

Augmentation of chemokine production by severe acute respiratory syndrome coronavirus 3a/X1 and 7a/X4 proteins through NF- κ B activation

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Received 5 September 2006; revised 6 November 2006; accepted 16 November 2006

Available online 27 November 2006

Edited by Hans-Dieter Klenk

Abstract Severe acute respiratory syndrome (SARS) is characterized by rapidly progressing respiratory failure resembling acute/adult respiratory distress syndrome (ARDS) associated with uncontrolled inflammatory responses. Here, we demonstrated that, among five accessory proteins of SARS coronavirus (SARS-CoV) tested, 3a/X1 and 7a/X4 were capable of activating nuclear factor kappa B (NF- κ B) and *c-Jun* N-terminal kinase (JNK), and significantly enhanced interleukin 8 (IL-8) promoter activity. Furthermore, 3a/X1 and 7a/X4 expression in A549 cells enhanced production of inflammatory chemokines that were known to be up-regulated in SARS-CoV infection. Our results suggest potential involvement of 3a/X1 and 7a/X4 proteins in the pathological inflammatory responses in SARS.

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Keywords: SARS; ARDS; Inflammation; NF- κ B; Transcription; IL-8

1. Introduction

Severe acute respiratory syndrome (SARS) is characterized by dyspnea with rapidly progressing changes on radiography in the later stages of the illness [1,2]. Pathological findings of the lungs in SARS resemble those in acute/adult respiratory distress syndrome (ARDS) associated with various clinical conditions [3]. It has been proposed that ARDS is the outcome of an uncontrolled inflammatory response and that nuclear

factor kappa B (NF- κ B) is a critical transcription factor involved in the pathogenesis of ARDS [4,5].

SARS coronavirus (SARS-CoV) genome contains open reading frames (ORFs) for several accessory proteins without sequence similarity to known coronavirus proteins. These include 3a (originally called X1 or ORF3), 3b (X2 or ORF4), 6 (X3 or ORF7), 7a (X4 or ORF8), and 8b (X5 or ORF11) [6–8]. Here, we investigated whether these accessory gene products of SARS-CoV were able to induce inflammatory responses.

2. Materials and methods

2.1. Construction of lentivirus vectors expressing SARS-CoV proteins
DNA fragments corresponding to the SARS-CoV 3a/X1, 3b/X2, 6/X3, 7a/X4, and 8b/X5 genes (identical to the Urbani strain, GenBank accession number AY278741) were amplified by polymerase chain reaction (PCR) from cDNA from the Hanoi 01-03 strain [9] of SARS-CoV using the specific primers 5'-CACCATGGATTTGTT-TATGAGA-3' (forward) and 5'-CAAAGGCACGCTAG-TAGTCGTCG (reverse) for 3a/X1; 5'-CACCATGATGCCAACTA-CTTGTGTTTGC-3' (forward) and 5'-ACGTACCTGTTTCTTCC-GAAACG-3' (reverse) for 3b/X2; 5'-CACCATGTTTCATCTT-GTTGACTTCC-3' (forward) and 5'-TGGATAATCTAACTCCAT-AGGTTC-3' (reverse) for 6/X3; 5'-CACCATGAAAATTATTCT-CTTCTG-3' (forward) and 5'-TCTGTCTTTCTTAATGGT-GAAGC-3' (reverse) for 7a/X4; 5'-CACCATGTGCTGAAGAT-CCTGTGAAG-3' (forward) and 5'-ATTTGTTTCGTTTATTTAAA-ACAACAAG-3' (reverse) for 8b/X5. Each amplified fragment was inserted into the pENTR™/D-TOPO vector (Invitrogen, Carlsbad, CA), and was subsequently transferred into a modified lentivirus expression vector (pLenti6/V5-DEST, Invitrogen) containing an intron of human beta-globin (Fig. 1A) by site-specific recombination using the Gateway Cloning System (Invitrogen). They were designated as pLenti/V5/X1, pLenti/V5/X2, pLenti/V5/X3, pLenti/V5/X4, and pLenti/V5/X5, respectively. An enhanced green fluorescent protein (EGFP)-expressing vector (pLenti/V5/GFP) was also constructed as a control.

2.2. Plasmids

A reporter plasmid expressing luciferase (κ B-Luc) driven by five tandem NF- κ B binding sites derived from IL-2 receptor α [10] was provided by Dr. Junichi Fujisawa (Kansai Medical School, Osaka, Japan). Reporter plasmids for the wild-type (-133-Luc) and various mutant (AP-1-Luc, NF- κ B-Luc, and NF-IL6-Luc) interleukin 8 (IL-8) promoters [11] were provided by Dr. Naofumi Mukaida (Kanazawa University, Kanazawa, Japan). The HA-tagged expression vectors

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Abbreviations: ARDS, acute/adult respiratory distress syndrome; EGFP, enhanced green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; HA, hemagglutinin; IL-8, interleukin 8; JNK, Jun N-terminal kinase; NF- κ B, nuclear factor kappa B; ORF, open reading frame; RANTES, regulated on activation normal T cell expressed and secreted; SARS-CoV, severe acute respiratory syndrome coronavirus

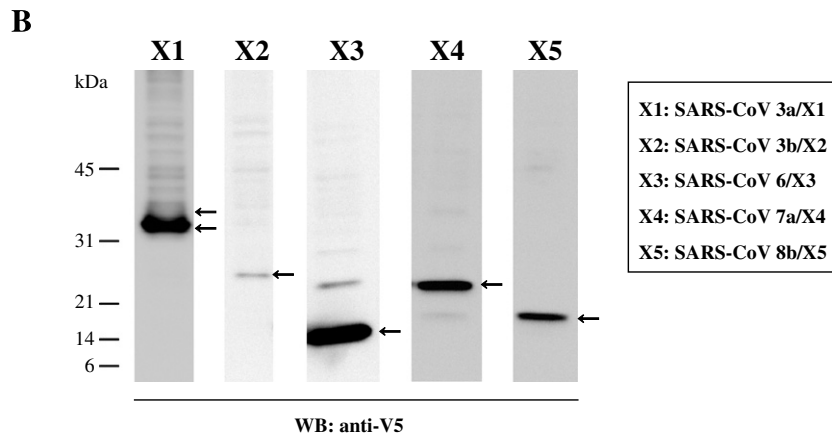
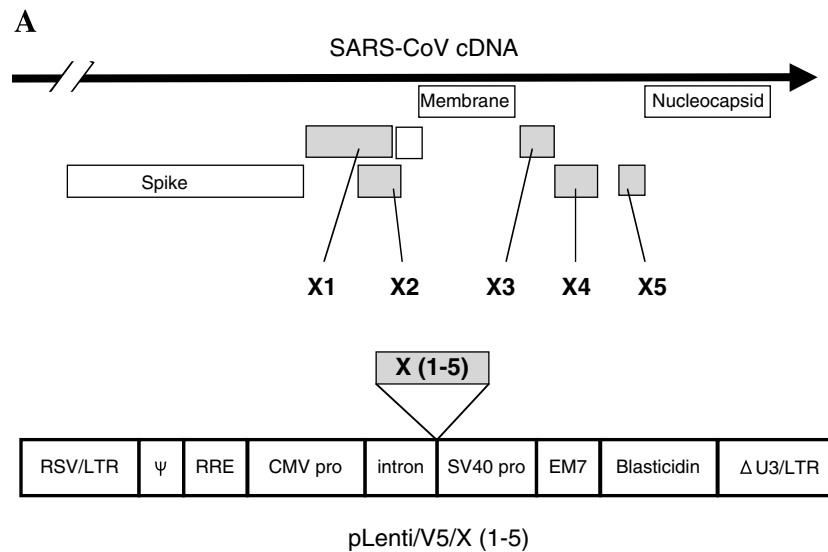


Fig. 1. Expression of SARS-CoV accessory proteins. (A) Locations of SARS-CoV 3a/X1 (X1), 3b/X2 (X2), 6/X3 (X3), 7a/X4 (X4), and 8b/X5 (X5) gene fragment amplified (gray squares) (top) and the construct of the resulting lentivirus vectors expressing SARS-CoV genes (pLenti/V5/X1-X5) (bottom) are schematically shown. (B) Western blot analysis of cell lysates from HEK293T cells transfected with the indicated SARS-CoV gene expression plasmid (pLenti/V5/X1-X5) was carried out using an anti-V5 antibody. Arrows indicate predicted sizes of each product.

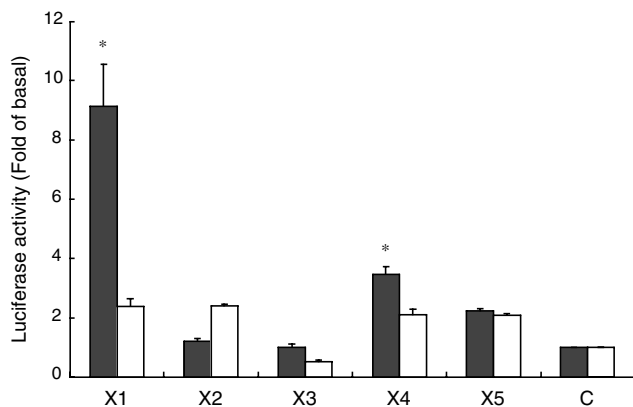


Fig. 2. Activation of NF- κ B by SARS-CoV accessory gene expression. (A) The NF- κ B reporter plasmid (κ B-Luc) (closed bar) and phRL-CMV (open bar) were cotransfected with pLenti/V5/X1-X5 or control pLenti/V5/GFPvectors (X1-5, C) into HEK293T cells, and luciferase activities were measured approximately 40 h after transfection. Data were expressed as means \pm S.D. ($n = 3$) of the relative values against GFP controls. Similar results were obtained in three independent experiments. * $P < 0.05$ vs GFP controls.

HA-JNK1 and HA-JNK3 were provided by Dr. Hidenori Ichijo (The University of Tokyo, Tokyo, Japan). A control plasmid expressing renilla luciferase driven by the cytomegalovirus promoter (phRL-CMV) was purchased from Promega (Madison, WI).

2.3. Western blotting

Cell lysates were prepared in lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10% glycerol, 1% Triton X-100 and protease inhibitor cocktail [CALBIOCHEM, La Jolla, CA]), and proteins (20 μ g) were separated by electrophoresis on a Tris-Glycine minigel (Invitrogen), transferred to nitrocellulose filters, and reacted with antibodies followed by visualization with the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Piscataway, NJ). Densitometric analysis was performed on scanned filters using ImageJ 1.37v software (<http://rsb.info.nih.gov/ij/>).

2.4. Antibodies

Anti-V5 antibody (Invitrogen) and anti-phospho Jun N-terminal kinase (JNK) antibody (Cell Signaling Technology, Beverly, MA) were used for Western blotting. Immunoprecipitation was performed with anti-HA (Roche) or anti-V5 antibodies as previously described [12].

2.5. Reporter assays

Luciferase-expressing various reporter plasmids (200 or 300 ng) together with renilla luciferase-expressing phRL-CMV (20 ng) were co-

transfected with vectors expressing SARS-CoV genes into HEK293T or A549 cells (2×10^5 cells) using Lipofectamine2000 (Invitrogen) or Fugene6 (Roche), respectively. Luciferase and renilla luciferase activities were measured from cell lysates 30 or 40 h after transfection, using the Luciferase assay system (Promega) and the Renilla luciferase assay system (Promega), respectively.

2.6. Enzyme-linked immunosorbent assay (ELISA)

The amounts of IL-8 and regulated on activation normal T cell expressed and secreted (RANTES) in the culture supernatants of A549 cells were measured by Quantikine human IL-8 and RANTES ELISA kits (R&D Systems), respectively, 48 h after transfection.

3. Results and discussion

3.1. Expression of SARS-CoV accessory proteins

Lentivirus expression vectors for SARS-CoV 3a/X1, 3b/X2, 6/X3, 7a/X4, and 8b/X5 genes were constructed as shown in Fig. 1A. These expression vectors were transfected into

HEK293T cells, and expression of coding proteins with predicted sizes were confirmed by Western blotting (Fig. 1B). The expression level of 3b/X2 protein was always lower than the other SARS-CoV proteins tested in this system. Anti-V5 antibody detected two bands of 3a/X1 protein presumably due to a posttranslational modification as recently reported [13].

3.2. Activation of NF- κ B and JNK by SARS-CoV accessory proteins

We first examined effects of SARS-CoV genes on NF- κ B, the major transcription factors activated in ARDS [5], using a reporter plasmid expressing luciferase (κ B-Luc) [10]. As shown in Fig. 2, expression of 3a/X1 and 7a/X4 significantly enhanced NF- κ B mediated transcription (9.1 and 3.5-folds, respectively) in HEK293T cells ($P < 0.05$). The effects of 3b/X2, 6/X3 and 8b/X5 were not significant compared to the GFP control. We also determined the effect of SARS-CoV genes on mitogen-activated protein kinases that are also

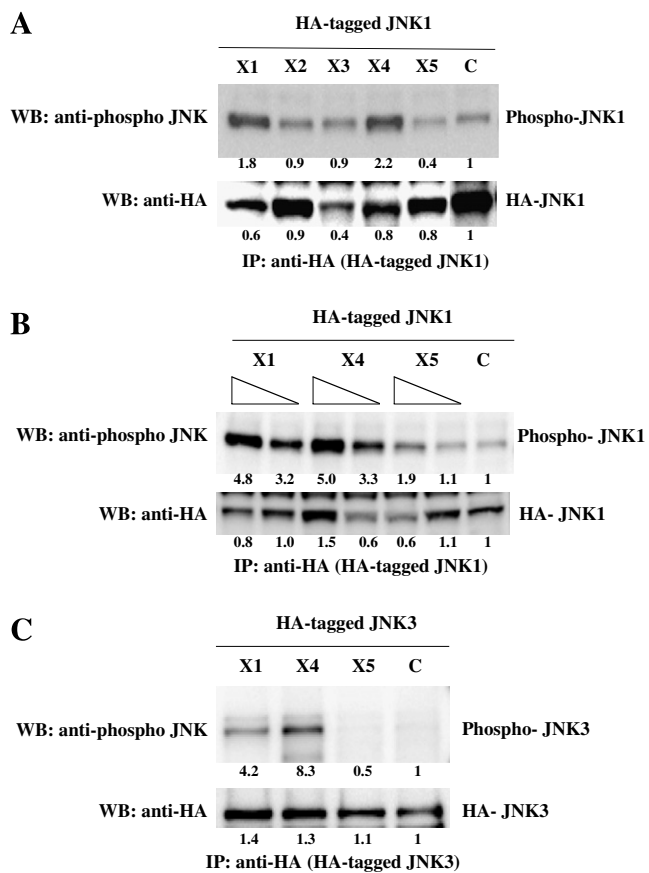


Fig. 3. Activation of JNK by SARS-CoV accessory gene expression. (A) Lysates from HEK293T cells co-transfected with HA-JNK1 plasmid (3 μ g) and pLenti/V5/X1- X5 or control pLenti/V5/GFP plasmid (X1-5, C) (3 μ g) were immunoprecipitated (IP) with mouse anti-HA antibody and subjected to Western blot (WB) analysis with rabbit anti-phospho JNK antibody. The filter was stripped and restained with anti-HA antibody. (B) A similar phosphorylation assay of HA-JNK1 with different doses of pLenti/V5/X1, X4, or X5 (3 or 1 μ g) plasmids. pCDNA3.1 (Invitrogen) plasmid was used to standardize transfection efficiency. (C) A phosphorylation assay on HEK293T cells that were co-transfected with HA-JNK3 plasmid (3 μ g) and the indicated lentivirus vectors (3 μ g). The values at the bottom end of each lane represent relative densities against control bands.

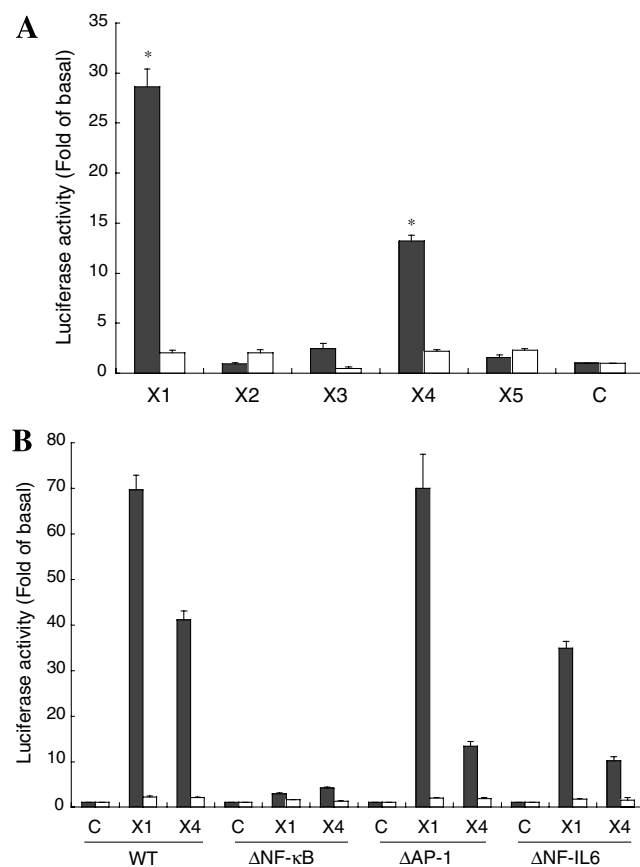


Fig. 4. SARS-CoV 3a/X1 and 7a/X4 augment IL-8 promoter activity in HEK293T cells. (A) The IL-8 promoter reporter plasmid (closed bar) and pRL-CMV (open bar) was co-transfected with pLenti/V5/X1-X5 or control pLenti/V5/GFP plasmid (X1-5, C) into HEK293T cells, and luciferase activities were measured. * $P < 0.05$ vs GFP controls. (B) The wild-type (WT) or mutant (Δ NF- κ B, Δ AP-1, and Δ NF-IL6) IL-8 promoter reporter plasmids (closed bar) together with pRL-CMV (open bar) were cotransfected with control or pLenti/V5/X1 plasmids (C, X1 or X4) into HEK293T cells. Luciferase activities were measured approximately 40 h after transfection. Data were expressed as means \pm S.D. ($n = 3$) of the relative values against GFP controls.

associated with chemokine production [14,15]. HA-tagged JNK1 expressed in HEK293T cells was markedly phosphorylated by 3a/X1 and 7a/X4 but not by 3b/X2, 6/X3, or 8b/X5 (Fig. 3A and B). 3a/X1 and 7a/X4 also activated JNK3 (Fig. 3C). There was no obvious activation of ERK and p38 α by any SARS-CoV genes tested (data not shown).

3.3. Augmentation of IL-8 promoter activity by SARS-CoV accessory proteins

We next examined whether SARS-CoV proteins were capable of activating the promoter of IL-8 that is a representative chemokine involved in ARDS [16] and regulated by NF- κ B and MAP kinases including JNK [17]. It has been shown that

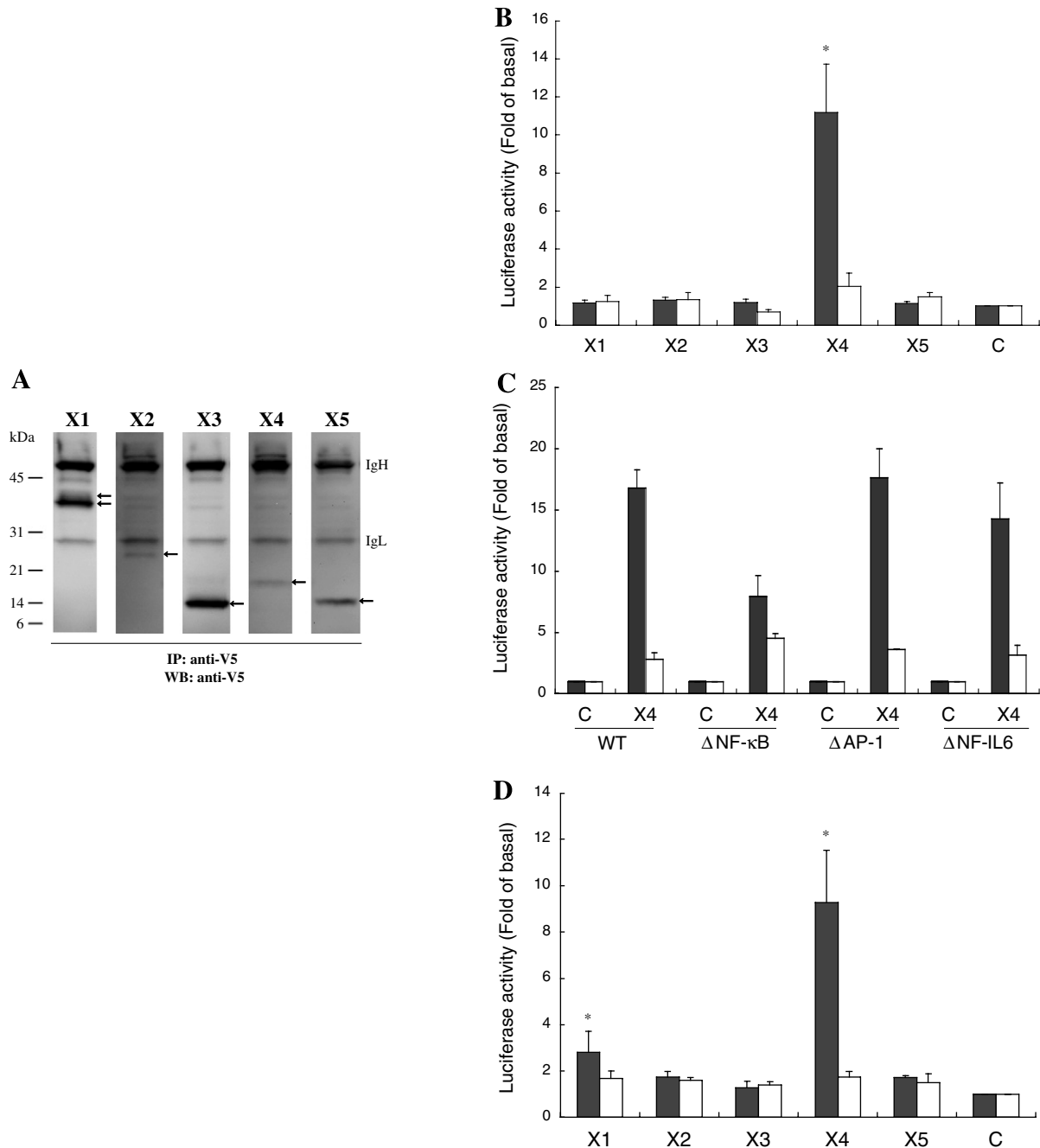


Fig. 5. Effects of SARS-CoV accessory gene expression on IL-8 promoter and NF- κ B activities in A549 cells. (A) Cell lysates from A549 cells transfected with the indicated SARS-CoV gene expression plasmids (pLenti/V5/X1-X5) were immunoprecipitated (IP) with an anti-V5 antibody and then subjected to Western blot (WB) analysis using the same antibody. Arrows indicate predicted sizes of each product. IgH and IgL, immunoglobulin heavy and light chains, respectively. (B) The wild-type IL-8 promoter reporter plasmid (closed bar) and phRL-CMV (open bar) were cotransfected with pLenti/V5/X1-X5 or control plasmids (X1-5, C) into A549 cells, and luciferase activities were measured. (C) The wild-type (WT) or mutant (Δ NF- κ B, Δ AP-1, and Δ NF-IL6) IL-8 promoter reporter plasmids (closed bar) and phRL-CMV (open bar) were co-transfected with control or pLenti/V5/X4 plasmids (C, X4) into A549 cells, and luciferase activities were measured. (D) The κ B-Luc (closed bar) and phRL-CMV (open bar) were co-transfected with pLenti/V5/X1-X5 or control plasmids (X1-5, C) into A549 cells. Luciferase activities were measured approximately 40 h (B, C) and 30 h (D) after transfection. Data were expressed as the means \pm S.D. ($n = 3$) of the relative values against GFP controls. * $P < 0.05$ vs GFP controls.

the IL-8 level is elevated in the plasma of SARS patients [18]. When the reporter plasmids expressing luciferase under the control of the human wild-type IL-8 promoter (-133-Luc) [11] were co-transfected in HEK293T cells, IL-8 promoter activity was enormously augmented by expression of 3a/X1 (28.7-fold) and 7a/X4 (13.2-fold). The effects of 3b/X2, 6/X3, and 8b/X5 were not significant (Fig. 4A). Augmentation of the IL-8 promoter activity by 3a/X1 and 7a/X4 was abolished by a mutation at the NF- κ B site in the IL-8 promoter, indicating that this effect was mainly mediated through NF- κ B (Fig. 4B).

3.4. Effects of SARS-CoV accessory proteins in A549 cells

We next examined the effect of SARS-CoV gene products in human lung cancer-derived A549 cells [19]. The expression levels of SARS-CoV proteins in transiently transfected A549 cells were lower than those in HEK293T cells, but were detectable following immunoprecipitation with anti-V5 antibody (Fig. 5A). In a reporter assay using A549 cells, 7a/X4 showed the greatest effect on the IL-8 promoter activity (11.2-fold) among tested (Fig. 5B). Although 3a/X1 showed a 2.2-fold increase in IL-8 promoter activity at an early time point, such as 24 h after transfection (data not shown), it was no longer significant 40 h after transfection in A549 cells. A mutation at the NF- κ B site affected 7a/X4-mediated activation of the IL-8 promoter (Fig. 5C), indicating that the effect of 7a/X4 in A549 cells was also mainly mediated through the NF- κ B site. A reporter assay using the κ B-Luc plasmid showed that 7a/X4 enhanced NF- κ B activity 9.3-fold while 3a/X1 enhanced it 2.8-fold in A549 cells (Fig. 5D).

Thus, 3a/X1 and 7a/X4 were capable of activating NF- κ B and IL-8 promoter, but such effects were predominantly elicited by 3a/X1 in HEK293T cells and by 7a/X4 in A549 cells.

3.5. Enhancement of inflammatory chemokine production by SARS-CoV 3a/X1, and 7a/X4

Finally, we examined whether 3a/X1, and 7a/X4 actually induced inflammatory chemokine production in A549 cells. As shown in Table 1, although A549 cells spontaneously produced IL-8, expression of 7a/X4 further increased the levels of IL-8 production. 3a/X1 also enhanced IL-8 production, but its statistical significance was variable (Table 1). Production of another chemokine RANTES, that is controlled at least by NF- κ B [20] and up-regulated in SARS-CoV-infected cells [21], was significantly induced by 3a/X1 and 7a/X4 (Table 1).

Table 1
Enhancement of IL-8 and RANTES production by SARS-CoV 3a/X1 and 7a/X4

Chemokines	Experiment	Chemokine production (pg/ml) in A549 cells expressing		
		GFP (control)	3a/X1	7a/X4
IL-8	Exp. 1	685 ± 9	N.D.	1027 ± 111*
	Exp. 2	591 ± 16	615 ± 13	719 ± 20*
	Exp. 3	1345 ± 56	1767 ± 263	1976 ± 324*
	Exp. 4	537 ± 50	708 ± 29*	673 ± 61*
	Exp. 5	481 ± 35	641 ± 38*	616 ± 44*
RANTES	Exp. 1	101 ± 5	N.D.	442 ± 57*
	Exp. 2	80 ± 18	192 ± 11*	362 ± 15*
	Exp. 3	12 ± 6	120 ± 35*	175 ± 54*

ND, not determined.

* $P < 0.05$ vs GFP controls.

Both 3a/X1 and 7a/X4 proteins are expressed in SARS-CoV-infected cells [22]. 3a/X1 protein is a cell membrane-associated protein, potentially secreted and incorporated into the virion [23–25]. 7a/X4 protein is located in the cytoplasm [26]. Recent studies reported that over-expression of 3a/X1 and 7a/X4 induced apoptosis of the cell [27,28]. Our results indicated that these cell-associated SARS-CoV accessory gene products could activate NF- κ B and JNK, and might be strong candidates to induce pathological inflammatory responses in SARS.

Acknowledgement: We wish to thank Drs. Junichi Fujisawa (Kansai Medical School, Osaka, Japan), Naofumi Mukaida (Kanazawa University, Kanazawa, Japan), and Hidenori Ichijo (The University of Tokyo, Tokyo, Japan) for providing plasmids. This study was supported by the Special Coordination Funds for Promoting Science and Technology of Japan Science and Technology.

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