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Characterization of protein–protein interactions between the nucleocapsid protein and membrane protein of the SARS coronavirus

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Abstract

The human coronavirus, associated with severe acute respiratory syndrome (SARS-CoV), was identified and molecularly characterized in 2003. Sequence analysis of the virus indicates that there is only 20% amino acid (aa) identity with known coronaviruses. Previous studies indicate that protein–protein interactions amongst various coronavirus proteins are critical for viral assembly. Yet, little sequence homology between the newly identified SARS-CoV and those previously studied coronaviruses suggests that determination of protein–protein interaction and identification of amino acid sequences, responsible for such interaction in SARS-CoV, are necessary for the elucidation of the molecular mechanism of SARS-CoV replication and rationalization of anti-SARS therapeutic intervention. In this study, we employed mammalian two-hybrid system to investigate possible interactions between SARS-CoV nucleocapsid (N) and the membrane (M) proteins. We found that interaction of the N and M proteins takes place *in vivo* and identified that a stretch of amino acids (168–208) in the N protein may be critical for such protein–protein interactions. Importantly, the same region has been found to be required for multimerization of the N protein (He et al., 2004) suggesting this region may be crucial in maintaining correct conformation of the N protein for self-interaction and interaction with the M protein.

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Coronaviruses are a family of enveloped viruses with a single-stranded positive-sense RNA genome approximately 30 kb in length (Holmes, 2001). The human coronavirus, associated with severe acute respiratory syndrome (SARS-CoV), was identified in April 2003 during a worldwide outbreak of atypical pneumonia, which started in Guangdong Province of the People's Republic of China (Drosten et al., 2003; Ksiazek et al., 2003). SARS-CoV is markedly different from other members of the Coronaviridae family and is the only coronavirus currently known to cause severe morbidity and mortality in humans (Lee et al.,

2003). The complete SARS-CoV genome has since been sequenced and all viral proteins including the membrane glycoprotein and nucleocapsid phosphoprotein identified (Marra et al., 2003).

In the coronaviruses, the nucleocapsid (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 (Hiscox et al., 2001; He et al., 2003). The M protein was reported to be the most abundant viral protein expressed during infection and a key protein in the assembly of both naked and enveloped virus particles (Kuo and Masters, 2002). However,

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the SARS-CoV nucleocapsid protein and membrane protein share little amino acid (aa) homology with those of others in the family and the many potential roles of this suspected multifunctional proteins are yet to be determined. Similarly, the 25-kDa membrane (M) glycoprotein also has low homology with other coronavirus M proteins.

The protein–protein interaction between Coronaviridae N and M proteins has been reported in several *in vitro* studies on mouse hepatitis virus (Narayanan and Makino, 2001; Kuo and Masters, 2002) and transmissible gastroenteritis (Escors et al., 2001). Although interaction of M–N has been observed, the amino acid sequence of the N protein involved in these viral protein–protein interaction has not been identified. Determination of M–N interaction and identification of the amino acid sequences involved in this process would help shed light on the molecular mechanism of the virus assembly and provide valuable information pertaining to rationalization of future anti-viral strategies.

To better understand the process of SARS-CoV viral assembly, we performed an *in vivo* mammalian two-hybrid assay (Clontech, Palo Alto, CA) to investigate the interaction between the N and M proteins. Mammalian two-hybrid systems are one of the most commonly used assays for the detection of *in vivo* protein–protein interactions that we have successfully employed in our studies on multimerization of SARS-CoV N protein (He et al., 2004). Because this assay is performed *in vivo* as opposed to other *in vitro* biochemical methods, viral proteins are prone to be in their native conformations and therefore interactions are more likely to be biologically accurate (Luo et al., 1997). The N and M proteins were obtained from PCR amplification of cDNA,

reverse transcribed from the SARS-CoV RNA isolated from a clinical specimen. The N protein gene was cloned in frame into the pM vector (Clontech, Palo Alto, CA), which carries a Gal4 DNA binding domain (BD) (pM-N), while the M protein was cloned in pVP16 vector (pVP16-M), which carries a herpesvirus VP16 DNA activation domain (AD). In the case of an interaction between the two proteins, the BD and AD domains form a transcriptional activation complex and activate the secreted alkaline phosphatase (SEAP) reporter gene provided on a third transfected vector (pG5SEAP; Clontech, Palo Alto, CA). The intensity of protein–protein interaction, as reflected by reporter activities, was measured by a chemiluminescent assay.

Mammalian two-hybrid analysis of N and M interaction was carried out by co-transfection of 0.4 μg of pM-N and 0.4 μg of pVP16-M vectors along with 0.2 μg pG5SEAP reporter vector into 3×10^5 Huh7 cells. The transfection reagent Effectene (Qiagen, Valencia, CA) was used to deliver the plasmids into the cells according to the manufacturer's instructions. The Huh7 cell line is derived from human hepatocellular carcinoma cells that are commonly used for gene expression studies (Degawa-Yamauchi et al., 2002; Zhang et al., 2002). Control samples are listed in Fig. 2. Transformed cells were monitored for protein–protein interaction by assaying culture medium for SEAP activity (Great Escape SEAP; Clontech). As indicated in Fig. 1, the pM-N + pVP16-M cotransfected sample showed an approximately over 5 times higher chemiluminescence activity compared to controls (pM-N + pVP16, and pM + pVP16-M co-transfections). The results obtained from the mammalian two hybrids system indicate that interaction between M and

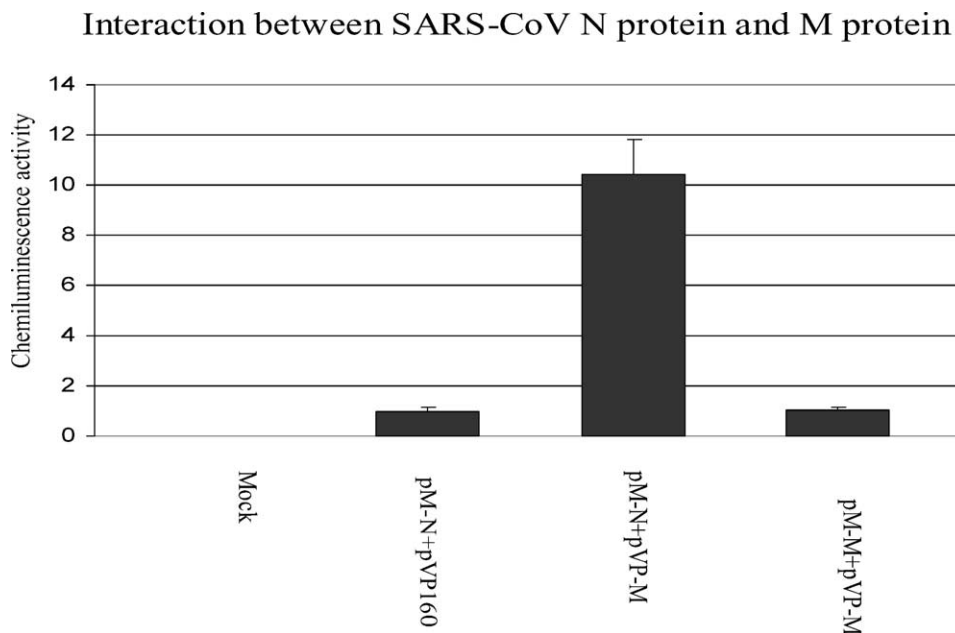


Fig. 1. Mammalian two-hybrid assay on SARS-CoV nucleocapsid protein and membrane protein. Constructs labeled in the figure were co-transfected into Huh7 cells. Forty-eight hour post-transfection, cells were harvested and subjected to the SEAP assay. Chemiluminescence values were used to measure the interaction activities.

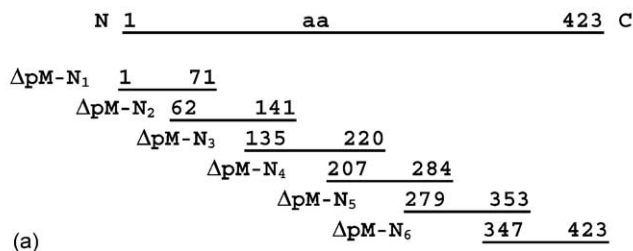
N does take place, consistent to findings in other coronaviral systems (Narayanan and Makino, 2001; Escors et al., 2001; Kuo and Masters, 2002).

To determine the specific region of the nucleocapsid protein where N–M protein interaction occurs, we constructed a series of N protein deletions in the 1269 bp sequence (Fig. 2a). PCR products of the truncated nucleocapsid sequences were ligated in frame into the pM vector. The truncated constructs were Δ pM-N1, Δ pM-N2, Δ pM-N3, Δ pM-N4, Δ pM-N5, and Δ pM-N6. Each truncated construct was co-transfected with pVP-M and the pG5SEAP reporter plasmid separately into Huh7 cells. Forty-eight hour after transfection, SEAP assay was performed to quantify the level of chemiluminescence activity. As shown in Fig. 2b, Δ pM-N3 construct (aa, 135–220) showed the highest activity level among all six constructs, while Δ pM-N1, Δ pM-N2, and Δ pM-N4 resulted in moderate activity levels. In addition, Δ pM-N5 and Δ pM-N6, which correspond to the carboxy region of the N protein, shared the lowest SEAP activity. The result suggested that the region containing aa 135–220 may be crucial for the N and M protein interaction.

To further map the regions of interaction within the SARS-CoV N protein, a series of eight sequentially deleted mutants were generated (Fig. 3a). These mutants were cloned into mammalian two-hybrid vector pM. The eight sequential truncation mutants of the SARS-CoV N protein, designated from S-DpM-N1 to S-DpM-N8, were co-transfected with pG5SEAP reporter vector and pVP-N that contains full-length N gene. We also used a different cell line in this assay, Vero cells that are susceptible to SARS-CoV infection. Forty-eight hours post-transfection, chemiluminescence-based assay was conducted to measure the protein–protein interaction activity through the SEAP reporter gene. As shown in Fig. 3b, deletion of aa 168–208 resulted in a loss of SEAP activity, consistent to results shown in Fig. 2.

In conclusion, we have shown interaction between the SARS-CoV M and N does take place in vivo. Furthermore, a region in the middle of the N genes appears to be important for M–N interaction, an observation that is different from what has been reported in MHV, a coronavirus that may have the closest genetic resemblance to SARS-CoV among all coronaviruses. In MHV, it is the carboxy terminus of the

Arrangement of truncated nucleocapsid (N) products.



Interaction between Truncated N Protein Constructs and M Protein

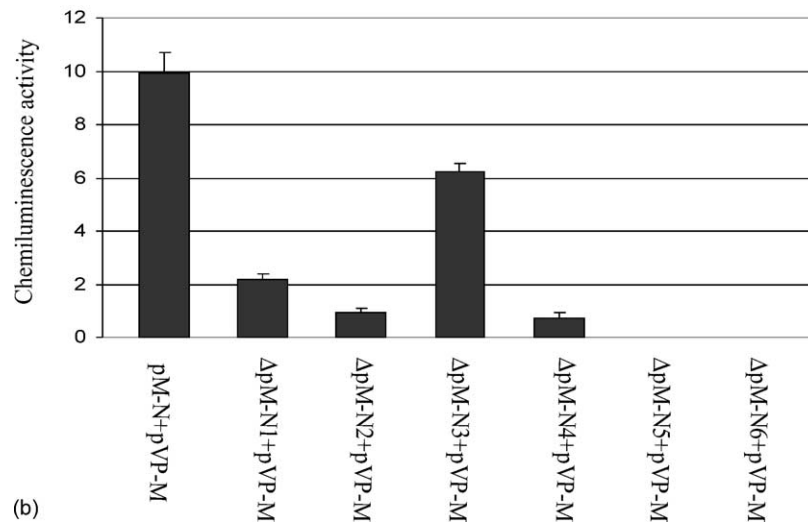


Fig. 2. Mutation analysis of the SARS-CoV nucleocapsid (N) protein. Schematic description of the DpM-N related mutants (a), and SEAP chemiluminescence values of the mammalian two hybrid assay on protein–protein interaction between SARS-CoV membrane (M) protein and the N protein truncation mutants (b).

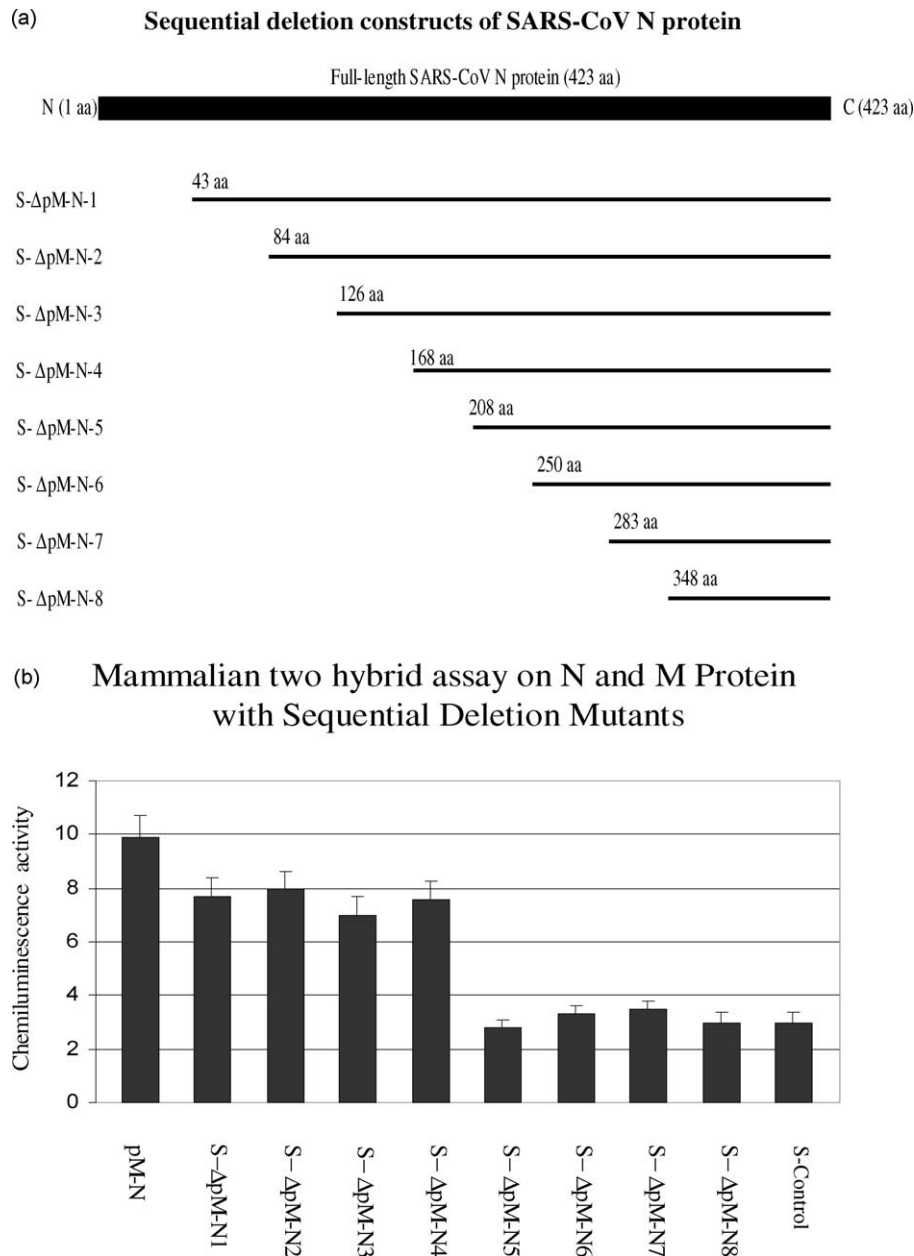


Fig. 3. Sequential deletion analysis of the SARS-CoV nucleocapsid (N) protein. Schematic description of the S-ΔpM-N related mutants (a), and SEAP chemiluminescence values of the mammalian two-hybrid assay on protein–protein interaction between SARS-CoV membrane (M) protein and the N protein sequential deletion mutants (b). The mutants were constructed using pM vector as labeled in (b). Each mutant was then co-transfected with the full-length N gene in pVP16 vector (pVP-N). The pM vector co-transfected with pVP-N was used as the negative control.

N protein not the central region, which has been implicated previously in M protein binding by indirect mutational analysis (Kuo and Masters, 2002).

The importance of M–N interaction in viral interaction appears to be an essential process for coronaviral assembly as coronaviruses differ from other enveloped viruses such as paramyxoviruses and rhabdoviruses in that they lack the structural matrix protein that commonly links the envelope to the nucleocapsid. Thereby, it can be envisaged that replacing the matrix protein in the role of core stabilization through nucleocapsid binding appears to be one of the func-

tions of the coronavirus M protein. A recent study on MHV suggests a multiple binding role for the nucleocapsid protein in virus assembly and morphogenesis (Narayanan et al., 2003). The M protein has also been found to have a viral RNA binding site that interacts with the RNA packaging signal (Narayanan et al., 2003). Through the complex of viral RNA, nucleocapsid protein, and M protein, it is evident that the assembly of a mature coronavirus is a multi-step process involving protein–protein and protein–RNA interactions.

Interaction between M and N in coronaviruses has been reported in the literature, however, most of these studies were

focused on identifying the binding domains in the M, but not the N gene (Narayanan et al., 2003). Although SARS-CoV nucleocapsid protein shares low homology with others in the coronavirus family, our current study revealed that the M–N protein–protein interaction exists in SARS-CoV as it does in other members of the Coronaviridae family, reinforcing a notion that M–N interaction forms the crucial structure for coronaviruses. Interestingly enough, aa 168–208 have also been found to be indispensable for multimerization of N proteins (He et al., 2004), suggesting that this region is pivotal for maintaining the native structure of the N protein so that physical interaction can take place for both N–N self-interaction and N–M interaction. In other words, it could be envisaged that this region may not serve as contact site for either N–N or N–M interaction, future studies using techniques such as cross-linking and peptide mapping could be helpful in identifying the exact role of this region. It should be noted, however, that the region (168–208) may be critical in maintaining M and N interaction, other regions in the N protein may provide accessory roles in facilitating this process since all truncated and deletion mutants demonstrated relatively lower chemiluminescence than that of the full-length N protein (Fig. 3b). More studies are ongoing in our laboratories to further investigate the functional role of this region using other experimental approaches such as reverse genetics. Taken together, our data has shown for the first time that the SARS-CoV N and M proteins can interact with each other in cells susceptible to SARS-CoV infection and amino acids 168–208 of the N-protein appear to be necessary for one of the critical steps of viral assembly and maturation process.

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