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NOTE

Nucleocapsid Amino Acids 211 to 254, in Particular, Tetrad Glutamines, are Essential for the Interaction Between the Nucleocapsid and Membrane Proteins of SARS-Associated Coronavirus

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GST pull-down assays were used to characterize the SARS-CoV membrane (M) and nucleocapsid (N) interaction, and it was found that the amino acids 211-254 of N protein were essential for this interaction. When tetrad glutamines (Q) were replaced with glutamic acids (E) at positions of 240-243 of the N protein, the interaction was disrupted.

Keywords: SARS-CoV, membrane protein, nucleocapsid protein, M-N interaction, GST pull-down assay

The outbreak of severe acute respiratory syndrome (SARS) in 2003 was associated with a newly discovered coronavirus, SARS-associated coronavirus (SARS-CoV). This is an enveloped positive-strand RNA virus that encodes four major structural proteins: spike (S), membrane (M), envelope (E), and nucleocapsid (N) (Rota *et al.*, 2003). The N protein of SARS-CoV contains 422 amino acids (aa) and plays an important role in virus particle assembly. It can bind viral RNA to form a helical core structure (Hsieh *et al.*, 2005) and form dimers through self-association by the SR-rich motif (aa183-197) (He *et al.*, 2004). The most abundant viral constituent of SARS-CoV is the M protein, a 25 kDa protein containing three transmembrane segments flanked by a short amino-terminal ectodomain and a large carboxy- terminal endodomain. Based on the information from other known coronaviruses, the M protein is assumed to be a key player in the assembly of virions (de Haan *et al.*, 1998).

Assembly of virus particles is an essential step for a productive viral replication cycle and assembly of enveloped viruses requires complex interactions between the lipid envelope, envelope proteins, and internal viral components. The M-N interaction has been described for the mouse hepatitis virus (Narayanan and Makino, 2001; Kuo and Masters, 2002) and transmissible gastroenteritis virus (Escors *et al.*, 2001). Recently, the M-N interaction of SARS-CoV in the absence of viral RNA was validated *in vivo* (He *et al.*, 2004) and the binding site in M protein was localized to amino acids 194-205 (Fang *et al.*, 2005). In this study, using the GST pull-down assay we characterized the M-N interaction of SARS-CoV and found that amino acids 211-254 in the N protein, in particular tetrad glutamines (^{240}QQQQ^{243}), are essential for this interaction.

The cDNA of SARS-CoV M protein (aa 90-221) was amplified with pGEM®-T-M as a template and cloned into the prokaryotic expression vector pET-His

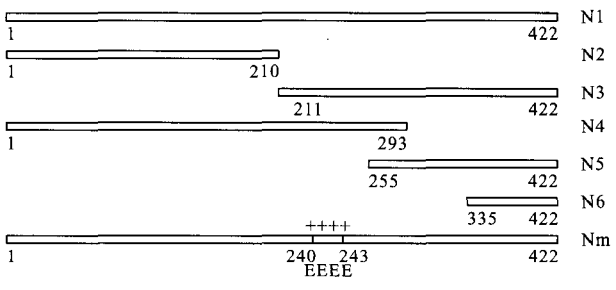

Fig. 1. Schematic representation of truncated sequences and site mutation of SARS-CoV N protein. Numbers below the bars indicate the first or last amino acid in the deletion mutations. Mutant names are indicated in the right column.

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Table 1. Primers for amplifying the M gene, and truncated and site-mutant fragments of N gene

Forward	
MF	ATGTGGGGATCCATGGCTTAGCTACTTC
N1 (N2, N4) F	TGAGCAGGATCCGTCATGGGAGCCATTAAATTGGATGAC
N3F	GTATTAGGATCCATGGCTAGCGGAGGT
N5F	CAAGCTGCAGAGATCTGTCATGGCATCTAAAAAGCCTCG
N6F	GCACGTGGATCCGTCATGGGAGCCATTAAATTGGATGAC
NmF	AAAGGCGAAGAAGAAGAAGGCCAAACTGTCACT
Reverse	
MR	GCCTTGCTAGCGAATTCCTGTACTAGCA AAG
N1 (N3, N5, N6) R	ACACTTGAATTCTGCCTGAGTTGAATC
N2R	AGCTAAGAATTCTCGAGCAGGAGAATTTCC
N4R	GATGATGAATTCGATTAGGTCTTGGTCC

Note: The restriction sites (underlined) were as follows: *Bam*HI in the MF, N1 (N2, N4) F, N3F and N6F, *Bg*lII in the N5F and *Eco*RI in the reverse primers.

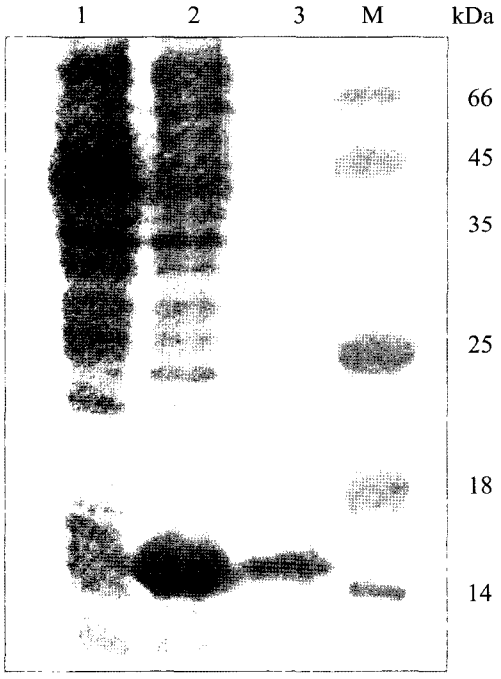

Fig. 2. SDS-PAGE analysis of purified His-tagged SARS-CoV M (90-211). Lanes 1 and 2: crude lysate from bacterial cultures containing the pET-His-M, before (lane 1) and after (lane 2) 3 h induction. Lane 3: Ni-NTA affinity column purified recombinant M protein. M: protein markers.

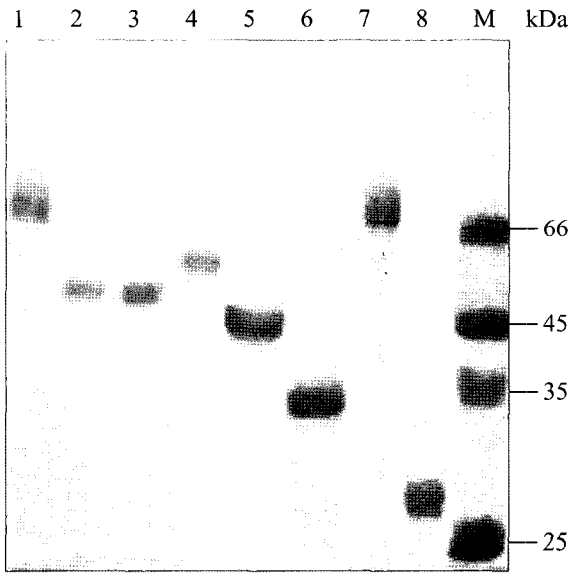

Fig. 3. SDS-PAGE analysis of various truncated and site mutants of GST-N fusion proteins. Different forms of GST-N proteins were expressed in *E. coli* strains, purified by Ni-NTA affinity column, fractionated by 10% SDS-PAGE. Lane 1: GST-N1, Lane 2: GST-N2, Lane 3: GST-N3, Lane 4: GST-N4, Lane 5: GST-N5, Lane 6: GST-N6, Lane 7: GST-Nm, Lane 8: GST, M: protein markers.

to produce pET-His-M. Using pGEM®-T-N (Timani *et al.*, 2004) as a template, the truncated N genes were amplified and cloned into the pET-GST vector to produce a series of prokaryotic expression plasmids (Fig. 1). This vector introduced a GST tag and a small hexahistidine tag at the N-terminus and the C-terminus of the target protein respectively. The substitution mutation of N gene (^{240}QQQQ^{243} → ^{240}EEEE^{243}) was obtained by two rounds of PCR using three pairs of primers (N1F and NmR, NmF and N1R, N1F and N1R). All primers are listed in Table 1. The recombinant plasmids were confirmed by direct sequencing.

The recombinant plasmids were transformed into the BL21 *E. coli* strain (DE3). After 3 h induction with IPTG at 37°C, high-level expression of recombinant proteins were observed, compared with uninduced cell lysates (data not shown). The His-tagged GST-fusion N proteins and M protein were purified on a Ni-NTA affinity column (Qiagen) and concentrated by dialysis against polyethyleneglycol 6000. As shown in Fig. 2 and Fig. 3, the different truncated versions of GST-N proteins and M (aa 90-211) were successfully purified.

Anti-M rabbit antiserum was generated by immunizing a rabbit with purified SARS-CoV M protein (aa 90-211) from *E. coli* cultures. Western blot analysis was performed to confirm that antiserum could strongly and specifically react with recombined M protein (data not shown).

Equal amounts (approximately 5 μg) of GST or GST fusion recombinant N proteins were immobilized on 20 μl of GST resin, and then incubated with 1-2 μg of recombinant M protein in GBT buffer for 2 h at 4°C. The bound proteins were eluted and detected by Western blot with anti-SARS-CoV M protein rabbit antiserum (1:500) and visualized by enhanced chemiluminescence (Amersham). As shown in Fig. 4, we found that the recombinant GST-N1, GST-N3, and GST-N4 could bind to M protein, while other mutant proteins GST-N2, GST-N5, GST-N6, and GST-N_m could not. As a negative control, M protein bound to neither GST nor GST resin. Our results showed that 44 amino acids of N protein from 211 to 254 were essential for the M-N interaction of SARS-CoV. When ^{240}QQQQ^{243} was replaced with ^{240}EEEE^{243} in the N protein, the M-N interaction was disrupted, suggesting that these four basic amino acids in the N protein were vital for the M-N interaction of SARS-CoV.

Using the mammalian two-hybrid method, He *et al.* (2004) proposed that amino acids 168-208 of N protein contributed to the M-N interaction of SARS-CoV. However, using the yeast two-hybrid method, Luo *et al.* (2006) demonstrated that the amino acids 351-422 of N protein were vital to this interaction. GST pull-down assay is widely used as a confirmed method to analyze protein-protein interaction, but it cannot characterize the interaction *in vivo*. Our findings from the GST pull-down assay are not compatible with the above mentioned reported results. The contradiction is probably due to the use of different methods and systems. Furthermore, the Proteam software program was used to analyze different properties of M and N proteins of SARS-CoV. It identified that the regions for the M-N protein interaction, amino acids 211-254 of N and 194-205 of M, display high polarity and hydrophilic properties as well as the formation of β-sheets. It is possible that the M-N interaction of SARS-CoV is through hydrophilic interaction. In conclusion, we found that the amino acids 211-254 of N protein contribute to the M-N interaction SARS-CoV and four glutamines are critical to this interaction. Our current work will hopefully help to clarify the molecular mechanism of the SARS-CoV M-N interaction and provide valuable information for future anti-viral strategies.

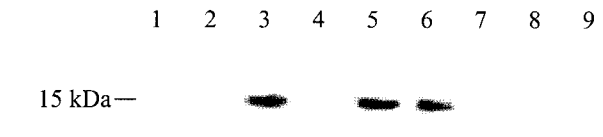

Fig. 4. GST pull-down assay. Lane 1: GST, M protein and GST resin; Lane 2: M protein and GST resin; Lane 3: GST-N1, M protein and GST resin; Lane 4: GST-N2, M protein and GST resin; Lane 5: GST-N3, M protein and GST resin; Lane 6: GST-N4, M protein and GST resin; 7: GST-N5, M protein and GST resin; 8: GST-N6, M protein and GST resin; 9:GST-Nm, M protein and GST resin.

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