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Short communication

Intracellular localization of the SARS coronavirus protein 9b: Evidence of active export from the nucleus

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Abstract

Open reading frame 9b (ORF 9b) encodes a 98 amino acid group-specific protein of severe acute respiratory syndrome (SARS) coronavirus (CoV). It has no homology with known proteins and its function in SARS CoV replication has not been determined. The N-terminal part of the 9b protein was used to raise polyclonal antibodies in rabbits, and these antibodies could detect 9b protein in infected cells. We analyzed the sub-cellular localization of recombinant 9b protein using fluorescence microscopy of live transfected cells and indirect immunofluorescence of transfected fixed cells. Our findings indicate that the 9b protein is exported outside of a cell nucleus and localizes to the endoplasmic reticulum. Our data also suggest that the 46-LRLGSQLSL-54 amino acid sequence of 9b functions as a nuclear export signal (NES).

Keywords: SARS coronavirus; Protein 9b; Nuclear export

Severe acute respiratory syndrome (SARS) first appeared in Guangdong Province, Southern China in November 2002. This newly emerging infectious disease quickly spread to 29 countries on five continents along international air travel routes, causing large-scale outbreaks in Hong Kong, Singapore and Toronto in early 2003.

A novel coronavirus was identified as the etiological agent of SARS (Peiris et al., 2003). The SARS coronavirus (CoV) is an enveloped virus with a positive single-stranded RNA genome of 29,727 nucleotides (Marra et al., 2003; Rota et al., 2003). The genome contains open reading frames (ORFs) which can encode the replicase, four main structural proteins, as well as proteins that vary in length from 39 to 274 amino acids with no homology to other coronaviruses. These proteins are often referred to as group-specific, because despite the absence of homology, they can be found in each of the four groups of coronaviruses while their sequence and their genes location differ among the groups.

Expression of group-specific proteins does not appear to be essential for CoV replication in cultured cells (Haijema et al., 2003). However, there are indications that strains in which these genes have been deleted may be attenuated in in vivo models of CoV infection (Ortego et al., 2003) and that expression of SARS CoV group-specific proteins by an attenuated murine CoV may substantially increase its virulence (Pewe et al., 2005). These observations suggest a possible role for these group-specific proteins in CoV pathogenesis. The objective of the present study was the characterization of the novel SARS-CoV group-specific protein 9b.

The SARS-CoV ORF 9b (also known as ORF13) overlaps with the nucleocapsid gene and encodes a 98-amino-acid protein. Antibodies against the 9b protein were found in the sera of convalescent patients (Guo et al., 2004; Qiu et al., 2005; Zhong et al., 2005), and expression of this protein was demonstrated in diseased organs and SARS-CoV infected cells by immunohistochemistry (Chan et al., 2005). Recently, the crystal structure of 9b has been resolved (Meier et al., 2006). The protein has a novel fold, a dimeric tent-like beta structure with an amphipathic surface, and a central hydrophobic cavity that binds lipid molecules.

In our study, the ORF 9b gene was amplified from RNA of SARS-CoV-infected Vero E6 cells (strain Tor 2) using a one step RT PCR kit (Qiagen) (see Table 1 for primer sequences)

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Table 1
Primers used in this study

Primer	Sequence[a]	Sense	Application
ORF13FOR	CG**GGATCC**ATGGACCCCAATCAAACCAA	+	pcDNA-9b
ORF13STOP	G**GAATTC**TCATTTTGCCGTCACCACCACGA	−	pcDNA-9b
ORF13GEXF	G**GAATTC**TGATGGACCCCAATCAAACCAA	+	pGEX-9bN and p9b-EYFP
ORF13XHO	GGTCATCTGGACCACTATTG	−	pGEX-9bN
13REVBCL	T**TGATCA**TTTGCCGTCACCACCACGAACG	−	p9b-EYFP

[a] Restriction sites introduced into primers are in bold.

and cloned into a pcDNA3 (Invitrogen) vector into *Bam*HI and *Eco*RI sites to create pcDNA-9b. Next, a 196-bp fragment of the gene was amplified by PCR (primers are listed in the Table 1), digested with *Eco*RI and *Xho*I and subcloned in frame with the glutathione-*S*-transferase (GST) gene in *Eco*RI and *Xho*I sites of the vector pGEX5X2 (Amersham Biosciences) to create pGEX-9bN. Expression of the recombinant GST-9b fusion protein in bacteria was induced in the presence of 0.1 mM isopropyl beta-D-thiogalactopyranoside. Proteins from the induced bacterial cultures were separated by SDS-polyacrylamide gel electrophoresis and the GST-9b fusion protein was eluted from the gel by the "crush and soak" method (Sambrook et al., 1989). This gel-purified protein was used to immunize New Zealand White rabbits in order to generate anti-9b serum. The generated rabbit polyclonal antibodies were used for detection of expression of ORF 9b gene in cultured cells infected with SARS CoV. Near-confluent monolayer of cultured Vero E6 cells was inoculated with SARS-CoV at a multiplicity of infection (MOI) of 0.1 plaque forming units per cell. Forty-eight hours post infection the cells were harvested and lysed in 1% SDS.

The expression of the 9b protein in African green monkey kidney cells of Vero E6 lineage (derivative of the ATCC line CCL-81) was demonstrated by Western blot analysis. Total proteins from infected cells were separated by 15% polyacrylamide SDS gel electrophoresis in Tris–Tricine buffer, immobilized on a nitrocellulose membrane and probed with polyclonal rabbit serum raised as described above against recombinant 9b protein (Fig. 1). A unique protein band was detected in the extracts from infected Vero E6 cells (Fig. 1, Panel B, lane 1) while such a band was not present in protein extracts from non-infected cells (Panel B, lane 2), which indicated that ORF 9b was expressed during SARS-CoV infection and could be detected by specific rabbit polyclonal antibodies. Using these antibodies, the protein of a similar electrophoretic mobility was detected in HEK 293 cells infected with recombinant adenovirus expressing the 9b protein (Fig. 1, Panel A, lane 1). Adenovirus expressing the 9b protein was constructed using AdenoEasy Ad5 homologous recombination kit (CLONTECH). The observed electrophoretic mobility of the protein extracted from the infected cells was between 10 and 15 kDa which is close to the 11 kDa calculated molecular weight of the 9b protein. To our knowledge, this is the first Western blot analysis of the 9b protein.

Since biological processes are directed and regulated in different cellular compartments, it is commonly agreed that obligatory intracellular parasites such as viruses would depend on appropriate trafficking of host and viral molecules important for completion of the viral replication cycle and respective metabolic pathways. It would be interesting to study the subcellular localization of the 9b protein in the SARS-CoV-infected cells using anti-9b antibodies for immunofluorescence. Unfortunately, anti-9b rabbit serum we developed in this study appeared to show a background cross-reactivity with proteins in uninfected cultured cells (data not shown), which made it unsuitable for conclusive demonstration of the cellular localization of the 9b in the SARS-CoV infected cells. Therefore, we examined cellular localization of recombinant 9b tagged with enhanced

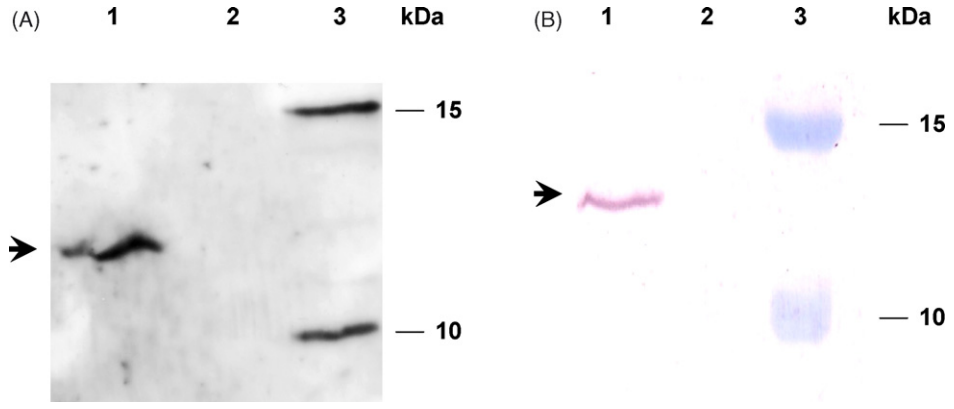

Fig. 1. Immunoblot of total cellular proteins of cells expressing 9b and probed with anti-9b rabbit serum. (A) Expression of 9b by 293 HEK cells infected with adenovirus encoding 9b; lane 1, total protein extract from 293 HEK cells infected with recombinant adenovirus encoding 9b; lane 2, total protein extract from uninfected 293 HEK cells; lane 3, Precision Plus Protein Standard (BioRad). (B) Expression of the 9b by Vero E6 cells infected with SARS coronavirus. Lane 1, total protein extract from Vero E6 cells infected with SARS-CoV; lane 2, total protein extract from uninfected Vero E6 cells; lane 3, PageRuler Prestained Protein Ladder Plus (Fermentas). Size of the protein standards indicated on the right. Arrow indicates the position of the unique 9b-specific protein band.