non-tagged ORF8 failed to do so (Fig 3). Similarly, the non-tagged ORF8 also failed to activate p38.

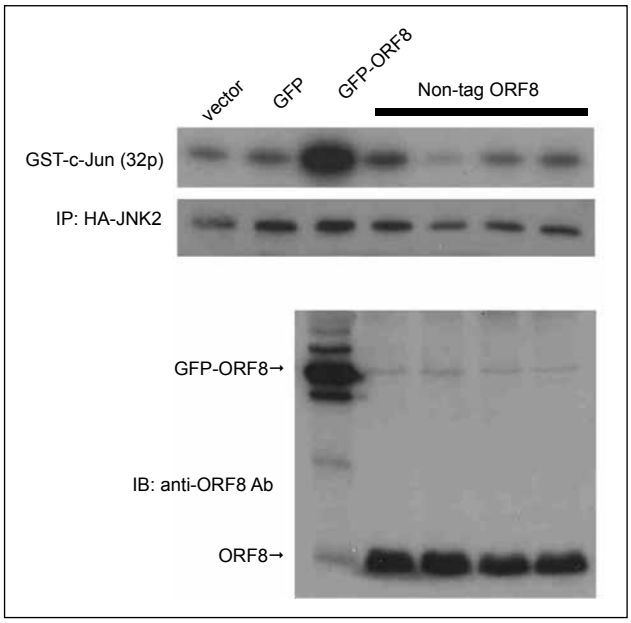

Fig 3. ORF8 without tag does not activate JNK
HEK293 cells are co-transfected with constructs encoding HA-JNK2 and non-tagged ORF8. HA-JNK is immunoprecipitated from cell lysates and subjected to protein kinase assays. The expression of ORF is detected by our locally produced antibodies

Discussion

To study the cellular function of novel proteins in the absence of suitable antibodies, it has been a widely adopted practice to fuse a unique tag (eg HA, FLAG, Myc, etc) to either end of the protein of interest. This facilitates detection of the protein in cells and cell lysates and in isolation of the protein of interest by immunoprecipitation. In recent years, GFP has become a very popular tag, as it enables imaging of live cells, instant knowledge of subcellular localisation patterns, and tracking of the fate of GFP-positive cells over a long period of time. Although protein tagging is a fast and useful technique (especially for proteins without suitable antibodies), addition of a tag to a protein can create artefacts due to changes in protein localisation and folding, interference or disruption of interaction of the protein with other partners, etc. Therefore, caution is necessary for findings derived from tagged proteins.

Owing to the high mortality rate caused by SARS-CoV, it is essential to understand the molecular mechanisms underlying the pathogenesis. We undertook the project at a time when many key reagents (including the antibodies againt ORF8) were not available. GFP-tagged ORF8 was found to potently induce cell death, which was consistent with other reports.[3,4] In addition, GFP-tagged ORF8 strongly activated the JNK and p38 MAPK pathways in host cells. Non-tagged ORF failed to induce these changes; this suggested that artefacts were generated in the GFP fusion proteins. Before obtaining the ORF8 antibody, a segment of ORF8 gene encoding aa 17-94 was used as the bait in the yeast two-hybrid screening. Several interesting clones were found. Owing to the lack of a suitable biological assay for the native ORF8, these clones were not further characterised.

In addition to ORF8, ORF3 (also known as ORF3a), another novel ORF that was found to be expressed in SARS-CoV-infected cells was also extensively studied. Similarly, GFP-ORF3 induced cell death and activated both JNK and p38 in host cells. In contrast, the non-tagged ORF3 failed to induce cell death and activate JNK and p38 as well as IkB kinase.

Conclusions

The native (non-tagged) ORF8 and ORF3 did not significantly induce cell death, nor did they activate JNK and p38 MAPK pathways. We believe that results in several reports on ORF8 and ORF3 were most likely due to artefacts generated by inappropriate fusion of an epitope tag at either end of the viral proteins.[3,4] Therefore, caution should be exercised in interpreting results derived from epitope-tagged proteins. Suitable antibodies to the protein of interest should be prepared to facilitate the study of the native proteins.

Acknowledgement

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