Peptide Domain Involved in the Interaction between Membrane Protein and Nucleocapsid Protein of SARS-associated Coronavirus

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Severe acute respiratory syndrome (SARS) is an emerging infectious disease associated with a novel coronavirus (CoV) that was identified and molecularly characterized in 2003. Previous studies on various coronaviruses indicate that protein-protein interactions amongst various coronavirus proteins are critical for viral assembly and morphogenesis. It is necessary to elucidate the molecular mechanism of SARS-CoV replication and rationalize the anti-SARS therapeutic intervention. In this study, we employed an in vitro GST pull-down assay to investigate the interaction between the membrane (M) and the nucleocapsid (N) proteins. Our results show that the interaction between the M and N proteins does take place in vitro. Moreover, we provide an evidence that 12 amino acids domain (194-205) in the M protein is responsible for binding to N protein. Our work will help shed light on the molecular mechanism of the virus assembly and provide valuable information pertaining to rationalization of future anti-viral strategies.

Keywords: Membrane protein, Nucleocapsid protein, Protein interaction and GST resin pull-down assay, SARS-CoV

Introduction

Severe acute respiratory syndrome (SARS), also called atypical pneumonia, is a human severe respiratory infectious disease that emerged recently in Asia, North America and Europe. A novel coronavirus named “SARS-associated coronavirus” (SARS-CoV) was recently identified as the causative agent of SARS (Drosten et al., 2003; Ksiazek et al., 2003; Peiris et al., 2003). Severe morbidity and mortality rate is up to 10% in the SARS-CoV infected patients (Lee et al., 2003). The virions of the SARS-CoV are 80-140 nm in diameter, with projections surrounding the periphery (Ksiazek et al., 2003). The SARS-CoV is identified as positive-stranded RNA virus with approximately 29,727 nucleotides genomes packaged in helical nucleocapsids. The genome organization is similar to that other coronaviruses, where phylogenetic analyses and sequence comparisons showed that the SARS-CoV is not closely resembled to any of previously characterized coronaviruses. There are four open reading frames (ORF) downstream of replicase (rep) that are predicted to encode the four structural proteins spike (S), envelope (E), membrane (M), and nucleocapsid (N), which are common to all known coronaviruses. Besides, SARS-CoV also encodes several uncharacterized non-structural proteins (Marra et al., 2003; Rota et al., 2003).

The M protein of SARS-CoV consists of 221 amino acids in length and the amino acid sequence has low homology with M proteins of other members of coronaviruses. Based on the available information from other known coronaviruses, the 25 kDa M protein is an important and the most abundant structural protein and can induce antibody-dependent complement-mediated neutralization reaction (Woods et al., 1987). The M protein also plays a predominated role in the assembly of both naked and enveloped virus particles (Kuo and Masters, 2002). Research has demonstrated that the M protein contains highly conserved glycosylated sequences, and its glycosylation may be related to the interaction between virus and host cells (de Haan et al., 2002, 1998). In addition, the M protein is characterized as having three domains; these include a short N-terminal ectodomain, a triple-spanning transmembrane domain, and a C-terminal endodomain (Armstrong et al., 1984).

The N protein of SARS-CoV is 422 amino acids in length, sharing only 20-30% homology with the N proteins of other members of coronaviruses. Based on the available information, the N protein is an extensively phosphorylated, highly basic structural protein, which is known to bind viral RNA to form the helical core structure (Davies et al., 1981). Several
functions including viral packaging, viral core formation, and signal transduction have been attributed to the coronavirus nucleocapsid protein (Hiscox et al., 2001; He et al., 2003).

The protein-protein interaction between Coronavirusidae M and N proteins has been reported in several in vitro studies on mouse hepatitis virus (MHV) (Narayan and Makino, 2001; Kuo and Masters, 2002) and transmissible gastroenteritis virus (TGEV) (Escors et al., 2001). Although the interaction between M and N proteins of SARS-CoV has been observed in vitro, the amino acid sequence of the M protein involved in this viral protein-protein interaction has not been identified yet. It is undoubted that determination of M-N interaction and identification of the amino acid sequences in this process will enlighten the molecular mechanism of the virus assembly which leads to future anti-viral strategies.

In this study, GST pull-down assay has been employed to investigate the interaction between the N and M proteins. As is expected, the interaction between the N and M proteins has been successfully observed in vitro and 12 amino acids peptide (194-205) in the C-terminal endosomal domain of M protein was found to be responsible for this interaction.

Materials and methods

Plasmid construction Plasmid pET-GST was used as a prokaryotic expression vector for GST fusion proteins, and this vector introduces a GST-Tag and a small hexahistidine-Tag at the N-terminus and the C-terminus respectively, to facilitate subsequent purification. Truncated versions of M protein coding DNA fragments (M1, M2, M3, and M4) were obtained by polymerase chain reaction (PCR) using full length pGEM®-M as a template (gifted from Dr. Ying Zhu, Wuhan university, Wuhan, China), and the target DNA fragments were cloned into pET-GST double digested with BamHI and EcoRI using standard cloning method, generating a 5’ oligonucleotide with an artificial BamHI site and an initiation codon and a 3’ oligonucleotide with EcoRI site. The recombinant plasmids pET-GST-M (90-221), pET-GST-M2 (90-205), pET-GST-M3 (90-193) and pET-GST-M4 (90-180) were constructed using a pair set of primers, (M-F and M-R1), (M-F and M-R2), (M-F and M-R3) and (M-F and M-R4), respectively (Table-1) and transformed into DH5α strains. Positive colonies were analyzed, confirmed by PCR and sequenced. Construction of pET-His-N recombinant plasmid for prokaryotic expression of SARS-CoV N protein was described previously (Timani et al., 2004).

Expression and purification of recombinant protein The recombinant plasmids were transformed into E. coli strains BL21 (DE3). Protein expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C for 3 hours.

<table>
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<th>Table 1. Primers for M gene sequential deletion</th>
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<tr>
<td>M-F ATG TGG GGA GAT CAT GGG CTT TAG CTA CTIC</td>
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<tr>
<td>M-R-1 GGCT GCT GAG CCA ATC CTT GTC TAC AG</td>
</tr>
<tr>
<td>M-R-2 GCC ATG GAA ATC TTA AAT TTA GTT TCA ATAC GGG T</td>
</tr>
<tr>
<td>M-R-3 CTA ATG GAT TGC AAG AACC TTG AAT AAG GCC</td>
</tr>
<tr>
<td>M-R-4 GCA GTC GAA ATC TCT AAT TTA GTG AAT AAG GAA G</td>
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Note: The restriction sites are underline; it is BamHI in the forward primer and EcoRI in the reverse primers.

GST pull-down assay Equal amounts (approximately 1 μg) of GST or GST fusion recombinant M proteins were immobilized on 20 μl of GST resin, then incubated with 100 ng of SARS-CoV N protein in GBT buffer [10% glycerol, 50 mM NaCl, 5 mM Imidazole, 1 mM NaF, and 1 mM PMSF], then sonicated and centrifuged at 12,000 g at 4°C for 30 min. The clear supernatant was applied onto Ni-NTA affinity column (Qiagen, Chatsworth, USA) then washed with gradient washing buffers (20 mM Tris-HCl pH 7.9, 300 mM NaCl, 5, 20, 40, 60, 100 mM Imidazole). Purified recombinant protein was eluted with elution buffer (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 200 mM Imidazole) and concentrated by dialysis against polyethyleneglycol 6000. The expression and purification of N protein were described previously (Timani et al., 2004).

Western blotting The proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (PVDF) by standard methods. After blocking with PBBT (14 mM NaCl, 2.7 mM KCl, 10 mM NaHPO4, 1.8 mM L KH2PO4, 0.02% Tween-20) containing 5% non-fat milk for 1 h at 4°C, the membrane was incubated for 2 h at room temperature with anti-SARS-CoV N protein rabbit antibody (1:750, unpublished data). After washing at least 4 times with GBT buffer, the bound proteins were eluted by heating at 100°C for 10 min with 2 x SDS-PAGE disruption mix and were resolved by 12% SDS-PAGE, then detected by Western blotting.

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<th>Table 2. The binding domain in the M protein as being responsible for the interaction with nucleocapsid protein. The numbers indicated the position of the first amino acids in the binding site sequences</th>
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<tr>
<td><strong>The binding site sequence</strong></td>
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<tr>
<td>TGEV</td>
</tr>
<tr>
<td>MHV</td>
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<tr>
<td>SARS-CoV</td>
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Interaction between N-M Proteins of SARS-CoV

appropriate volume of ECL solution (Amersham, Arlington Heights, USA) and reacted at room temperature for about 1 min, then put onto a sensitization film for 1-3 min to develop and fix.

Result

Expression and purification of recombination proteins

Different truncated forms of GST-M proteins (GST-M1, GST-M2, GST-M3 and GST-M4) with a C-terminal hexahistidine-tag were expressed in BL21(DE3) and purified by Ni-NTA affinity column. After 3 h induction with IPTG at 37°C, high level expression of recombination proteins were observed when compared with uninduced cells lysates. The His-tagged GST-fusion proteins were eluted with elution buffer (20 mM Tris-Cl pH 7.9, 500 mM NaCl, 200 mM Imidazole) and concentrated by dialysis against polyethyleneglycol 6000 resulting in single bands of expected molecular mass. These data demonstrated that the different truncated versions of GST-M proteins were successfully expressed in E. coli and purified to homogeneity (Fig. 2).

The interaction of SARS-CoV M protein with N protein

We examined the possible interaction between SARS-CoV M and N proteins in vitro and identified the minimal sequence for this interaction. First, pull-down assay was carried out with GST-M1 (90-221) and N protein. The result demonstrated that N protein interacted with N-terminal truncated M protein in GST-fused form. As a negative control, N protein bound neither GST nor GST resin. This result was in consensus with previous report by He et al. (2004) and demonstrated that the binding region didn’t lie in the N-terminal of M protein. In order to identify the M-N binding domain of SARS-CoV, we employed GST pull-down with a set of C-terminal truncated forms of GST-M proteins with N protein. We found that the deletion mutant GST-M2 (90-205) bound N protein, while other mutant proteins GST-M3 (90-193), GST-M4 (90-180) did not bind. The analysis of GST pull-down results showed that the binding region occurred in the C-terminal of M protein and a region composed of 12 amino acids from 195 to 206 has been found to be the minimal sequence for the interaction between SARS-CoV M protein and N protein as shown in Fig. 3.

Discussion

In our research we employed GST pull-down assay to investigate the molecular interaction between the SARS-CoV M and N proteins and confirmed it did take place in vitro. Furthermore, series of truncated forms of GST-fused M
proteins were expressed in E. coli and purified by Ni-NTA affinity column. Using GST pull-down assay, we found that a region in the carboxy terminal of M protein appears to be vital for M-N interaction and the interaction site has been identified to the residues 194 to 205 of the SARS-CoV M protein. An early study by Sturman et al. (1980) was the first to show a direct and temperature-dependent association between the M and N proteins purified from MHV. Nearly two decades later, Lili et al. used targeted recombination with MHV for the systematic genetic analysis of coronavirus M-N proteins interaction and they found residues 201 to 224 of the MHV M protein were the contributory parts for the interaction of M-N proteins can interact with each other directly and temperature-dependent association between the M-N proteins, reinforcing a notion that M-N interaction forms the crucial structure for coronaviruses. However, the intermolecular interaction of SARS-CoV M and N protein remains unclear. Recently He et al. employed the mammalian two-hybrid system to investigate the possible interaction between SARS-CoV M and N proteins and they found that interaction takes place in vivo and identified that a stretch of amino acids 168 to 208 which includes SR rich domain in the N protein may be critical for such protein-protein interactions. The same region has also been found to be indispensable for multimerization of the N proteins (He et al., 2004). In other words, it can be assumed that this region may not serve as contact site for either N-N or M-N interaction. Overall, the SARS-CoV N protein is highly basic but there is concentration of acidic residues in its carboxy-terminal domain; this general pattern is common to all coronaviruses N proteins (Laude and Masters, 1995). Residues 195 to 206 of the SARS-CoV M protein concentrate with basic amino acids. We hypothesize that the residues 195 to 206 of the SARS-CoV M protein are electrostatically linked with one or more glutamate residues in carboxy-terminal domain of N protein. To test this, we are currently carrying out a comprehensive mutagenesis of the charged residues of the carboxy terminus of N protein. In conclusion, our data has shown that the SARS-CoV M and N proteins can interact with each other in vitro and the minimal sequence for the interaction has been mapped to the residues (194-206aa) in SARS-CoV M protein. This region of the M protein appears to be important for one of the critical steps of viral assembly and maturation process. Our work would help shed light on the molecular mechanism of the virus assembly and provide valuable information, which regards to rationalize the future anti-viral strategies.

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References


