ISSN 1672-9145 **Acta Biochimica et Biophysica Sinica** 2005, **37**(9): 607–612 **CN** 31-1940/Q

Activation of NF-κB by the Full-length Nucleocapsid Protein of the SARS Coronavirus

Qing-Jiao LIAO[#], Lin-Bai YE*, Khalid Amine TIMANI[#], Ying-Chun ZENG, Ying-Long SHE, Li YE, and Zheng-Hui WU

State Key Laboratory of Virology, College of Life Science, Wuhan University, Wuhan 430072, China

Abstract The severe acute respiratory syndrome coronavirus (SARS-CoV) is the major causative agent for the worldwide outbreak of SARS in 2003. The mechanism by which SARS-CoV causes atypical pneumonia remains unclear. The nuclear factor kappa B (NF-κB) is a key transcription factor that activates numerous genes involved in cellular immune response and inflammation. Many studies have shown that NF-κB plays an important role in the pathogenesis of lung diseases. In this study, we investigated the possible regulatory interaction between the SARS-CoV nucleocapsid (N) protein and NF-κB by luciferase activity assay. Our results showed that the SARS-CoV N protein can significantly activate NF-κB only in Vero E6 cells, which are susceptible to SARS-CoV infection, but not in Vero or HeLa cells. This suggests that NF-κB activation is cell-specific. Furthermore, NF-κB activation in Vero E6 cells expressing the N protein is dose-dependent. Further experiments showed that there is more than one function domain in the N protein responsible for NF-κB activation. Our data indicated the possible role of the N protein in the pathogenesis of SARS.

Key words severe acute respiratory syndrome coronavirus (SARS-CoV); nucleocapsid protein; NF-κB

Severe acute respiratory syndrome (SARS) is a newly emerging infectious disease that has spread to many countries. The causative agent of SARS has been identified as a novel coronavirus, namely, the SARS-associated coronavirus [1,2]. The SARS-CoV is an enveloped, positive-sense RNA virus with a genome comprising about 30,000 nucleotides predicted to contain 13–15 open reading frames (ORFs). A sequence comparison with corresponding ORFs of other known coronaviruses has revealed a pattern of gene organization similar to typical coronaviruses [3,4]. The high viral virulence resulting in a significant mortality rate of infected patients has created widespread scientific interest in understanding the mechanisms of pathogenicity of this virus.

The SARS-CoV nucleocapsid (N) protein (NP) is a 46 kDa structural protein and shares little homology with other members of the coronavirus family. Besides its nucleocapsid assembly during the viral life cycle, the N protein has also been reported to activate the activator protein 1 (AP1) signal transduction pathway and induce apoptosis in COS-1 cells in the absence of growth factors [5,6].

The nuclear factor κB (NF-κB) belongs to a highly conserved Rel-related protein family, which includes RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50) and NF-κB2 (p100/p52). The p50/p65 heterodimer, commonly referred to NF-κB, is the most abundant and ubiquitous. NF-κB is the key transcription factor that activates many genes involved in cellular immune response and inflammation, such as interferon-β, tumor necrosis factor (TNF)-α, interleukin (IL)-2, IL-6 and IL-8 [7].

It has been reported that NF-κB plays an important role in the pathogenesis of many lung diseases [8]. The clinical symptom of SARS patients is atypical pneumonia characterized by progressive respiratory failure leading to lung fibrosis and the formation of cysts [9].

In order to understand the role of NF-κB in SARS-CoV infection, we studied the regulatory interaction between the SARS-CoV N protein and NF-κB. Our results showed

Received: March 8, 2005 Accepted: May 19, 2005

[#] These authors contributed equally to this work

*Corresponding author: Tel, 86-27-68752372; Fax, 86-27-68764763; E-mail, linbaiye@whu.edu.cn

DOI: 10.1111/j.1745-7270.2005.00082.x

that the full-length N protein can significantly increase NF-κB activity only in the Vero E6 cells and this activation is dose-dependent.

Materials and Methods

Plasmid construction

The recombinant plasmids with different deletion mutations of the N protein gene were constructed by inserting the corresponding DNA fragments into the eukaryotic expression vector pcDNA3 under the immediate early *CMV* promoter. The DNA fragments were amplified by PCR from pGEMT-NP [10] using the primers in **Table 1**. Primers 1a and 2a were used for pcNP containing the full-length N protein gene. Primers 1a and 2b were used for pcN1–225 containing 675 bp of the N protein gene at the 5′ end. Primers 1b and 2a were used for pcN226–422 containing 591 bp of the N protein gene in the 3′ end. Primers 1b and 2c were used for pcN226–300 containing the middle 225 bp of the N protein gene. Primers 1c and 2a were used for pcN355–422 containing 201 bp of the N protein gene at the 3' end. All the constructed plasmids were confirmed by restriction digestion and sequencing.

Table 1 Primers used in this study

Primer	Sequence (5′→3′)
1a	TGAGCA*GGATCC*GTC**ATG**TCTGATAATT-GACCCC (*Bam*HI)
1b	GCAGCT*GGATCC*GTC**ATG**GACAGATTGA-ACGGCTTG AG (*Bam*HI)
1c	CAGCT*GGATCC*GTC**ATG**GGAGCCATTAA-ATTCGATGAC (*Bam*HI)
2a	CGCACG*GAATTC***TTA**TGCCTGAGTTGA-ATCAGC (*Eco*RI)
2b	GGCACC*GAATTC***TTA**TAGCAGCAATACC-GCGAG (*Eco*RI)
2c	GTCGCC*GAATTC***TTA**TTTGTGATCAGTT-CCTTG (*Eco*RI)

The restriction enzyme sites were indicated in italic. ATG and TTA in bold represent the start codon and stop codon, respectively.

Cell culture and transfection

Vero, HeLa and Vero E6 cells obtained from China Centre for Type Culture Collection (Wuhan, China) were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, USA) containing 10% fetal bovine serum (Hyclone, Logan, USA) at 37 °C, with 5% CO_2. The DNA transfection experiment was performed using Lipofectamine™ 2000 (Gibco) according to the manufacturer's instructions.

Western blot analysis

The total cell proteins were separated by 15% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride membrane 24 h after transfection. The blots were first blocked with 5% non-fat milk in Tris buffer saline (TBS) containing 0.1% Tween-20, and then probed with the first antibodies, rabbit anti-N protein poly antibodies or anti-actin antibody (sc-1616; Santa Cruz Biotechnology, Santa Cruz, USA), for 1 h at 37 °C. After extensive washing, secondary antibodies conjugated with horseradish peroxidase (HRP) were applied onto the blots for at least 1 h at 37 °C. The blots were washed 5 times with TBS containing 0.1% Tween-20. Reagents for enhanced chemiluminescence were applied to the blots and the light signals were detected by X-ray film.

Luciferase activity assay

The pNF-κB-Luc vector (Stratagene, La Jolla, USA) containing the *Photinus pyralis* (firefly) luciferase reporter gene driven by the basic promoter element (TATA box) plus five repeats of κB *cis*-enhancer element (TGGGGACTTTCCGC) was used in this experiment. Approximately 5×10^4 cells were plated onto a 24-well tissue culture plate 24 h before transfection. The cells were co-transfected with 0.4 μg of pNF-κB-Luc plus 0.4 μg of pcNP-mutant or 0.05, 0.10, 0.20 and 0.40 μg of pcNP. The dose of transfected plasmids was kept at 0.8 μg using the pcDNA3 vector. Cells co-transfected with pNF-κB-Luc and the pcDNA3 vector were used as the negative control. Cells were harvested 24 h after transfection and were then lysed in the reporter lysis buffer (Promega, Madison, USA). The luciferase activity was measured by a TD-20/20 luminometer (Turner BioSystems, Sunnyvale, USA) and normalized using the protein concentration of the cell lysates. Each experiment was repeated at least 3 times.

Statistical analysis

Data were expressed as mean±SD. The activation of NF-κB was considered to be statistically significant if the relative luciferase activity showed an increase higher than

2 folds.

Results

Identification of SARS N protein and its mutants

The expressions of the N protein in three cell lines and N protein mutants in the Vero E6 cell line were measured by Western blotting. As shown in **Fig. 1**, the N protein was expressed with the correct molecular weight of 46 kDa in HeLa, Vero and Vero E6 cells [**Fig. 1(A)**], and N protein mutants were expressed with corresponding molecular weights (N protein, 46 kDa; N1–225, 25 kDa; N226–422, 21 kDa; N355–422, 10 kDa; and N226–300, 9 kDa) in Vero E6 cells [**Fig. 1(B)**]. The expression levels of different N protein mutants were a little different at the same transfection concentrations because they interacted differently with the antibodies (anti-N protein rabbit serum). This phenomenon was also observed when we performed an indirect-immunofluorescence assay for the localization of N protein mutants (data not shown).

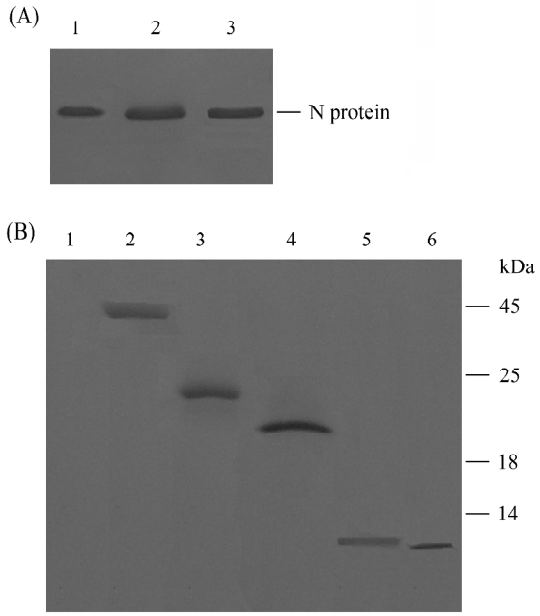

Fig. 1 Detection of the N protein and its mutants by Western blot analysis

(A) Expression of N protein in three cell lines. 1, HeLa; 2, Vero; 3, Vero E6. (B) Expression of N mutants in Vero E6 cells. Cells transfected with different recombinant plasmids were harvested 24 h post-transfection, and cells transfected with pNF-κB-Luc and the pcDNA3 vector only were used as the negative control. Cells were lysed and analyzed using 15% SDS-PAGE and subjected to immunoblot analysis with the corresponding antibodies as described in "Materials and Methods". 1, control; 2, pcNP; 3, pcN1–225; 4, pcN226–422; 5, pcN355–422; 6, pcN226–300. The molecular weight marker is shown on the right.

Activation of NF-κB in Vero E6 cells by N protein

The effect of the N protein on NF-κB activity in HeLa, Vero and Vero E6 cells was investigated by the luciferase activity assay. In Vero E6 cells, the expression of the N protein resulted in a significant increase in NF-κB activity compared with the control (**Fig. 2**). With the increase in the concentration of pcNP, the NF-κB activity was 2-fold to 8-fold higher than that of the control [**Fig. 3(A)**], clearly showing that activation of NF-κB by the SARS-CoV N protein is dose-dependent. In contrast, the increase in NF-κB activity in HeLa cells was not obvious, being only about 2-fold higher than that of the control (**Fig. 2**). In Vero cells transfected with pcNP, there was almost no change in the NF-κB activity compared with the control (<2 folds, **Fig. 2**). The expression of the N protein in Vero E6 cells transfected with different concentrations of pcNP plasmids was also detected by Western blotting. The result clearly showed that the expression level of the N protein is linearly related to the amount of pcNP plasmid transfected [**Fig. 3(B)**]. On the basis of these results, we concluded that the N protein can activate NF-κB only in Vero E6 cells and that this activation is dose-dependent.

Function domain of the N protein for NF-κB activation

To determine the function domain of the N protein for NF-κB activation, we constructed four N protein mutants [**Fig. 4(A)**]. First, we examined two mutants, N1–225 and N226–422. N1–225 contains the N-terminal part of

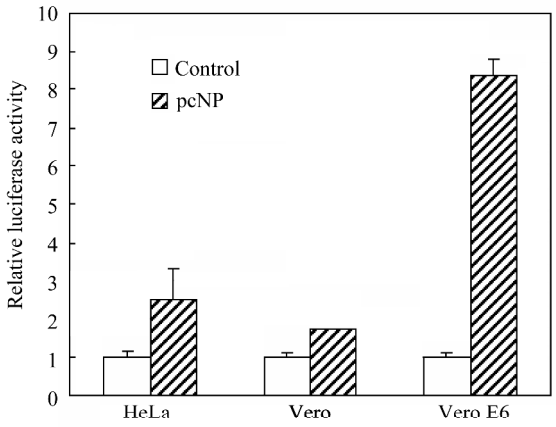

Fig. 2 NF-κB activation by the N protein

Different cell lines were co-transfected with 0.4 μg of pNF-κB-Luc and 0.4 μg of pcNP. Cells co-transfected with pNF-κB-Luc and the pcDNA3 vector were used as the negative control. Cells were harvested 24 h after transfection and assayed for luciferase activity. Data were expressed in mean±SD (n=3).

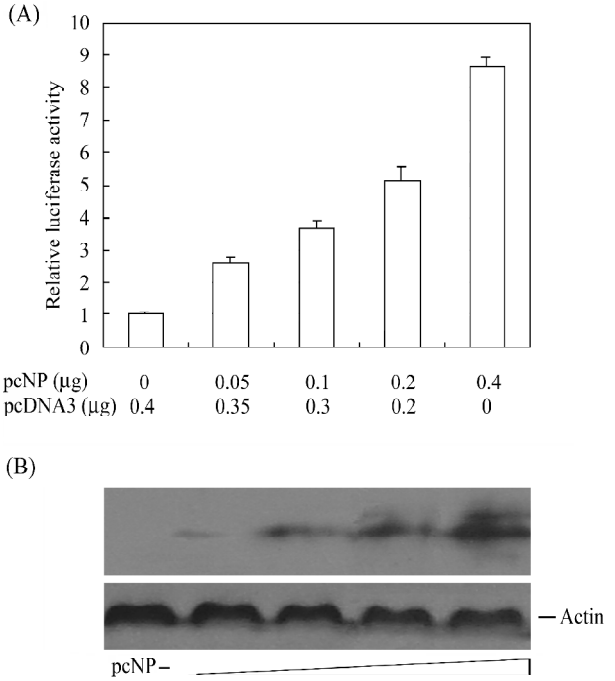

Fig. 3 NF-κB activation by different amounts of N protein

(A) Vero E6 cells were co-transfected with 0.4 μg of pNF-κB-Luc and 0.05, 0.10, 0.20 and 0.40 μg of pcNP. Cells co-transfected with pNF-κB-Luc and the pcDNA3 vector were used as the negative control. Cells were harvested 24 h after transfection and assayed for luciferase activity. Data were expressed in mean±SD (n=3). (B) Expression levels of N protein in the Vero E6 cells transfected with different concentrations of plasmids. The actin band showed the same amount of proteins loaded in each well.

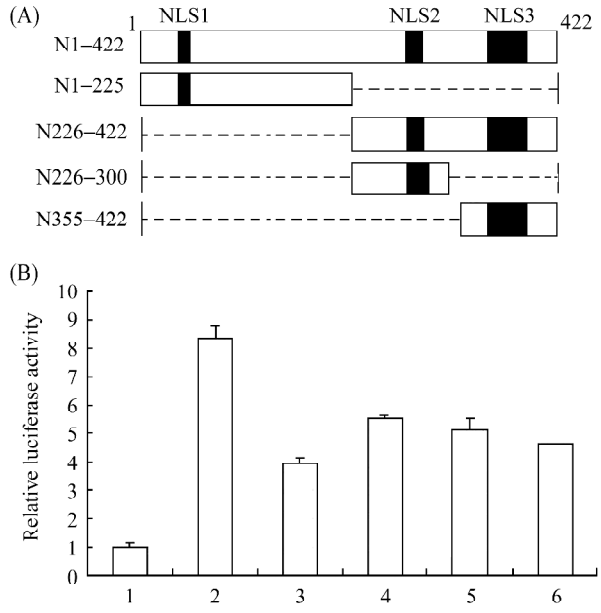

Fig. 4 NF-κB activation by different NP mutants

(A) Different N mutants. (B) Vero E6 cells were co-transfected with 0.4 μg of pNF-κB-Luc and 0.4 μg of pcNP mutations. Cells co-transfected with pNF-κB-Luc and the pcDNA3 vector were used as the negative control. Cells were harvested 24 h after transfection and assayed for luciferase activity. Data were expressed in mean±SD (n=3). 1, control; 2, pcNP; 3, pcN1–225; 4, pcN226–422; 5, pcN355–422; 6, pcN226–300.

the N protein with an SR-rich region which may be responsible for the N protein phosphorylation. N226–422 contains the C-terminal fragment of the N protein. As indicated in **Fig. 4(B)**, N226–422 exhibited a higher increase in NF-κB activity. This suggests that the function domain might be located in the C-terminal of the N protein, so we examined two other mutants, N226–300 containing the middle part of the N protein and N355–422 containing the C-terminal of the N protein. We found it very strange that these two mutants both had an increase in NF-κB activity [**Fig. 4(B)**]. It seems that all the N protein mutants can activate NF-κB, but the increase in activity in every mutant is lower than that of the full-length N protein. It has been reported that the N protein can be cleaved into several small fragments in virus-infected cells by caspases [11]. NF-κB activation may be crucial for virus replication or proliferation; to minimize the loss of the N protein through degradation caused by the host factor, there are several function domains in the N protein responsible for NF-κB activation and the activation caused by the N protein is the synergistic effect of all the function domains.

Discussion

In this study, we have shown that the SARS-CoV N protein significantly activates NF-κB in Vero E6 cells, but not in HeLa or Vero cells, and that this activation is dose-dependent. This is in accordance with the results of a previous study, which showed that the SARS-CoV N protein can not activate NF-κB in both Vero and Huh-7 cells [5]. This suggests that the activation of NF-κB by the N protein is cell-specific. The Vero E6 cell line is highly susceptible to virus infection. There must be some specific cellular factors which support SARS-CoV proliferation and participate in the activation of NF-κB by the N protein in Vero E6 cells. Further experiments have to be conducted to find these factors.

In addition, we also found that the full-length N protein had the highest NF-κB activity. This suggests that there is more than one function domain in the N protein responsible for NF-κB activation and that the activation caused by the N protein is a result of the synergistic effect of all the function domains. Furthermore, the localization of these mutants is different. The full-length N protein and N355–422 are located mainly in the cytoplasmid, while

N1–225, N226–300 and N226–422 are located in both the cytoplasmid and nucleolus, and the latter two can also be found in the nucleoli (data not shown). The mechanism by which NF-κB is activated by these mutants is unclear. Some mutants may bind directly with the κB binding site in the promoter of the target gene or form a complex with other nuclear factors and then bind with the κB binding site. Other mutants may activate NF-κB through signal pathways.

Three nuclear localization signal (NLS) motifs in the N protein were identified using PSORT II [**Fig. 4(A)**]. We found that the NF-κB activation caused by the N protein is slightly related to the NLS motifs. The more NLS motifs the mutant has, the higher the NF-κB activity that can be detected. The full-length N protein has three NLS motifs and has the highest NF-κB activity, while N1–225 and N355–422 have only one NLS motif and have the lowest NF-κB activity. NLS is a basic amino acid-rich sequence and can be recognized by proteins of the importin superfamily that mediate transport across the nuclear envelope [12]. But it is unclear as to how NLS affects NF-κB activation; the basic characteristic of NLS may play an important role.

NF-κB is a critical regulator of the immediate early pathogen response, playing an important role in promoting inflammation and regulating cell proliferation and survival [13]. NF-κB is highly activated at sites of inflammation in diverse diseases and it induces the transcription of pro-inflammatory cytokines (e.g. IL-1β, TNF-α and IL-6), chemokines (e.g. IL-8) and adhesion molecules, including intercellular adhesion molecule 1 (ICAM-1), matrix metalloproteinases (MMPs), cyclooxygenase 2 (COX2) and inducible nitric oxide synthase (iNOS) [14]. Because of the multiple functions of NF-κB, many viruses, including several human pathogens, such as the human immunodeficiency virus (HIV)-1, human T-cell leukemia virus (HTLV)-1, herpes simplex virus (HSV)-1, hepatitis C virus (HCV) and Epstein-Barr virus, have evolved different strategies to modulate the activity of NF-κB [15–19]. Some of them modulate the NF-κB activity through the binding of the viral particles to the NF-κB receptor, while others modulate the activity through viral proteins. The activation of NF-κB may be a strategy used by viruses to control the host cells in order to facilitate the early release of virus progeny or help the emerging virus evade the host immune system.

NF-κB has been shown to regulate the production of acute inflammatory mediators in a variety of cells and animal models developed to elucidate the pathobiology of lung diseases, including acute respiratory distress syndrome (ARDS), systemic inflammatory response syndrome (SIRS), asthma, respiratory viral infections, occupational and environmental lung disease and cystic fibrosis [8]. A high IL-8 expression resulting from NF-κB activation is always observed in cell or animal models of these lung diseases [20–22]. In addition, there are some emerging clinical data related to NF-κB activation in the pathogenesis of ARDS, SIRS and asthma. In ARDS and SIRS, NF-κB activation in alveolar macrophages and other types of lung cells very likely modulates neutrophilic alveolitis and lung injury [23]. In asthma, NF-κB activation in airway epithelial cells and other types of cells may affect initiation or maintenance of the inflammatory phenotype that characterizes the disease [24].

SARS is characterized by a persistent fever and respiratory symptoms with lung consolidation, lymphopenia and respiratory failure in life-threatening cases [9]. SARS sequelae, such as transendothelial migration of polymorphonuclear cells into the lung tissues, multiple organ dysfunction and ARDS, have been postulated to be associated with cytokine and chemokine dysregulation [25]. High IL-8 and IL-2 levels have been observed in SARS patients [26]. This indicates that NF-κB might also play an important role during SARS infection.

Although the basic function of the N protein of a virus is to bind with the genome and form a virus core, many viruses' N proteins, such as the HCV core protein and hepatitis B virus (HBV) X protein, have shown a regulatory effect on NF-κB activation [18,27]. In this article, the regulatory effect of the SARS-CoV N protein on NF-κB was studied. Our results show that the full-length N protein activates NF-κB activity only in Vero E6 cells and that the activation is dose-dependent. This indicates that the SARS-CoV N protein may be involved in the pathogenesis of SARS and this finding can be used in the deve-lopment of therapeutics for the treatment of SARS.

References

1 Drosten C, Gunther S, Preiser W, van der Werf S, Brodt HR, Becker S, Rabenau H *et al*. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N Engl J Med 2003, 348: 1967–1976

2 Ksiazek TG, Erdman D, Goldsmith CS, Zaki SR, Peret T, Emery S, Tong S *et al*. A novel coronavirus associated with severe acute respiratory syndrome. N Engl J Med 2003, 348: 1953–1966

3 Rota PA, Oberste MS, Monroe SS, Nix WA, Campagnoli R, Icenogle JP, Penaranda S *et al*. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. Science 2003, 300: 1394–1399

4 Marra MA, Jones SJ, Astell CR, Holt RA, Brooks-Wilson A, Butterfield YS, Khattra J *et al*. The genome sequence of the SARS-associated coronavirus. Science 2003, 300: 1399–1404

5 He R, Leeson A, Andonov A, Li Y, Bastien N, Cao J, Osiowy C *et al*. Activation of AP-1 signal transduction pathway by SARS coronavirus nucleocapsid protein. Biochem Biophys Res Commun 2003, 311: 870–876
6 Surjit M, Liu B, Jameel S, Chow VT, Lal SK. The SARS coronavirus nucleocapsid protein induces actin reorganization and apoptosis in COS-1 cells in the absence of growth factors. Biochem J 2004, 383: 13–18
7 Li Q, Verma IM. NF-κB regulation in the immune system. Nat Rev Immunol 2002, 2: 725–734
8 Christman JW, Sadikot RT, Blackwell TS. The role of nuclear factor-κB in pulmonary diseases. Chest 2000, 117: 1482–1487
9 Peiris JS, Yuen KY, Osterhaus AD, Stohr K. The severe acute respiratory syndrome. N Engl J Med 2003, 349: 2431–2441
10 Timani KA, Ye L, Ye L, Zhu Y, Wu Z, Gong Z. Cloning, sequencing, expression, and purification of SARS-associated coronavirus nucleocapsid protein for serodiagnosis of SARS. J Clin Virol 2004, 30: 309–312
11 Ying W, Hao Y, Zhang Y, Peng W, Qin E, Cai Y, Wei K *et al*. Proteomic analysis on structural proteins of severe acute respiratory syndrome coronavirus. Proteomics 2004, 4: 492–504
12 Hicks GR, Raikhel NV. Protein import into the nucleus: An integrated view. Annu Rev Cell Dev Biol 1995, 11: 155–188.
13 Karin M, Cao Y, Greten FR, Li ZW. NF-κB in cancer: From innocent bystander to major culprit. Nat Rev Cancer 2002, 2: 301–310
14 Bonizzi G, Karin M. The two NF-κB activation pathways and their role in innate and adaptive immunity. Trends Immunol 2004, 25: 280–288
15 Bour S, Perrin C, Akari H, Strebel K. The human immunodeficiency virus type 1 Vpu protein inhibits NF-κB activation by interfering with βTrCP-mediated degradation of IκB. J Biol Chem 2001, 276: 15920–15928
16 O'Mahony AM, Montano M, van Beneden K, Chen LF, Greene WC. Human T-cell lymphotropic virus type 1 tax induction of biologically active NF-κB requires IκB kinase-1-mediated phosphorylation of RelA/p65. J Biol Chem 2004, 279: 18137–18145
17 Goodkin ML, Ting AT, Blaho JA. NF-κB is required for apoptosis prevention during herpes simplex virus type 1 infection. J Virol 2003, 77: 7261–7280
18 Watashi K, Hijikata M, Marusawa H, Doi T, Shimotohno K. Cytoplasmic localization is important for transcription factor nuclear factor-κB activation by hepatitis C virus core protein through its amino terminal region. Virology 2001, 286: 391–402
19 Luftig M, Yasui T, Soni V, Kang MS, Jacobson N, Cahir-McFarland E, Seed B *et al*. Epstein-Barr virus latent infection membrane protein 1 TRAF-binding site induces NIK/IKKα-dependent noncanonical NF-κB activation. Proc Natl Acad Sci USA 2004, 101: 141–146
20 Mastronarde JG, He B, Monick MM, Mukaida N, Matsushima K, Hunninghake GW. Induction of interleukin (IL)-8 gene expression by respiratory syncytial virus involves activation of nuclear factor (NF)-κB and NF-IL-6. J Infect Dis 1996, 174: 262–267
21 Simeonova PP, Luster MI. Asbestos induction of nuclear transcription factors and interleukin 8 gene regulation. Am J Respir Cell Mol Biol 1996, 15: 787–795
22 DiMango E, Ratner AJ, Bryan R, Tabibi S, Prince A. Activation of NF-κB by adherent *Pseudomonas aeruginosa* in normal and cystic fibrosis respiratory epithelial cells. J Clin Invest 1998, 101: 2598–2605
23 Schwartz MD, Moore EE, Moore FA, Shenkar R, Moine P, Haenel JB, Abraham E. Nuclear factor-kappaB is activated in alveolar macrophages from patients with acute respiratory distress syndrome. Crit Care Med 1996, 24: 1285–1292
24 Hart LA, Krishnan VL, Adcock IM, Barnes PJ, Chung KF. Activation and localization of transcription factor, nuclear factor-κB, in asthma. Am J Respir Crit Care Med 1998, 158: 1585–1592
25 Chang YJ, Liu CY, Chiang BL, Chao YC, Chen CC. Induction of IL-8 release in lung cells via activator protein-1 by recombinant baculovirus displaying severe acute respiratory syndrome-coronavirus spike proteins: Identification of two functional regions. J Immunol 2004, 173: 7602–7614
26 Lee CH, Chen RF, Liu JW, Yeh WT, Chang JC, Liu PM, Eng HL *et al*. Altered p38 mitogen-activated protein kinase expression in different leukocytes with increment of immunosuppressive mediators in patients with severe acute respiratory syndrome. J Immunol 2004, 172: 7841–7847
27 Wang T, Wang Y, Wu MC, Guan XY, Yin ZF. Activating mechanism of transcriptor NF-kappaB regulated by hepatitis B virus X protein in hepatocellular carcinoma. World J Gastroenterol 2004, 10: 356–360

Edited by
Bing SUN