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<th>Severe acute respiratory syndrome coronavirus nucleocapsid protein does not modulate transcription of the human FGL2 gene</th>
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Severe acute respiratory syndrome (SARS) is a highly lethal infectious disease that spread in China and globally in 2003. The primary aetiologic agent has been identified as SARS coronavirus (SARS-CoV; Peiris et al., 2003), a distant member of group 2 coronaviruses (Gorbalenya et al., 2004). Closely related SARS-CoV-like viruses have also been found in various bats (Lau et al., 2005).

Coronaviruses are large, enveloped and positive-stranded RNA viruses that cause a number of diseases in humans and animals (Cheng et al., 2007). The coronavirus nucleocapsid (N) protein is a relatively conserved structural protein that binds genomic RNA and plays an important role in viral RNA synthesis, viral assembly and formation of the RNA replication complex (Narayanan et al., 2003; Almazán et al., 2004). Intracellularly, coronavirus N protein localizes to the cytoplasm, in which it interacts with M protein to form an icosahedral core (Hurst et al., 2005). In addition, N proteins from mouse hepatitis virus (MHV) and several other coronaviruses have also been shown to localize to the nucleus to exert an impact on cell cycle progression (Wurm et al., 2001; Chen et al., 2002). In line with this, MHV N protein regulates the expression of cellular genes such as Fgl2 (Ning et al., 1999). Fgl2 encodes a prothrombinase termed fibrinogen-like protein 2, which causes vascular thrombosis, fibrin deposition and pneumocyte necrosis. MHV N protein stimulates Fgl2 transcription through cellular transcription factors, implicating a role in the pathogenesis of MHV-associated hepatitis (Ning et al., 2003).

While nucleolar localization has also been suggested (You et al., 2007), SARS-CoV N protein is more frequently found in the cytoplasm (Rowland et al., 2005; You et al., 2005; Fan et al., 2006). Interestingly, the gene regulatory function of this protein has also been documented in the context of AP-1-, NF-κB- and CCAAT/enhancer binding protein (C/EBP)-dependent transcription (He et al., 2003; Yan et al., 2006; Zhang et al., 2007), interferon (IFN) production (Kopecky-Bromberg et al., 2007) and transforming growth factor-β signalling (Zhao et al., 2008). Because fibrosis and vascular thrombosis in the lung are also observed commonly in patients with SARS (Nicholls et al., 2003), SARS-CoV N protein has been proposed to regulate the expression of the human FGL2 gene (Robertson, 2003). Indeed, a recent work has demonstrated that FGL2 transcription is stimulated by SARS-CoV N protein in transfected cells (Han et al., 2008). Interestingly, the association of single nucleotide polymorphisms in the FGL2 locus with nasopharyngeal shedding of SARS-CoV and clinical severity has recently been suggested (Chen et al., 2006).

The important implications in pathogenesis prompted us to re-examine the regulation of FGL2 transcription by SARS-CoV N protein in both transfected and infected cells. As a first step, we expressed SARS-CoV N protein in
HEK293 cells. cDNA encoding SARS-CoV N protein was PCR-amplified from a molecular clone of the SARS-CoV subgenome (Chan et al., 2006) and subcloned into the pCDNA3.1 vector. Western blot analysis indicated that N protein was abundantly expressed in transfected cells (Fig. 1a, compare lanes 1 and 2, and lanes 3 and 4). We then verified the subcellular localization of N protein by using confocal immunofluorescence microscopy as described previously (Chin et al., 2007). Consistent with previous reports (Rowland et al., 2005; You et al., 2005; Fan et al., 2006), N protein localized predominantly to the cytoplasm of transfected cells (Fig. 1b, transfected cells are indicated by arrows, compare with neighbouring non-transfected cells).

**Fig. 1.** Expression and gene regulatory activity of SARS-CoV N protein in cultured cells. (a) Western blot analysis. HEK293 cells were mock-transfected with empty vectors (lanes 2 and 4) or transfected with pCDNA3.1-V5-N (lane 1) or pCDNA3.1-N (lane 3). Lysed cells were immunoblotted with mouse monoclonal anti-V5 (Invitrogen; lanes 1 and 2) and rabbit polyclonal anti-N (Imgenex; lanes 3 and 4). Experiments were repeated twice and similar results were obtained. (b) Confocal microscopic analysis of subcellular localization. Vero cells were transfected with pCDNA3.1-V5-N (i–iii) and pCDNA3.1-N (iv–vi) and stained with mouse monoclonal anti-V5 and mouse anti-N (Imgenex), respectively. Nuclei were stained with propidium iodide (PI). Arrows indicate transfected cells. Results are representative of four independent experiments. Bar, 30 μm. (c) Gene regulatory activity. TLR3-expressing HEK293 cells were transfected with pISRE-Luc and progressively increasing amounts (200, 400 and 600 ng; white, black and hatched bars, respectively) of expression vectors (Chan et al., 2006; Kok & Jin, 2006) for the indicated viral proteins (S, SARS-CoV S; E, SARS-CoV E; N, SARS-CoV N; NS1, influenza A virus NS1). Twenty-four hours after transfection, cells were stimulated with 1 μg poly (I:C) ml⁻¹ (pIC) for 12 h. The mock-treated group (Mock) was treated with DMSO only. Results represent mean ± SD from three independent experiments. *P<0.05 by Student’s t-test, indicating that expression of neither S nor E protein significantly influenced ISRE activity. †P<0.05 by Student’s t-test, indicating that the expression of either N or NS1 protein significantly inhibited ISRE-dependent expression of the luciferase reporter.
We next investigated whether SARS-CoV N protein expressed in HEK293 cells might have gene regulatory activity as it does in other cells (He et al., 2003; Yan et al., 2006; Kopecky-Bromberg et al., 2007; Zhang et al., 2007; Zhao et al., 2008). We used reporter plasmid pISRE-Luc, in which the expression of firefly luciferase is driven by IFN-stimulated response elements (ISRE). Because the expression level of endogenous TLR3 in HEK293 cells was very low, an expression vector for TLR3 (pCDNA3.1-TLR3; Kok & Jin, 2006) was stably transfected into these cells. Dual luciferase activity was assayed as described previously (Chin et al., 2007; Choy et al., 2008) by normalizing the readouts of firefly luciferase to those of Renilla luciferase expressed from plasmid pRLSV40 (Promega) cotransfected into the cells. Expression of N protein significantly repressed ISRE-dependent transcription in a dose-dependent manner (Fig. 1c), exactly as demonstrated by Kopecky-Bromberg et al. (2007). The extent of repression induced by N protein was comparable to that in the case of influenza A virus NS1, another well-characterized viral IFN antagonist (Krug et al., 2003; Kok & Jin, 2006; Siu et al., 2009). In further support of the specificity of effect, the repression was not seen when SARS-CoV S or E protein was expressed (Fig. 1c). Thus, SARS-CoV N protein expressed in HEK293 cells was able to repress ISRE transcriptional activity, which controls IFN production. Although only ~25% of the cells were transiently transfected, more than 95% of the transfected cells were found to express both luciferase and the indicated viral protein, as verified by confocal microscopy. Similar results were also obtained from Vero cells (data not shown).

The stimulation of the FGL2 promoter by SARS-CoV N protein has implications in not only the pathogenesis of SARS but also the development of therapeutics (Robertson, 2003; Han et al., 2008). To shed light on whether and how SARS-CoV N protein might activate transcription of the FGL2 gene, we constructed the reporter plasmid pFGL2-Luc, in which the expression of firefly luciferase is under the control of the FGL2 promoter. This promoter, containing nt −1000 to +10 of the human FGL2 gene, was PCR-amplified from genomic clone RP11-467H10 (ImaGenes). To confirm the activity of this construct, we cotransfected it into HEK293 cells with an expression plasmid for Sp1, a known activator of the FGL2 promoter (Liu et al., 2003, 2006). Human Sp1 cDNA was derived from IMAGE clone 5928633 (ImaGenes) and subcloned into pCDNA3.1. A more than threefold stimulation of reporter expression by Sp1 demonstrated that pFGL2-Luc sensitively reflected intracellular activity of the FGL2 promoter (Fig. 2a). Consistent with this result and a previous report (Liu et al., 2006), treatment of transfected cells with IFN-γ led to approximately 4.5-fold activation of reporter expression (Fig. 2a). Although only ~20% of the cells were transiently transfected, more than 90% of the transfected cells were found to express both luciferase and Sp1/N protein, verified by confocal microscopy.

However, when we cotransfected pFGL2-Luc and an expression plasmid for SARS-CoV N protein into HEK293 cells, no induction of reporter activity was observed (Fig. 2b). To further characterize the influence of N protein on the expression of endogenous FGL2, we analysed the steady-state amounts of FGL2 transcript in HEK293 cells overexpressing Sp1 or N protein by semiquantitative RT-PCR as described previously (Kok et al., 2007; Siu et al., 2008). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene was used as an RT-PCR control. The relative amounts of FGL2 mRNA normalized to the GAPDH transcript were obtained by using Scion Image software. Primers for RT-PCR will be provided upon request. While the expression of Sp1 correlated with an elevation of the FGL2 transcript over the basal level (Fig. 3a, compare lane 2 with lane 1), expression of N protein did not alter the relative amount of FGL2 mRNA in transfected HEK293 cells (Fig. 3a, lanes 3–5 compared with lane 1). We then analysed protein expression by using rabbit polyclonal anti-Sp1, goat

![Fig. 2.](http://vir.sgmjournals.org) Expression of SARS-CoV N protein did not stimulate the FGL2 promoter. HEK293 cells were transfected with pFGL2-Luc (100 ng) plus progressively increasing amounts (100, 200, 400 and 600 ng) of Sp1 expression plasmid (a) or SARS-CoV N protein expression plasmid (b). Ctrl, Control cells transfected with pFGL2-Luc and empty vector. As a positive control, another group of cells was treated with 1000 U IFN-γ for 16 h. Results represent mean ± SD from three independent experiments. P values (Student's t-test) indicate the significance of the influence of either Sp1 or N protein expression on FGL2 promoter activity.
Polyclonal anti-FGL2 and mouse monoclonal anti-α-tubulin (Santa Cruz). The accumulation of Sp1 protein in the cells was consistently associated with an abrupt increase in FGL2 protein level (Fig. 3b, lane 2 compared with lane 1), whereas increased N protein expression had no influence on the steady-state amounts of FGL2 (Fig. 3b, lanes 3–5 compared with lane 1). Similar observations were also made in Vero cells, where expression of N protein did not induce the expression of FGL2 transcript or FGL2 protein in transfected and infected cells. (a and b) HEK293 cells were mock-transfected with 1 µg pCDNA3.1 empty vector (Vec, lane 1) or transfected with 1 µg pCDNA3.1-SP1 (Sp1, lane 2) or with progressively increasing amounts (0.5, 0.75 and 1 µg) of pCDNA3.1-N (lanes 3–5). Semi-quantitative RT-PCR (a) and Western blotting (b) were performed 48 h after transfection. (c) Vero cells were transfected with increasing amounts (1, 1.5 and 2 µg) of pCDNA3.1-N and semi-quantitative RT-PCR was carried out. (d) Infection of HEK293/ACE2 (■) and Vero (▲) cells with SARS-CoV (strain GZ50) at an m.o.i. of 5 in serum-free DMEM. (e–g) FGL2 expression was not induced in SARS-CoV-infected HEK293/ACE2 (e), Vero (f) or Calu3 (g) cells. Cells were either mock-infected (lanes 1–3) or infected with SARS-CoV at an m.o.i. of 5 (lanes 4–6). Both detached and attached infected cells were harvested at 24, 48 and 72 h.p.i. Proteins were immunoblotted with antibodies against SARS-CoV N, FGL2 and α-tubulin (e, g) or analysed by semi-quantitative RT-PCR (f). Results are representative of three independent experiments. In (a), (c) and (f), the ratio of FGL2 : GAPDH are given underneath the lane numbers.
not affect the level of FGL2 mRNA (Fig. 3c). Thus, SARS-CoV N protein did not induce the expression of FGL2 transcript or protein in transfected cells.

To clarify the influence of SARS-CoV N protein on the expression of FGL2, we also examined the expression of FGL2 protein in HEK293/ACE2, Vero and Calu-3 cells infected with SARS-CoV strain GZ50 (m.o.i. of 5). The virus was propagated in Vero cells in a biosafety level 3 laboratory as described by Chan et al. (2006). Viral RNA copies were determined by quantitative RT-PCR as described by Du et al. (2008). HEK293/ACE2 cells stably express the SARS-CoV receptor angiotensin converting enzyme 2 (Narayanan et al., 2008). Calu-3 cells are polarized lung epithelial cells susceptible to SARS-CoV infection and serve as a good model for the study of SARS-CoV pathogenesis (Tseng et al., 2005; Yoshihawa et al., 2009). Calu-3 cells were kindly provided by Dr Pingbo Huang (Hong Kong University of Science and Technology) and Dr Wing Hung Ko (Chinese University of Hong Kong) and were cultured as described previously (Tseng et al., 2005; Sun et al., 2008; Yue et al., 2008). Consistent with previous reports (Chan et al., 2006; Narayanan et al., 2008), the viral growth curves indicated a higher yield of progeny virus in Vero cells than in HEK293/ACE2 cells (Fig. 3d). The expression of N protein or mRNA in infected HEK293/ACE2, Vero and Calu-3 cells was also verified by Western blotting or RT-PCR (Fig. 3e–g). Next, we compared the levels of FGL2 protein in mock- and SARS-CoV-infected HEK293/ACE2 cells at three different time points, but no significant difference was found (Fig. 3e, compare lanes 4–6 with 1–3). Likewise, the steady-state levels of FGL2 transcripts in mock- and SARS-CoV-infected Vero cells were similar (Fig. 3f, compare lanes 4–6 with 1–3). Finally, comparable amounts of FGL2 protein were also recovered from mock- and SARS-CoV-infected Calu-3 cells (Fig. 3g, lanes 4–6 compared with 1–3). Hence, the expression level of FGL2 mRNA and protein in infected cells did not vary.

Here, we showed that SARS-CoV N protein did not modulate the transcription of the human FGL2 gene. The expression of N protein and its ability to repress ISRE-dependent transcription in transfected cells were validated (Fig. 1). The activity of the pFGL2-Luc reporter plasmid was also confirmed with IFN-γ and cellular transcription factor Sp1 (Fig. 2a). Finally, the influence of N protein on FGL2 expression was assessed with a luciferase reporter assay (Fig. 2b), by RT-PCR (Fig. 3a, c and f) and by Western blotting (Fig. 3b, e and g). Collectively, our results did not support the regulation of FGL2 transcription by SARS-CoV N protein.

Our findings contradict a recent report on the induction of the FGL2 promoter through activation of C/EBPz by SARS-CoV N protein (Han et al., 2008). It is noteworthy that the human FGL2 promoter used in our study contains all of the cis regulatory elements described by Han et al., including the C/EBP site. In addition, the activity of our pFGL2-Luc construct was supported experimentally by the significant activation induced by cellular Sp1 transcription factor (Fig. 2a). Finally, mutations were not found in the N protein of SARS-CoV strain GZ50 used in this study (Guan et al., 2003). While we do not understand whether different experimental systems might explain different observations, we would like to point out that our work was carried out in transfected and infected HEK293, Vero and Calu-3 cells (Figs 2 and 3), while Han et al. used only transfected CHO, THP-1 and Vero cells in their study. We feel that our demonstration of the unaltered expression of FGL2 protein in infected HEK293/ACE2, Vero and Calu-3 cells (Fig. 3e–g) might be more biologically relevant to SARS-CoV.

SARS-CoV N protein is structurally and functionally related to MHV N protein. As such, both proteins are capable of multimerization and RNA binding (Narayanan et al., 2003; Luo et al., 2005; Zúñiga et al., 2007). In addition, both proteins have gene regulatory activity and can repress IFN production (Kopecky-Bromberg et al., 2007; Ye et al., 2007). However, SARS-CoV N protein also possesses properties that are not shared with its MHV counterpart. For example, SARS-CoV N protein localizes predominantly to the cytoplasm (Rowland et al., 2005; Surjit et al., 2005; You et al., 2005; Fan et al., 2006) and rarely to the nucleolus (Qinfen et al., 2004; Li et al., 2005; Tümir et al., 2005; Zeng et al., 2008), whereas MHV N protein is commonly found in the nucleolus (Wurm et al., 2001; Chen et al., 2002). Hence, SARS-CoV N protein probably functions in the cytoplasm to modulate cell signalling and IFN production, but does not directly regulate gene transcription in the nucleus. Nevertheless, our findings that SARS-CoV N protein did not activate FGL2 transcription suggest another important difference between SARS-CoV N protein and its MHV homologue. Our work also implies that FGL2 is unlikely to be involved in the pathogenesis of SARS. In this regard, further investigations are required to elucidate the molecular cause of fibrin deposition and vascular thrombosis in SARS.

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References


nucleocapsid protein is phosphorylated and localizes in the cytoplasm


