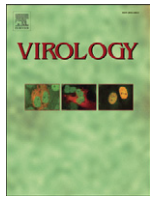




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Dissection and identification of regions required to form pseudoparticles by the interaction between the nucleocapsid (N) and membrane (M) proteins of SARS coronavirus

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ABSTRACT

When expressed in mammalian cells, the nucleocapsid (N) and membrane (M) proteins of the severe acute respiratory syndrome coronavirus (SARS-CoV) are sufficient to form pseudoparticles. To identify region(s) of the N molecule required for pseudoparticle formation, we performed biochemical analysis of the interaction of N mutants and M in HEK293 cells. Using a peptide library derived from N, we found that amino acids 101–115 constituted a novel binding site for M. We examined the ability of N mutants to interact with M and form pseudoparticles, and our observations indicated that M bound to NΔ(101–115), N1–150, N151–300, and N301–422, but not to N1–150Δ(101–115). However, pseudoparticles were formed when NΔ(101–115) or N301–422, but not N1–150 or N151–300, were expressed with M in HEK293 cells. These results indicated that the minimum portion of N required for the interaction with M and pseudoparticle formation consists of amino acids 301–422.

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Introduction

Severe acute respiratory syndrome (SARS), caused by a newly identified coronavirus (SARS-CoV), has been observed in about 30 countries, affecting more than 8,000 people and resulting in death in about 10% of cases (Guan et al., 2003). SARS-CoV was identified as a novel type of coronavirus, differing from known coronaviruses (Guan et al., 2003; Marra et al., 2003; Rota et al., 2003). The genome and functional receptor of SARS-CoV have been identified (Li et al., 2003), but no vaccine or drug treatment has yet been approved. Although bats carry viruses similar to SARS-CoV (Wang et al., 2006), its natural host has not yet been identified.

Coronaviruses have a positive single-stranded RNA genome of approximately 30 kb that encodes structural proteins, such as the spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins. The S protein encoded by SARS-CoV, a large type-I transmembrane glycoprotein, is necessary for binding and fusion to host cells. S protein consists of the N-terminal S1 domain, which binds human

angiotensin-converting enzyme 2 (ACE2), a functional receptor for SARS-CoV (Li et al., 2003), and a C-terminal S2 domain that mediates the fusion of viral and host cell membranes (Tripet et al., 2004; Xu et al., 2004).

The E protein, a type-II transmembrane glycoprotein, is the smallest structural protein in SARS-CoV (Wu et al., 2003). Although required for the formation of pseudoparticles in insect cells (Mortola and Roy, 2004), E is not necessary to form pseudoparticles in mammalian cells (Huang et al., 2004). E acts as a cation-selective ion channel (Wilson et al., 2004). rSARS-CoV-ΔE has been shown to replicate to titers 100- to 1000-fold lower than recombinant wild-type virus *in vivo*, although both viruses show the same morphology and stability at severe pH and temperature (DeDiego et al., 2007).

The M protein consists of a short N-terminal ectodomain harboring an N-glycosylation site, three transmembrane domains and a long cytoplasmic tail (Hu et al., 2003), and a C-terminal domain (residues 194–205 or 197–221) that binds to N (Fang et al., 2005; Luo et al., 2006b). M is essential for the formation of pseudoparticles (Huang et al., 2004). In the case of murine coronavirus, only M and E are required for formation of pseudoparticles (Bos et al., 1996; Vennema et al., 1996), in marked contrast with the case of SARS-CoV requiring M and N only to form pseudoparticles (Huang et al., 2004).

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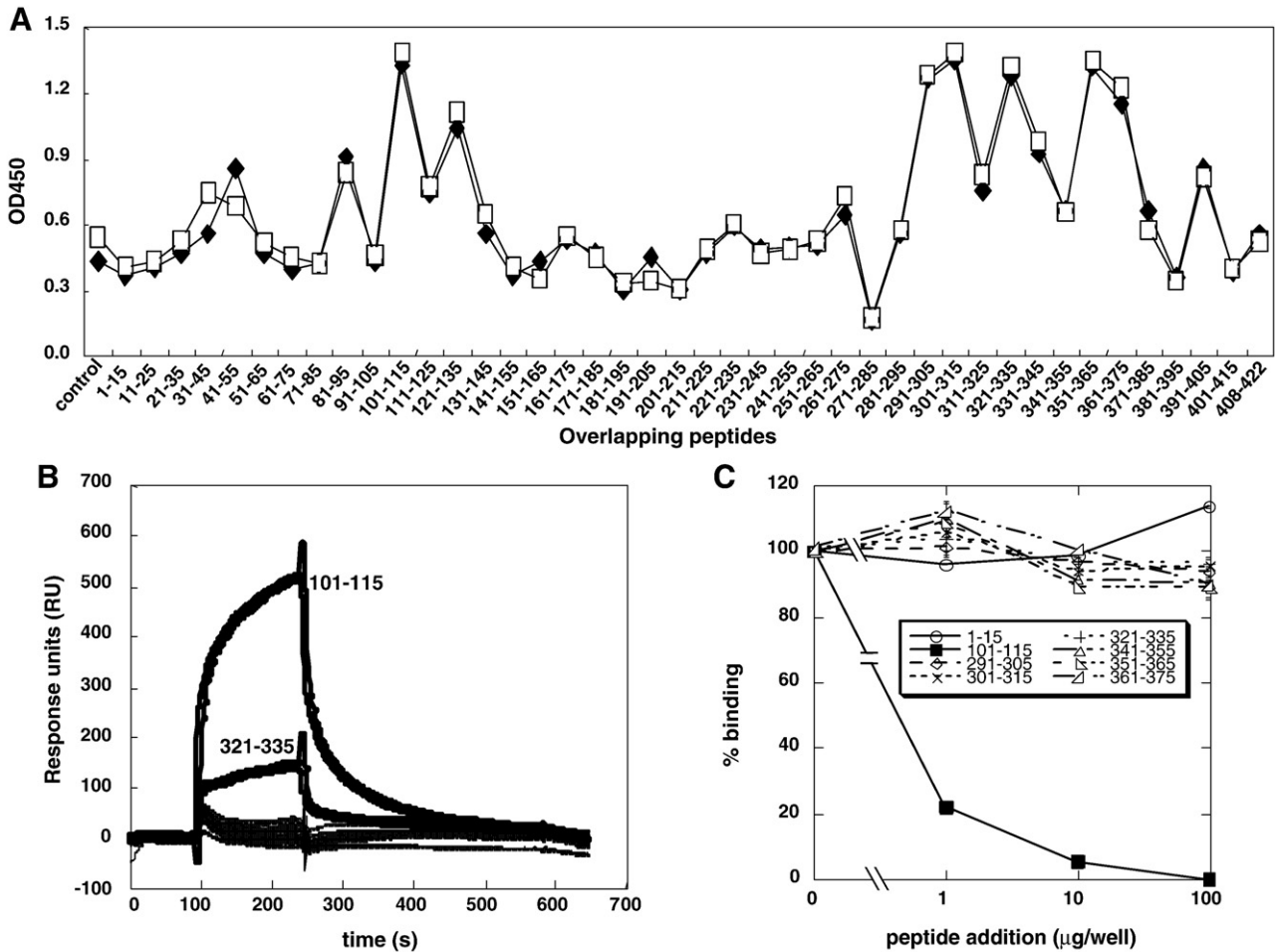


Fig. 1. Interaction of N-derived peptides with M protein. (A) Each peptide (10 $\mu\text{g}/\text{mL}$) was immobilized on ELISA plates. After blocking, the plates were incubated with M63 (final 1 $\mu\text{g}/\text{mL}$), and binding of M63 to N protein was detected with rabbit anti-M63 plus HRP-goat anti-rabbit IgG. The two data points for each peptide are the results of two independent experiments. (B) Each peptide (10 $\mu\text{g}/\text{mL}$) was injected into a BIAcore flow cell containing immobilized M116. The injection was started at 100 s, stopped at 240 s, and dissociated for 300 s. (C) Competitive inhibition of binding between N and M63 proteins by N-derived peptides. M63 was preincubated with each N peptide, and each mixture was added to a plate coated with N protein. Binding of M63 to N protein was detected as in (A). Binding in the absence of peptide was set at 100%. Data are presented as means \pm SD.

The N protein of coronaviruses is a major structural component that plays a role in virion assembly through its interactions with the viral genome and M protein (Hurst et al., 2005; Masters, 1992; Nelson and Stohlman, 1993; Narayanan et al., 2000; Narayanan et al., 2003). The N protein is also considered essential for viral RNA synthesis, although the cellular events affected by N are not yet known (Almazan et al., 2004). Crystal structures of C-terminal domain (amino acids 248–365) (Chen et al., 2007), N-terminal domain (amino acids 1–174)

(Saikatendu et al., 2007) and dimerization domain (amino acids 270–370) (Yu et al., 2006) have been reported. N contains a disordered region in the middle of the molecule (Chang et al., 2006; Zuniga et al., 2007), which does not form a particular structure otherwise it binds adequate binding partners.

Virion formation is the fundamental process in viral replication, and results from the interactions between viral proteins under physiological conditions. The expression of M, E, and S in Sf9 insect

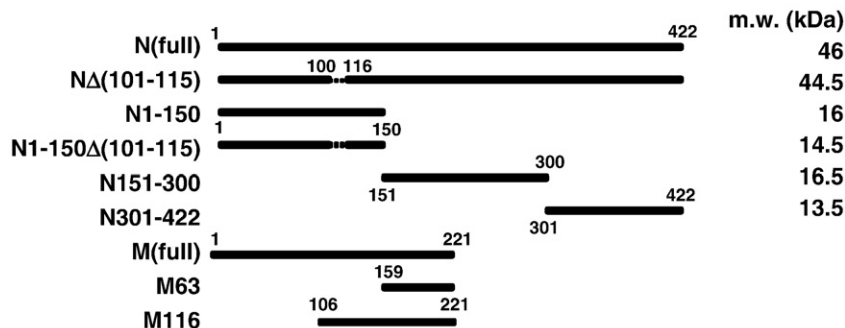


Fig. 2. Schematic diagram of the recombinant N and M proteins used in this study. N mutants are numbered according to the amino acid positions in the full-length N. M116 and M63 consist of the C-terminal 116 and 63 amino acids of M, respectively.

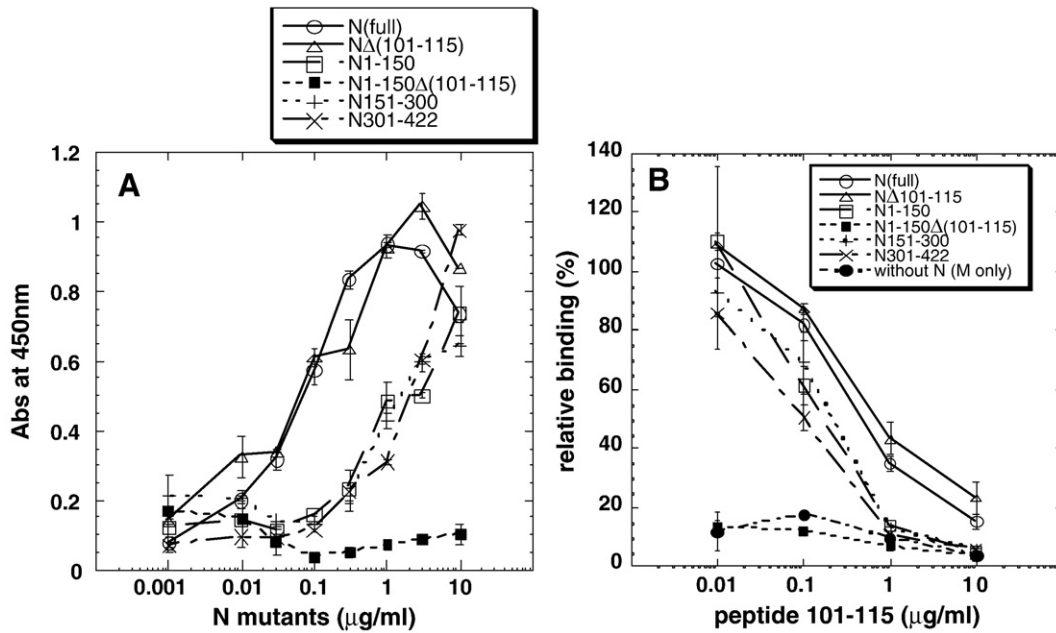


Fig. 3. Analyses of interactions between N mutants and M and the inhibition by peptide 101–115. (A) ELISA of the interaction between N mutants and M116. Each N peptide (0.01–10 μg/mL) was immobilized onto a plate. After blocking, the plate was incubated with M116 (1 μg/mL), and bound M116 was detected with rabbit anti-M63 and HRP-goat anti-rabbit IgG as described in Materials and methods. (B) Inhibition of the interaction between N mutants and M116 was observed with peptide 101–115. The experimental procedure was identical to that described in the legend to Fig. 1C. Data are presented as means ± SD.

cells was sufficient for formation of pseudoparticles (Mortola and Roy, 2004), whereas, only M and N were required in mammalian cells (Huang et al., 2004). Replication of SARS-CoV may be controlled by modifying the interaction between the M and N proteins. Using a

mammalian two-hybrid system, the region of N bound to M (amino acids 168–208) was found to contain a serine/arginine-rich motif (amino acids 184–196) (He et al., 2004b). The same region was necessary for self-multimerization of N (He et al., 2004a). Analysis

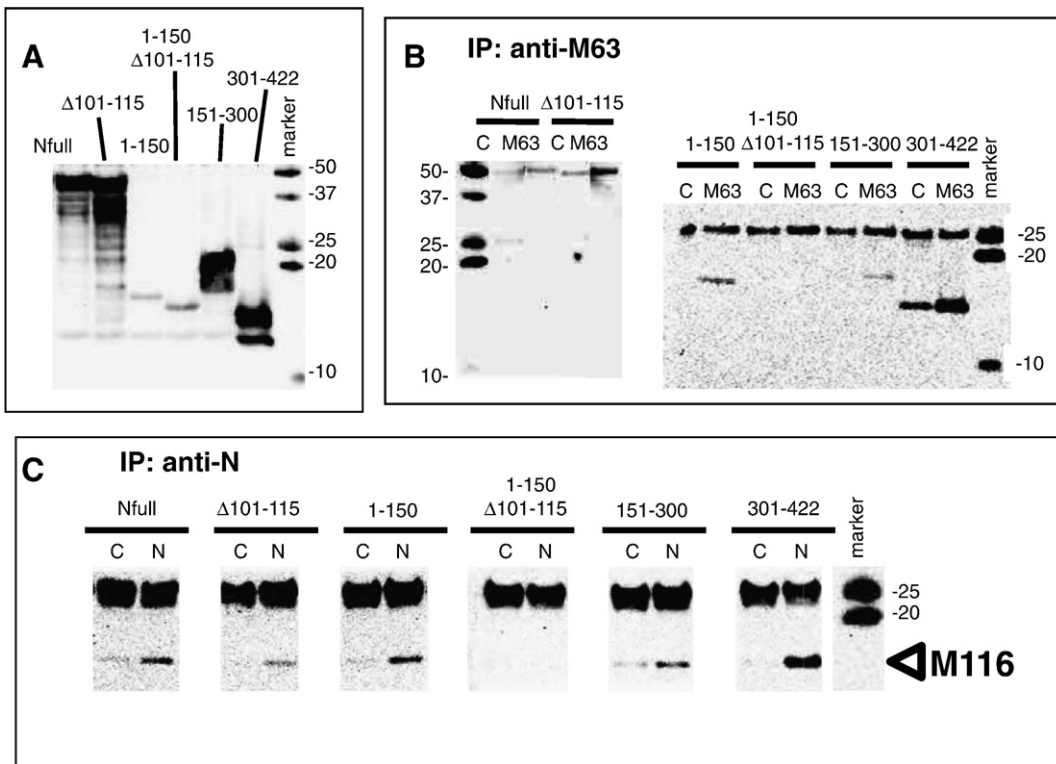


Fig. 4. Immunoprecipitation analyses of the interaction between N mutants and M116 in mammalian cells. (A) Western blotting of the lysates of HEK293 cells expressing N mutants 3 days post-transfection was performed as described in Materials and methods. (B,C) Immunoprecipitation analyses using control antiserum (C), anti-M63 (M63), or anti-N (N) antibodies of lysates of HEK293 cells coexpressing N mutants and M116. As both of the antibodies used for immunoprecipitation and detection of the target proteins were raised in rabbits, the immunoglobulin light chain (ca. 25 kDa) was observed due to cross-reaction with the 2nd antibody.

using a yeast two-hybrid system and surface plasmon resonance techniques showed that amino acids 351–422 of N interacted with amino acids 197–221 of M and that the C-terminal region of N (amino acids 343–402) was necessary for its oligomerization (Luo et al., 2006b).

In the present study, we identified a novel binding region for M in N and confirmed the interaction between this region of N and M in mammalian cells. We also found that interaction of the C-terminal portion of N with M was sufficient to form pseudoparticles in mammalian cells.

Results

Identification of regions in N that bind M

To identify the site(s) in the N protein that bind to M protein, we employed an overlapping peptide library covering the entire N protein. The peptides were immobilized onto ELISA plates, and binding of M63 to these peptides was analyzed using anti-M63 antibody. The M63 reacted with an N-terminal peptide corresponding to amino acids 101–115 and to 5 C-terminal peptides, corresponding to amino acids 291–305, 301–315, 321–335, 351–365, and 361–375 (Fig. 1A). In SRP analysis, two peptides corresponding to amino acids 101–115 (KMKELSPRWYFYLG) and 321–335 (IGMEVTPSGTWLTYH) showed binding to M116 (Fig. 1B). For peptide 101–115, the binding to M116 was dose-dependent, with a K_d of 0.73 μ M (data not shown).

To analyze whether the N-derived peptides compete with N itself in the interaction between M and N proteins, these peptides were incubated with M63 and subsequently added to ELISA plates containing immobilized N protein. Using anti-M63 antibody, we found that only peptide 101–115 significantly inhibited the interaction between M63 and N protein, whereas peptides 291–305, 301–315, 321–335,

341–355, 351–365, and 361–375 did not inhibit this interaction (Fig. 1C), nor did mixtures of peptides 291–305, 301–315, 341–355, 351–365, and 361–375 and peptides 91–105 and 111–125 (data not shown).

Binding of N mutants to M

The middle region of N contains a serine/arginine-rich motif, which has been reported to interact with M and to mediate N protein self-oligomerization (He et al., 2004b), whereas the C-terminal region of N contains domains involved in N protein self-oligomerization, RNA binding, and M protein binding (He et al., 2004a). The region comprised of amino acids 101–115 of N, however, has not been thought to interact with M. To dissect the interaction of N with M, several N protein mutants were constructed: N1–150, N151–300, N301–422, N1–150 Δ (101–115), and N(full) Δ (101–115) (Fig. 2). Using ELISA-based analyses (Fig. 3), M116 bound to N(full), N(full) Δ (101–115), and to a lesser extent to N1–150, N151–300, and N301–422, in a dose-dependent manner. In contrast, N1–150 Δ (101–115) did not show significant binding to M116. These results indicated that all three portions of N (1–150, 151–300, and 301–422) can bind M, and that the binding of N1–150 is mediated by residues 101–115.

Binding of N mutants to M in mammalian cells

We also analyzed the interaction of N mutants and M coexpressed in HEK293 cells by immunoprecipitation using antibodies against anti-M63 or anti-N proteins from lysate prepared from HEK293 cells expressed the N mutants and full-length M protein (Fig. 4). As the N mutants bound to the Protein G Sepharose without addition of any antibodies, we preincubated the cell lysate with Protein G Sepharose to reduce background binding. Immunoprecipitation experiments using anti-M63 antibody showed that N(full), N Δ (101–115), N1–150,

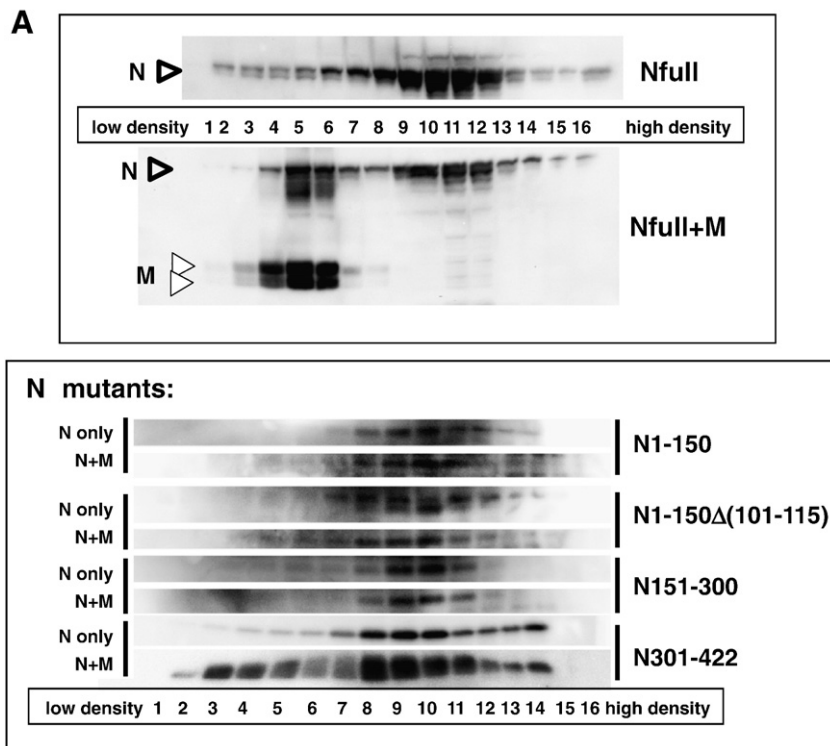


Fig. 5. Assembly of pseudoparticles in transfected HEK293 cells. (A) Gradient sedimentation analyses of pseudoparticles from cell lysates transfected with M and N mutants or transfected N mutants alone were performed 3 days after transfection, as described in Materials and methods. Fractions are numbered from the top of each tube (lower density). Arrows indicate the positions of M and N mutants. In the top panel, N and M were detected simultaneously by anti-M63 antiserum and anti-N serum, while only anti-N serum was used in the bottom panel. (B) Analysis of viral assembly of mutants of N and M proteins by TEM. TEM analysis was performed on HEK293 cells transfected with N mutants and M as described in Materials and methods. The areas in the white squares indicate the areas magnified and shown in the respective lower panels.

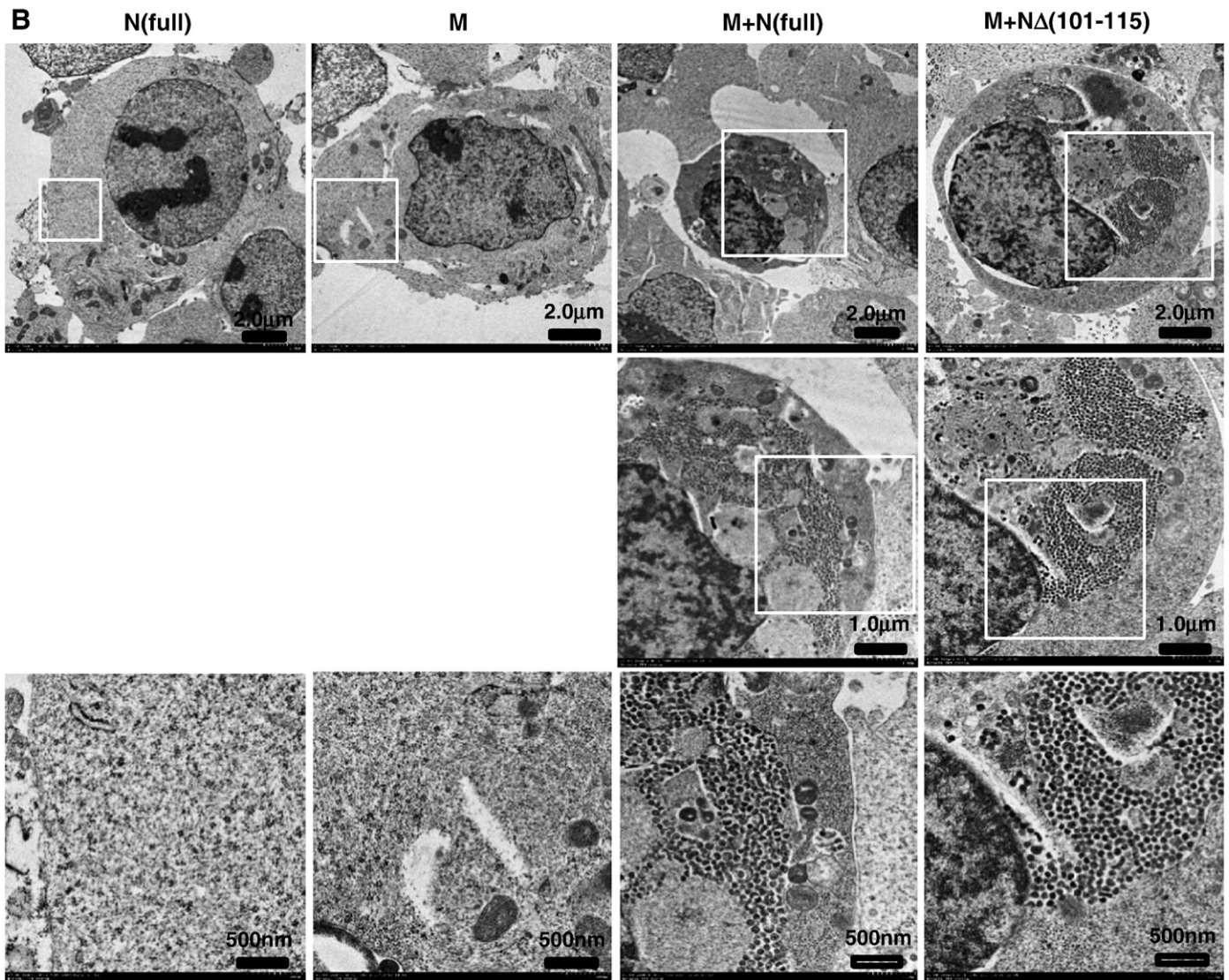


Fig. 5 (continued).

N151–300, and N301–422 were coprecipitated with M (Fig. 4B). Although N(full), NΔ(101–115), and N301–422 were immunoprecipitated with control serum even after pretreatment of the lysate with Protein G Sepharose, the amounts precipitated were increased using anti-M63. In contrast, only N1–150Δ(101–115) was not immunoprecipitated by anti-M63 antibody. Using anti-N antibody (Fig. 4C), M116 was immunoprecipitated along with N(full), NΔ(101–115), N1–150, N151–300, and N301–422, but not with N1–150Δ(101–115). These results suggested that all portions of N except N1–150Δ(101–115) can interact with M116 in mammalian cells.

Pseudoparticle formation by N mutants and M in 293 cells

We investigated whether the interaction of several N mutants and M protein would lead to formation of pseudoparticles (Fig. 5). In buoyant density gradient analysis, N was present around fractions 6–13 when N alone was expressed in HEK293 cells (Fig. 5A). In contrast, when N was coexpressed with M, N was present in fractions 4–6, the main fractions containing M, as well as in fractions 7–14, indicating the formation of pseudoparticles (Huang et al., 2004). When N mutants were expressed alone in 293 cells, they were detected in fractions 7–14. When coexpressed with M, N(full), NΔ(101–115), and

N301–422 were located in fractions 3–5, along with M. In contrast, N1–150, N1–150Δ(101–115), and N151–300 were not located in fractions containing M.

We used transmission electron microscopy (TEM) to determine whether pseudoparticles were formed by the coexpression of N mutants and M proteins in HEK293 cells (Fig. 5B). When N(full) and M were coexpressed in HEK293 cells, pseudoparticles of about 80 nm in size, the same size as SARS-CoV virions, appeared between the endoplasmic reticulum (ER) and Golgi bodies (Huang et al., 2004). When the N mutants N1–150, N1–150Δ(101–115), and N151–300 were coexpressed with M, pseudoparticles were not observed, whereas pseudoparticles were observed when NΔ(101–115) and N301–422 were coexpressed with M. The pseudoparticles observed were 60–80 nm in diameter (Fig. 5B). These results indicated that the C-terminal portion of N, consisting of amino acids 301–422, is necessary and sufficient for the formation of pseudoparticles with M.

Inhibition of the binding of N mutants to M by the 101–115 peptides

To determine the region in peptide 101–115 critical for the inhibition, we assessed the abilities of 10-mer peptides spanning amino acids 101–115 of N (Fig. 6A) to inhibit the interaction between N

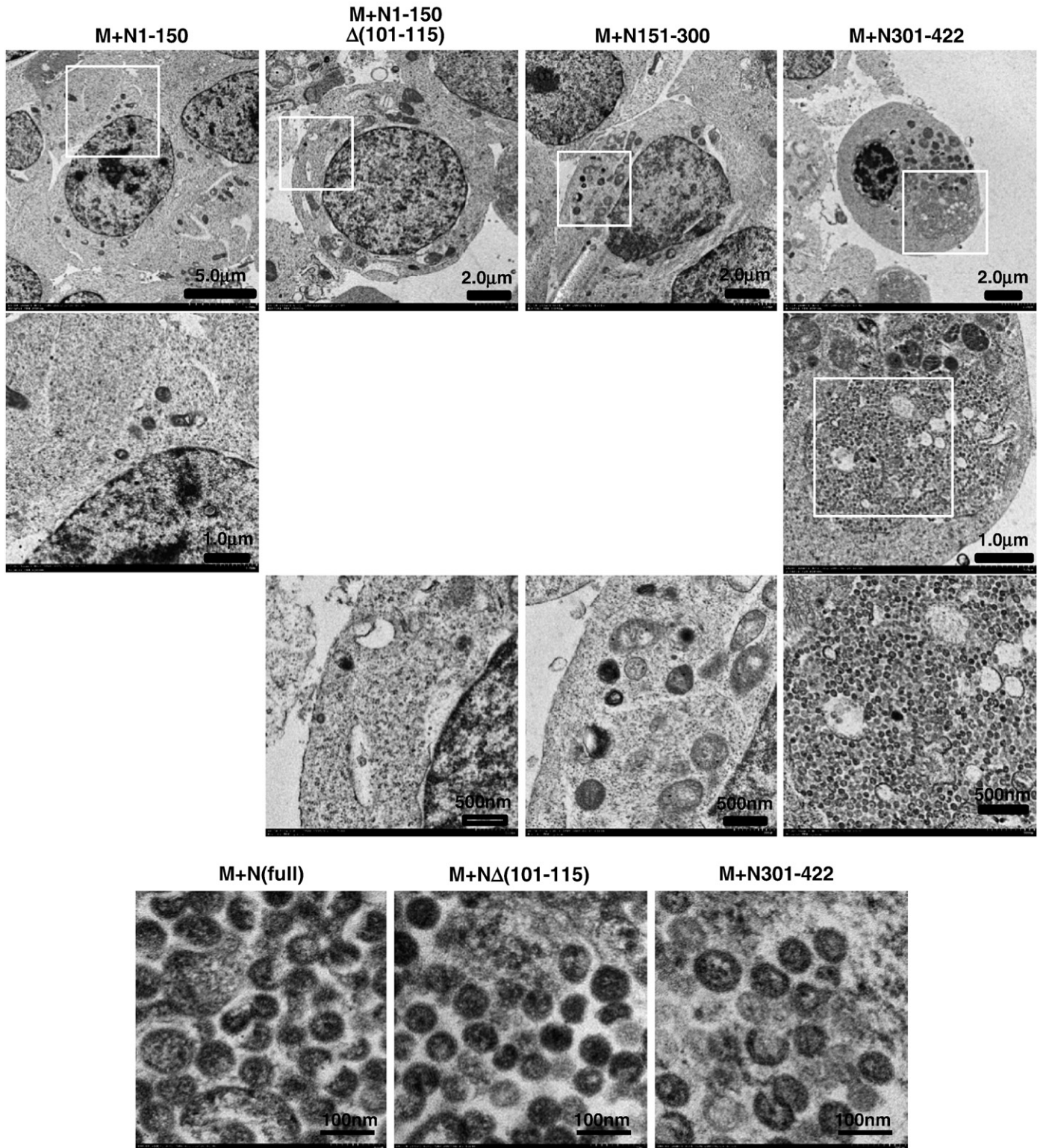


Fig. 5 (continued).

and M. We found that pep8 to pep10 significantly inhibited the interaction between N and M (Fig. 6B). Using peptides containing a shuffled amino acid sequence of pep10, we found that pep18 partially inhibited the M–N interaction, whereas pep16 and pep17 did not (Fig. 6C). These results indicated that the motif “PRWYFYLLG” was critical for inhibition, and that inhibition was at least partly sequence-specific and not nonspecific.

We also examined whether the 101–115 peptide interferes with the interaction between N mutants and M in ELISA format (Fig. 3B). Surprisingly, the 101–115 peptide inhibited the binding to M by N mutants that did not contain the corresponding sequence (NΔ101–115 and N1–150Δ101–115). As shown in Fig. 2, the peptides bound to M directly. Taken together, these results suggested that the mode of inhibition of the interaction between N and M by the 101–115 peptide

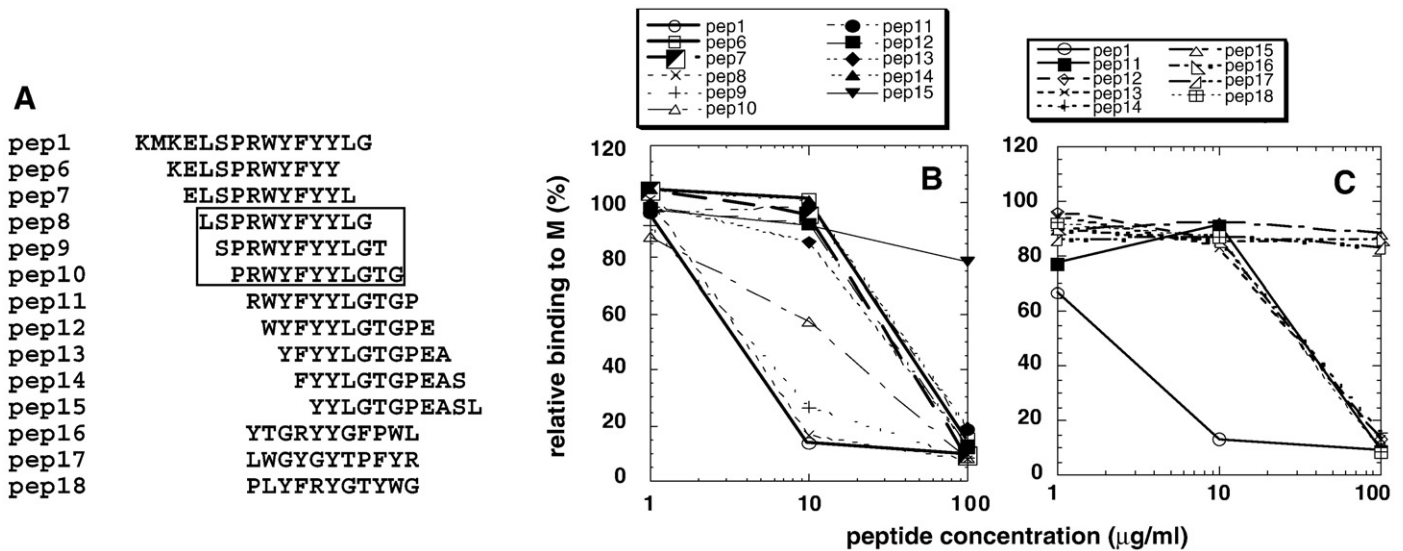


Fig. 6. Determination of the critical residues in N peptide 101–115 that inhibit the interaction between M and N. (A) Peptide sequences used in the analysis. Pep1 is the 101–115 peptide itself. Pep6 to Pep15 are 10-mer peptides spanning amino acids 101–115 of N. Pep16 to Pep18 are sequence-scrambled versions of Pep10. Amino acid sequences of Pep8 to Pep10, which showed strong inhibitory effects on the interaction between N and M in (B) are indicated in a box. (B) Ability of the 10-mer peptides derived from N peptide 101–115 to inhibit the interaction between N and M, as described in Materials and methods. (C) Abilities of the 10-mer scrambled versions of the peptides to inhibit the interaction between N and M. Binding in the absence of peptide was set at 100% (not shown). Data are presented as means \pm SD.

is occupation of the N binding site on M and not simple competition of 101–115 with the corresponding sequence in N.

Discussion

Due to the lack of an effective method of treatment or a vaccine for SARS, it is necessary to develop drugs that inhibit viral replication and/or transmission. One possible target is inhibition of virion formation. In mammalian cells, the interaction between N and M, the major structural proteins of SARS-CoV, is critical for the formation of pseudoparticles (Huang et al., 2004). To dissect this interaction at the sub-molecular level, we analyzed the interaction of M with peptides derived from N, and found that 101–115 showed significant binding to M. We also examined whether various N peptides could interact with M protein to form pseudoparticles. The results of the present study indicated that although N peptides 1–150, 151–300, and 301–422 can bind to M, only the latter is required for pseudoparticle formation.

The results of studies to assign the functional regions in N showed that N151–300 contains the SR-rich region that binds to M (168–208) (He et al., 2004b) as well as being important for the formation of N-dimers (Saikatendu et al., 2007). The N301–422 region contains a site (amino acids 351–422) that interacts with M sites (amino acids 343–363 and 382–402) for self-association (Luo et al., 2006a, 2006b). The N peptides 211–422 and 285–422 efficiently formed dimers, and dimeric N proteins associated into tetramers (Luo et al., 2006a). In the present study, biochemical analyses of recombinant proteins and coexpression analysis in mammalian cells showed that all regions of N interacted with M, and identified an as yet unreported region, amino acids 101–115, that also interacted with M.

Analysis of pseudoparticle formation by isolated proteins would provide critical clues into the process of virion formation *in vivo*. In mammalian cells, the formation of SARS-CoV pseudoparticles requires a minimum of two proteins, N and M (Huang et al., 2004). Using buoyant density gradient analysis and TEM, we observed pseudoparticle formation when M was coexpressed with N wild-type, N Δ (101–115), or N301–422 in HEK293 cells, indicating that the N protein segment of amino acids 301–422 was the minimum requirement for pseudoparticle formation. Based on these findings, we propose a

model for the assembly of SARS-CoV pseudoparticles by N and M (Fig. 7). N1–150 binds to M *via* the 101–115 region, as demonstrated in this study. N151–300 binds to M *via* the 168–208 region as reported previously (He et al., 2004b). N151–300 is also capable of forming homodimers but not oligomers. However, this binding is not sufficient to support pseudoparticle formation in mammalian cells (Fig. 5B). In contrast, N301–422 is capable of forming pseudoparticles. The differences among the N regions are probably based on the ability to form homooligomers. N301–422 contains a region capable of homotetramer formation (He et al., 2004a). The tetramers of N would provide scaffolds for the formation of pseudoparticles for M. Indeed, N colocalized with M in buoyant density gradients formed SDS-resistant oligomers (data not shown).

We found that peptides derived from amino acids 101–115 of N inhibited the interaction between N and M. However, this finding was partially contradictory as this region is fully dispensable for pseudoparticle formation. Based on the crystal structure, the peptide is located in the interior of N (Saikatendu et al., 2007). Thus, it is likely that the peptide region has a quite limited role in pseudoparticle formation.

Despite the limited role in pseudoparticle formation, the peptide showed a strong inhibitory effect on the interaction between N and M *in vitro*. We examined the abilities of these peptides to inhibit pseudoparticle formation by N and M *via* either peptide delivery or expression. However, these approaches were not successful (data not shown), which may have been due to ineffective delivery and insufficient expression of the peptides, respectively. Interestingly, the peptide (101–115) also inhibited the interaction between M and other N mutants, not containing the 101–115 region. Recombinant cytoplasmic region of the M protein used in this study consisted of only 116 amino acid residues. Thus, one possibility to explain this phenomenon is that M has only one site to bind N in the molecule and the site is shared by several regions in N for their interaction; the 101–115 peptide may occupy this site to inhibit access of N to M.

As the amino acid composition of the 101–115 peptide is relatively hydrophobic, it is possible that the observed inhibitory effect against the interaction between N and M may be nonspecific. However, scrambled versions of the peptide, especially pep16 and pep17, lost the ability to inhibit the interaction, indicating that the

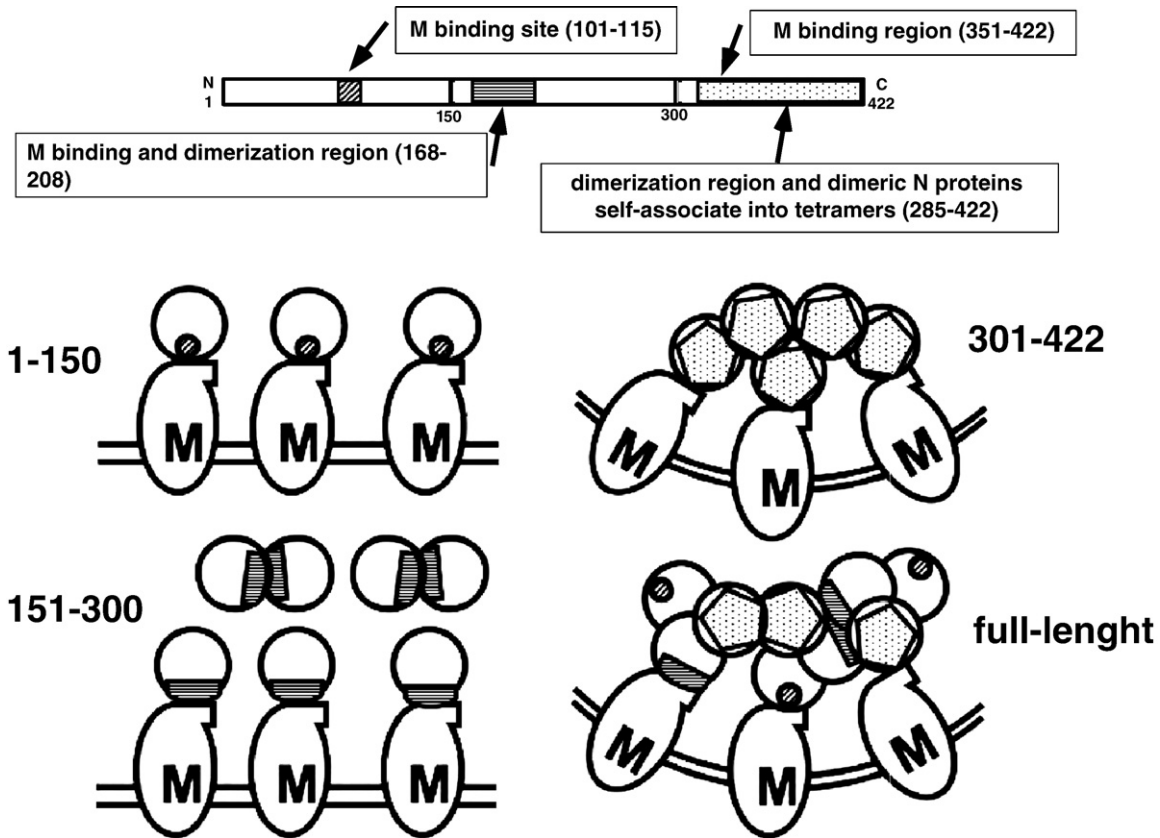


Fig. 7. Schematic model for the assembly of pseudoparticles by N and M of SARS-CoV. Three portions of N have been shown to interact with M, corresponding to residues 101–115 (hatched), 168–208 (striped), and 351–422 (stippled) (He et al., 2004a, 2004b; Luo et al., 2006b), but only interaction via the C-terminal of N led to formation of pseudoparticles. The 351–422 region can also form N tetramers, and thus may provide a scaffold for the stable interaction between N and M, allowing pseudoparticle formation.

inhibitory effect is at least partly specific for the sequence. The inhibitory effect observed with another scrambled version of the peptide, pep18, may have been because this peptide had a relatively large percentage of hydrophobic residues, which may be exchangeable to some extent.

In conclusion, we have shown here that the minimum requirement of N protein to form pseudoparticles with M are amino acid residues 301–422, and that a peptide derived from amino acids 101–115 of N may be a useful starting point to design molecule(s) to inhibit the formation of SARS-CoV virions. These findings will advance our understanding of the assembly of SARS-CoV and may facilitate the development of anti-SARS-CoV drugs.

Materials and methods

Preparations of mutant N proteins

All N-derived constructs used in this study are presented in Fig. 2. All of the plasmids expressing N mutants in *Escherichia coli* were prepared using pDEST17 (Invitrogen), which carries a recognition site for AcTEV protease (Invitrogen). pDEST17-SAR1262TEV encoding wild-type N protein was constructed by a commercial service (Invitrogen) and used as a template for PCR to amplify the various N-derivatives. A plasmid VRC8303 carrying codon-optimized N was kindly provided by Dr. Nabel of the National Institutes of Health (Huang et al., 2004) and used as a template for PCR to amplify N-derivatives for expression in mammalian cells. All constructs, with the exception of mutants carrying deletions of amino acids 101–115, were prepared by subcloning of the PCR products amplified using the primer pairs listed in Table S1 and *Pfx* polymerase (Invitrogen). To construct mutants with deletions of amino acids 101–115, N-terminal

and C-terminal fragments not containing the deletion were amplified separately by PCR using the primers listed in Table S1 and *Pfx* polymerase. The PCR products were subsequently purified, mixed, and used as a template for PCR amplification of the entire fragment carrying the deletion using *Taq* polymerase (Takara). For Gateway cloning (Invitrogen), the PCR products were cloned using a TOPO cloning kit (Invitrogen), digested from the resulting plasmids with *EcoRI* and subcloned into pDONR221 by BP recombination in accordance with the manufacturer's instructions. After antibiotic selection, the fragments were introduced into pDEST17 by LR recombination and used to transform TOP10 in accordance with the manufacturer's protocol. The resulting plasmids were introduced into *E. coli* strain BL21-AI (Invitrogen) to express the N mutants. PCR fragments for expression in mammalian cells were cloned using a TOPO cloning kit, digested with *Sall* and *NotI*, and introduced into the corresponding sites of the VRC vector.

The plasmids pDEST17-SAR-M-TEV63AA and pDEST17-SAR-M-TEV116AA, carrying the C-terminal 63 and 116 amino acids of M protein, respectively, and with a His-tags, were constructed by a commercial service (Invitrogen). To construct pQE2-M116, which carries an alternative version of M116 as a His-tagged protein, pDEST17-SAR-M-TEV116AA was used as a template for PCR amplification using the primer pair indicated in Table S1 and *Taq* polymerase. The PCR product was cloned using a TOPO cloning kit, and, following confirmation of the sequence, the insert was removed by digestion with *NdeI* and *NotI* and subcloned into the corresponding sites of pQE2 vector (Qiagen).

The plasmid VRC8301 carrying codon-optimized M was kindly provided by Dr. Nabel (Huang et al., 2004). To construct the plasmid pcDNA4/HisMaxB-hM116 for expression of M116 protein in mammalian cells, the corresponding DNA fragment was amplified by PCR

using VRC8301 as the template, the appropriate primer pair (Table S1), and *Pfx* polymerase. The PCR product was cloned into pCR-BluntII (Invitrogen), and the resulting plasmid was introduced into TOP10. After confirmation of the sequence, the fragment was excised by digestion with *EcoRI* and *NotI* and subcloned into the corresponding sites of pcDNA4/HisMaxB (Invitrogen).

Expression and purification of the N and M proteins

All recombinant proteins were affinity-purified using the His-tag introduced into the protein, and His-tags in the proteins used for biochemical analyses were removed enzymatically according to the manufacturer's instructions (see Supplement 1 for details).

Preparation of antibodies against N and M proteins

Aliquots of 100 μ g of purified N or M63 were used for immunization of rabbits with Freund's adjuvant (Difco). To prepare affinity-purified antibodies, M116 or N was coupled to NHS-activated sepharose (GE Healthcare) in accordance with the manufacturer's instructions. Then, 8 mL of serum was applied to each column, which was washed with 2CV of PBST. The antibody was eluted with 2 CV of 0.1 M glycine-HCl (pH 2.5), and the fractions were immediately neutralized with 1 M Tris-HCl (pH 8.8).

Mapping of the M protein binding site on the N protein

A set of 42 overlapping peptides (>70% purity) spanning the entire sequence of the N protein, each consisting of 15 amino acids overlapping the next peptide by 5 amino acids, were prepared by a commercial service (Biologica). The interaction of each of these peptides with M protein was assayed by an ELISA-based method. Briefly, the peptides (10 μ g/mL) were immobilized onto the wells of a 96-well microtiter plate (Corning) by incubation in 50 mM carbonate buffer (pH 9.0) containing 1 mM of the chemical cross-linker disuccinimidyl suberate (DSS; Pierce) at 37 °C for 1 h. After blocking with Superblock (Pierce), the plate was incubated for 0.5 h with 1 μ g/mL of M63 diluted in PBST, and washed three times with PBST. Binding of M63 was detected with the rabbit anti-M63 serum described above in combination with HRP-goat anti-rabbit IgG (GE Healthcare) and TMB (BioRad).

Surface plasmon response (SPR) analysis

The interactions of peptides derived from N protein with M116 were analyzed using a Biacore 3000 dual flow cell (Biacore). M116 was immobilized on a CM5 sensor chip by amine coupling at the level of 5500 response units (RU). As a negative control, a flow cell was immobilized with Tris-HCl at a parallel level. The response was the direct indication of the amount of analyte bound. The running and sample buffers were HBS-EP (Biacore). The analyte (70 μ L) was injected at 100 s followed by a dissociation phase (300 s). Each flow cell was regenerated with 10 μ L of 0.1% SDS. All experiments were carried out at a constant system flow rate of 20 μ L/min at 25 °C.

Analysis of M–N binding by ELISA

The interaction of M protein with several N mutant proteins was analyzed by ELISA as described above with minor modifications. Briefly, N mutant proteins (0.01–10 μ g/mL) were immobilized onto plates with 50 mM carbonate buffer (pH 9.0) for 1 h at 37 °C. After blocking, M116 (1 μ g/mL) was added for 0.5 h, and binding of M116 was detected with anti-M63 rabbit serum. To analyze the competition of N peptide (101–115) with N in the interaction between N and M, N (1 μ g/mL) was immobilized onto the microplates as describe above. Separately, M63 (1 μ g/mL) was incubated with peptides (>90% purity)

for 0.5 h and added to the plates blocked for 0.5 h. Binding of M63 was detected as described above.

Immunoprecipitation

HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal bovine serum. One day before transfection, aliquots of 3×10^6 cells were placed in 10-cm dishes, and the cells were subsequently transfected with 4 μ g of each plasmid using FuGENE 6 transfection reagent (Roche). After 3 days, the cells were washed with PBS, freeze-thawed in 1 ml of PBST, sonicated for 1 min, and centrifuged at 15,000 \times g for 5 min at 4 °C. Each supernatant was mixed with 30 μ L of Protein G Sepharose 4 Fast Flow (GE Healthcare) and rotated for 1 h at 4 °C, and centrifuged. Each supernatant was divided into 3 portions, to which were added 10 μ g of affinity-purified anti-N-antibody, anti-M-antibody, or control serum. Following rotation for 4 h at 4 °C, 30 μ L of Protein G Sepharose was added to each, and the mixtures were rotated for 1 h at 4 °C. The beads were washed 3 times with cold PBST and treated with SDS-PAGE buffer.

Pseudoparticle production and density gradient analysis

Pseudoparticle production was performed as described (Huang et al., 2004). Briefly, 293 cells transfected as described above were harvested, freeze-thawed three times in PBS, and pelleted in 15-mL conical tubes by centrifugation at 3100 rpm. Each cleared lysate was mixed with 2.5 mL of OptiPrep (Axis-Shield) and a density gradient was formed by centrifugation at 60,000 rpm for 3 h in a VTI65.2 rotor (Beckman). The fractions separated were subjected to immunoblotting using rabbit anti-M63 or rabbit anti-N serum.

Analysis of pseudoparticle formation by electron microscopy

Transfected 293 cells were harvested from plates by pipetting with DMEM and pelleted in 15-mL conical tubes by centrifugation at 1000 rpm. The supernatant was removed and 1 mL of fixing solution (0.2 M cacodylate buffer, pH 7.4, 3% glutaraldehyde, and 3% formaldehyde) was added. The specimens were mixed gently and analyzed using a TEM system (Hitachi) to visualize the pseudoparticles.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.virol.2008.07.012](https://doi.org/10.1016/j.virol.2008.07.012).

References

- Almazan, F., Galan, C., Enjuanes, L., 2004. The nucleoprotein is required for efficient coronavirus genome replication. *J. Virol.* 78, 12683–12688.
- Bos, E.C., Luytjes, W., Van Der Meulen, H.V., Koerten, H.K., Spaan, W.J., 1996. The production of recombinant infectious DI-particles of a murine coronavirus in the absence of helper virus. *Virology* 218, 52–60.
- Chang, C.K., Sue, S.C., Yu, T.H., Hsieh, C.M., Tsai, C.K., Chiang, Y.C., Lee, S.J., Hsiao, H.H., Wu, W.J., Chang, W.L., Lin, C.H., Huang, T.H., 2006. Modular organization of SARS coronavirus nucleocapsid protein. *J. Biomed. Sci.* 13, 59–72.
- Chen, C.Y., Chang, C.K., Chang, Y.W., Sue, S.C., Bai, H.I., Riag, L., Hsiao, C.D., Huang, T.H., 2007. Structure of the SARS coronavirus nucleocapsid protein RNA-binding

- dimerization domain suggests a mechanism for helical packaging of viral RNA. *J. Mol. Biol.* 368, 1075–1086.
- Dediego, M.L., Alvarez, E., Almazan, F., Rejas, M.T., Lamirande, E., Roberts, A., Shieh, W.J., Zaki, S.R., Subbarao, K., Enjuanes, L., 2007. A severe acute respiratory syndrome coronavirus that lacks the E gene is attenuated in vitro and in vivo. *J. Virol.* 81, 1701–1713.
- Fang, X., Ye, L., Timani, K.A., Li, S., Zen, Y., Zhao, M., Zheng, H., Wu, Z., 2005. Peptide domain involved in the interaction between membrane protein and nucleocapsid protein of SARS-associated coronavirus. *J. Biochem. Mol. Biol.* 38, 381–385.
- Guan, Y., Zheng, B.J., He, Y.Q., Liu, X.L., Zhuang, Z.X., Cheung, C.L., Luo, S.W., Li, P.H., Zhang, L.J., Guan, Y.J., Butt, K.M., Wong, K.L., Chan, K.W., Lim, W., Shortridge, K.F., Yuen, K.Y., Peiris, J.S., Poon, L.L., 2003. Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. *Science* 302, 276–278.
- He, R., Dobie, F., Ballantine, M., Leeson, A., Li, Y., Bastien, N., Cutts, T., Andonov, A., Cao, J., Booth, T.F., Plummer, F.A., Tyler, S., Baker, L., Li, X., 2004a. Analysis of multimerization of the SARS coronavirus nucleocapsid protein. *Biochem. Biophys. Res. Commun.* 316, 476–483.
- He, R., Leeson, A., Ballantine, M., Andonov, A., Baker, L., Dobie, F., Li, Y., Bastien, N., Feldmann, H., Strocher, U., Theriault, S., Cutts, T., Cao, J., Booth, T.F., Plummer, F.A., Tyler, S., Li, X., 2004b. Characterization of protein–protein interactions between the nucleocapsid protein and membrane protein of the SARS coronavirus. *Virus Res.* 105, 121–125.
- Hu, Y., Wen, J., Tang, L., Zhang, H., Zhang, X., Li, Y., Wang, J., Han, Y., Li, G., Shi, J., Tian, X., Jiang, F., Zhao, X., Wang, J., Liu, S., Zeng, C., Wang, J., Yang, H., 2003. The M protein of SARS-CoV: basic structural and immunological properties. *Genomics Proteomics Bioinformatics* 1, 118–130.
- Huang, Y., Yang, Z.Y., Kong, W.P., Nabel, G.J., 2004. Generation of synthetic severe acute respiratory syndrome coronavirus pseudoparticles: implications for assembly and vaccine production. *J. Virol.* 78, 12557–12565.
- Hurst, K.R., Kuo, L., Koetzner, C.A., Ye, R., Hsue, B., Masters, P.S., 2005. A major determinant for membrane protein interaction localizes to the carboxy-terminal domain of the mouse coronavirus nucleocapsid protein. *J. Virol.* 79, 13285–13297.
- Li, W., Moore, M.J., Vasilieva, N., Sui, J., Wong, S.K., Berne, M.A., Somasundaran, M., Sullivan, J.L., Luzuriaga, K., Greenough, T.C., Choe, H., Farzan, M., 2003. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* 426, 450–454.
- Luo, H., Chen, J., Chen, K., Shen, X., Jiang, H., 2006a. Carboxyl terminus of severe acute respiratory syndrome coronavirus nucleocapsid protein: self-association analysis and nucleic acid binding characterization. *Biochemistry* 45, 11827–11835.
- Luo, H., Wu, D., Shen, C., Chen, K., Shen, X., Jiang, H., 2006b. Severe acute respiratory syndrome coronavirus membrane protein interacts with nucleocapsid protein mostly through their carboxyl termini by electrostatic attraction. *Int. J. Biochem. Cell Biol.* 38, 589–599.
- Marra, M.A., Jones, S.J., Astell, C.R., Holt, R.A., Brooks-Wilson, A., Butterfield, Y.S., Khattra, J., Asano, J.K., Barber, S.A., Chan, S.Y., Cloutier, A., Coughlin, S.M., Freeman, D., Girn, N., Griffith, O.L., Leach, S.R., Mayo, M., McDonald, H., Montgomery, S.B., Pandoh, P.K., Petrescu, A.S., Robertson, A.G., Schein, J.E., Siddiqui, A., Smailus, D.E., Stott, J.M., Yang, G.S., Plummer, F., Andonov, A., Artsob, H., Bastien, N., Bernard, K., Booth, T.F., Bowness, D., Czub, M., Drebot, M., Fernando, L., Flick, R., Garbutt, M., Gray, M., Grolla, A., Jones, S., Feldmann, H., Meyers, A., Kabani, A., Li, Y., Normand, S., Stroher, U., Tipples, G.A., Tyler, S., Vogrig, R., Ward, D., Watson, B., Brunham, R.C., Krajdin, M., Petric, M., Skowronski, D.M., Upton, C., Roper, R.L., 2003. The genome sequence of the SARS-associated coronavirus. *Science* 300, 1399–1404.
- Masters, P.S., 1992. Localization of an RNA-binding domain in the nucleocapsid protein of the coronavirus mouse hepatitis virus. *Arch. Virol.* 125, 141–160.
- Mortola, E., Roy, P., 2004. Efficient assembly and release of SARS coronavirus-like particles by a heterologous expression system. *FEBS Lett.* 576, 174–178.
- Narayanan, K., Maeda, A., Maeda, J., Makino, S., 2000. Characterization of the coronavirus M protein and nucleocapsid interaction in infected cells. *J. Virol.* 74, 8127–8134.
- Narayanan, K., Kim, K.H., Makino, S., 2003. Characterization of N protein self-association in coronavirus ribonucleoprotein complexes. *Virus Res.* 98, 131–140.
- Nelson, G.W., Stohman, S.A., 1993. Localization of the RNA-binding domain of mouse hepatitis virus nucleocapsid protein. *J. Gen. Virol.* 74, 1975–1979.
- Rota, P.A., Oberste, M.S., Monroe, S.S., Nix, W.A., Campagnoli, R., Icenogle, J.P., Penaranda, S., Bankamp, B., Maher, K., Chen, M.H., Tong, S., Tamin, A., Lowe, L., Frace, M., Derisi, J.L., Chen, Q., Wang, D., Erdman, D.D., Peret, T.C., Burns, C., Ksiazek, T.G., Rollin, P.E., Sanchez, A., Liffick, S., Holloway, B., Limor, J., Mccaustland, K., Olsen-Rasmussen, M., Fouchier, R., Gunther, S., Osterhaus, A.D., Drosten, C., Pallansch, M.A., Anderson, L.J., Bellini, W.J., 2003. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. *Science* 300, 1394–1399.
- Saikatendu, K.S., Joseph, J.S., Subramanian, V., Neuman, B.W., Buchmeier, M.J., Stevens, R.C., Kuhn, P., 2007. Ribonucleocapsid formation of severe acute respiratory syndrome coronavirus through molecular action of the N-terminal domain of N protein. *J. Virol.* 81, 3913–3921.
- Triplet, B., Howard, M.W., Jobling, M., Holmes, R.K., Holmes, K.V., Hodges, R.S., 2004. Structural characterization of the SARS-coronavirus spike S fusion protein core. *J. Biol. Chem.* 279, 20836–20849.
- Vennema, H., Godeke, G.J., Rossen, J.W., Voorhout, W.F., Horzinek, M.C., Opstelten, D.J., Rottier, P.J., 1996. Nucleocapsid-independent assembly of coronavirus-like particles by co-expression of viral envelope protein genes. *EMBO J.* 15, 2020–2028.
- Wang, L.F., Shi, Z., Zhang, S., Field, H., Daszak, P., Eaton, B.T., 2006. Review of bats and SARS. *Emerg. Infect. Dis.* 12, 1834–1840.
- Wilson, L., Mckinlay, C., Gage, P., Ewart, G., 2004. SARS coronavirus E protein forms cation-selective ion channels. *Virology* 330, 322–331.
- Wu, Q., Zhang, Y., Lu, H., Wang, J., He, X., Liu, Y., Ye, C., Lin, W., Hu, J., Ji, J., Xu, J., Ye, J., Hu, Y., Chen, W., Li, S., Wang, J., Wang, J., Bi, S., Yang, H., 2003. The E protein is a multifunctional membrane protein of SARS-CoV. *Genomics Proteomics Bioinformatics* 1, 131–144.
- Xu, Y., Lou, Z., Liu, Y., Pang, H., Tien, P., Gao, G.F., Rao, Z., 2004. Crystal structure of severe acute respiratory syndrome coronavirus spike protein fusion core. *J. Biol. Chem.* 279, 49414–49419.
- Yu, I.M., Oldham, M.L., Zhang, J., Chen, J., 2006. Crystal structure of the severe acute respiratory syndrome (SARS) coronavirus nucleocapsid protein dimerization domain reveals evolutionary linkage between corona- and arteriviridae. *J. Biol. Chem.* 281, 17134–17139.
- Zuniga, S., Sola, I., Moreno, J.L., Sabella, P., Plana-Duran, J., Enjuanes, L. (2007). Coronavirus nucleocapsid protein is an RNA chaperone.