NOTE

Analysis of severe acute respiratory syndrome coronavirus structural proteins in virus-like particle assembly

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ABSTRACT

SARS-CoV has four major structural proteins: the N, S, M, and E proteins. To investigate the mechanism of SARS-CoV assembly, we cloned the genes encoding these four proteins into the eukaryotic expression vector pCAGGS and transfected them into 293T cells. When all four expression vectors were co-transfected VLP formed, as confirmed using electron microscopy. Using a rabbit polyclonal antibody specific to the N protein, N-protein-containing particles similar in size to the VLP were also observed by immunoelectron microscopy, indicating that the VLP contained the N protein. Co-immunoprecipitation analyses demonstrated an interaction between the N and M proteins, suggesting that N protein binds directly to M protein to be incorporated into VLP.

Key words particle formation, SARS-CoV, VLP.

A serious epidemic of SARS, an atypical pneumonia, occurred during 2002 and 2003 (1–3). The novel coronavirus that causes SARS (SARS-CoV) is an enveloped virus with a positive-strand RNA genome of approximately 30,000 nucleotides. The virus particle consists of an RNA genome and four major structural proteins: the N, S, M, and E proteins. Coronaviruses are thought to assemble in the ER–Golgi intermediate compartment through the interaction of structural proteins (4). However, information concerning the interactions of the SARS-CoV structural proteins is extremely limited.

The SARS-CoV N protein is a 46-kDa phosphoprotein that binds to genomic RNA to form a helical ribonucleocapsid that is enveloped by a lipid bilayer containing the S, M, and E proteins (5, 6). It has been proposed that the N protein functions in viral RNA replication and protein translation (7, 8). The S protein is a type-I glycoprotein that plays a role in viral entry by binding to the SARS-CoV receptor, ACE2, which is expressed on the surface of target cells (9). However, the S protein is not thought to be required for virus assembly (10). The M protein, a triple-spanning membrane protein, is the most abundant protein in the virion. The cytoplasmic C-terminal domain of the M protein interacts with the N protein, whereas the N-terminal domain is exposed on the extracellular side of the virion (11, 12). The E protein, which is present in small amounts, is a transmembrane protein that may function as a viroporin (13).

It has recently been reported that low levels of SARS-CoV VLP are released into the culture medium of mammalian cells (10, 14, 15). Because VLP formation resembles virion formation, the mechanism of SARS-CoV particle formation was investigated in the current study by cloning the coding regions for the S, M, E, and N structural proteins...
proteins into the eukaryotic expression vector pCAGGS (16). Primers were designed to add an N-terminal HA tag to the M protein, a C-terminal FLAG tag to the E protein, and a C-terminal HA tag to the S protein. The constructs expressing the M, E, S, and N proteins are designated HA-M-pCAG, E-FLAG-pCAG, S-HA-pCAG, and N-pCAG, respectively.

After co-transfecting 293T cells with HA-M-pCAG, E-FLAG-pCAG, S-HA-pCAG, and N-pCAG, we used EM to confirm the formation of VLP. One hundred nm diameter particles resembling SARS-CoV were visible by EM in the cytoplasm of transfected cells (Fig. 1a). The particles were localized in intracellular vesicles at the periphery of the nucleus (Fig. 1a, arrows) and as buds emerging from the intracellular membrane (Fig. 1b). Similar particles were not detected in normal 293T cells (data not shown).

To confirm the incorporation of N protein into the VLP, we performed an immuno-EM analysis using an N-protein-specific rabbit pAb. Immunofluorescence imaging confirmed that N protein was expressed in the cytoplasm of 293T cells co-transfected with the four expression plasmids (Fig. 1c). By immuno-EM analysis, N-protein-containing particles similar in size to the VLP were visible in intracellular vesicles (Fig. 1d, filled arrowheads), and N protein that was not incorporated into the particles was visible in the cytoplasm (Fig. 1d, open arrowheads). These results confirm that SARS-CoV VLP are produced in cells transfected with the expression plasmids for the M, E, S, and N proteins.

To facilitate the detection of low levels of the structural proteins, we established ELISA. In the N-ELISA, a rabbit anti-N-protein pAb (10 μg/ml) was used for N-protein capture, and an anti-N-protein mAb (Clone 122) (17) was used at 1:1000 dilution for N-protein detection. In the S-ELISA, an anti-S protein mAb (Clone 341C; Chemicon, Temecula, CA, USA) (18) was used at 1:500 dilution for S-protein capture, and a biotin-conjugated anti-HA-tag mAb (Sigma-Aldrich, St Louis, MO, USA) was used at 1:1000 dilution for S-protein detection.

To determine whether VLP are secreted from 293T cells that express the four structural proteins of SARS-CoV, we analyzed the culture medium of the transfected cells using equilibrium density centrifugation. The N- and S-protein peaks were detected in fraction 6 (Fig. 2) of the 20–50% sucrose gradient. The density of this fraction (1.157 g/cm³) was similar to that reported previously for VLP-containing fractions (14, 19, 10), indicating that the N and S proteins in fraction 6 were secreted as SARS-CoV VLP. The N protein, but not the S protein, was also detected in fractions 3 and 4 (density, 1.08–1.10 g/cm³; Fig. 2). The peaks of fractions 3 and 4 may represent aggregates of N protein released from broken cells. These results suggest that SARS-CoV VLP are formed in cells that express

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**Fig. 1. Analysis of SARS-CoV VLP formation in 293T cells by transmission EM.** (a) Electron micrograph of VLP (arrows) formed in 293T cells after cells were transfected with expression plasmids for the SARS-CoV M, E, S, and N proteins. Cytoplasm, (C); Golgi, (G); multi-vesicular body, (MVB); nuclei, (N). Scale bar = 200 nm. (b) Electron micrograph of VLP budding from the intracellular membrane. Scale bar = 200 nm. (c) N-protein expression analyzed by indirect immunofluorescence assay. Cells transfected with the M, E, S, and N expression plasmids were stained with rabbit anti-N-protein pAb. Scale bar = 25.0 μm. (d) Cells expressing M, E, S, and N proteins analyzed by immuno-EM with rabbit anti-N-protein pAb (10-nm colloidal gold). Filled and open arrowheads indicate N protein present in particles and cytoplasm, respectively. Scale bar = 200 nm.
SARS-CoV proteins in VLP assembly

In vitro research has shown that the N and M proteins of SARS-CoV interact with each other (11, 20, 21) and that this interaction is important for coronavirus assembly and N-protein incorporation into VLP (22–24). To demonstrate a direct interaction between the N and M proteins, we used immunoprecipitation. When the M and N proteins were co-expressed in 293T cells, they co-precipitated with either anti-N-protein pAb or anti-HA-tag mAb, which recognizes the HA-tagged M protein (Fig. 3a). The S and E proteins did not appear to be required for the N–M protein interaction (Fig. 3a). When either the M or N protein was expressed alone, anti-N-protein pAb did not immunoprecipitate the M protein, and anti-HA-tag mAb did not immunoprecipitate the N protein (Fig. 3a).

**Fig. 3. Interaction between N and M proteins in 293 T cells.** Briefly, E-FLAG-pCAG, (E, 3 μg); HA-M-pCAG, (M, 3 μg); N-pCAG, (N, 1 μg); and S-HA-pCAG (S, 2 μg), or a similar amount of empty pCAG vector (–) was transfected into 293T cells using TransIT-LT1 reagent. (a) Immunoprecipitation analysis. Transfected cells were lysed using lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS in phosphate-buffered saline), and cell lysates were immunoprecipitated with rabbit anti-N-protein pAb (20 μg/ml; upper two panels) or with anti-HA-tag mAb (lower two panels). The expression plasmids used for transfection are indicated at the bottom of the figure. The viral proteins were analyzed by Western blotting using anti-N-protein mAb (Clone 122) to detect N protein or anti-HA-tag mAb to detect M protein, as indicated to the left of the figure. (b) N-ELISA results. The ratio of the extracellular amount of N protein (N-ELISA extracellular OD value) to the total amount of N protein (sum of N-ELISA extracellular and intracellular OD values) was calculated for 293T cells that were transfected with the expression plasmids indicated on the x-axis. The results are expressed relative to those from cells that expressed the N protein alone (1). (c) S-ELISA results. The ratio of the extracellular amount of S protein to the total amount of S protein was calculated for each sample. The results are expressed relative to those from cells that expressed the S protein alone (1).

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The amount of extracellular N protein was significantly increased in cells co-transfected with N-pCAG and HA-M-pCAG (Fig. 3b). The ratio of extracellular N protein to total N protein (the sum of extra- and intracellular N protein) was 2.5 to 3.0 times higher in cells that co-expressed the N and M proteins than in cells that expressed the N protein alone (Fig. 3b). The amount of extracellular S protein was also significantly higher in the cells co-transfected with S-HA-pCAG, HA-M-pCAG, and N-pCAG (Fig. 3c) than in cells transfected with S-HA-pCAG alone. The ratio of extracellular to total S protein (the sum of extra- and intracellular S protein) in the cells that co-expressed the S, M, and N proteins was almost twice that in the cells that expressed the S protein alone (Fig. 3c). The results of the immunoprecipitation analysis suggest that a direct interaction between the M and N proteins is important for the secretion of N and S proteins into the culture medium.

The M and E proteins of MHV have been reported to be essential for virus particle formation and to be sufficient for the production of VLP (25); even the E protein alone has been reported to form VLP (26). Similar studies of SARS-CoV VLP formation have reported conflicting results. Huang et al. (10) have reported that the M and N proteins, but not the S and E proteins, are critical for particle formation, whereas Hsieh et al. (14) have suggested that the M and E proteins are sufficient for VLP formation. Mortola and Roy (15) have also reported that the M and E proteins, but not the S and N proteins, are required for VLP formation. The latter two reports are consistent with the findings reported for MHV (25).

Our results are in good agreement with those of Huang et al. (10), but not with those of Hsieh et al. (14) or Mortola and Roy (15). Like Huang et al. (10), we used a plasmid-based expression system, whereas Hsieh et al. (14) used a vaccinia-T7 polymerase system and Mortola and Roy (15) used a baculovirus system. Therefore, the discrepancies among the reported roles of the SARS-CoV structural proteins in particle formation might be attributable to the use of different experimental expression systems. When viral vectors are used, some other factors might compensate for the function of the N protein.

Coronavirus packaging of genomic RNA is dependent on a nucleotide sequence known as the PS (27, 28). Although packaging of MHV genomic RNA can occur independently of the MHV N protein (29), the MHV N protein does bind to a specific PS sequence located at the end of the 1b gene of the MHV RNA genome to facilitate its packaging into virus particles (24, 30, 31). Recently, the putative SARS-CoV PS was identified and localized in the genomic region spanning nucleotides 19715–20294, and N-protein expression was demonstrated to be required for packaging of the SARS-CoV genome into VLP (14, 32).

To validate these earlier reports using our VLP system, we constructed fusion RNA for use in a packaging experiment. This RNA, denoted as GFP-PS RNA (Fig. 4a) contains the SARS-CoV genomic region 18486–21485, which encompasses the putative PS (14). The EGFP gene was transcribed in vitro for use as negative-control RNA. Forty-four hours after transfection with plasmids expressing the SARS-CoV structural proteins, cells were transfected with the in vitro-transcribed RNA (2 μg/well) using a TransIT mRNA transfection kit (Mirus, Mississauga, Ontario, Canada) according to the manufacturer’s protocol. To elucidate which structural proteins were critical for the release of VLP containing GFP-PS RNA, RT-PCR was carried out using the culture medium from cells that expressed various combinations of the structural proteins (Fig. 4b). Before extraction of total RNA, the PEG-precipitated culture medium was resuspended in PBS supplemented with RNase A (20 μg/ml) and incubated for 30 min at room temperature to degrade free RNA.

GFP-PS RNA was detected in the culture medium of cells co-expressing both the N and M proteins (Fig. 4b, lanes 1–4), but not in that of cells lacking either the M
(lane 5) or the N (lane 6) protein, nor in that of cells expressing only the M (lane 7) or N (lane 8) protein. In contrast, the negative-control EGFP RNA was not detected in the culture medium of cells that co-expressed the M, E, S, and N proteins (data not shown). These results were reproducible in three independent experiments.

The N-ELISA also detected GFP-PS RNA when the N protein was released into the culture medium in the presence of M protein (Fig. 4, lanes 1–4), but not in the absence of M protein (lanes 5 and 8). RNase A-resistant GFP-PS RNA was detected only in the supernatant of cells that expressed both the N and M proteins, conditions that allow the release of the N protein into the culture medium (Fig. 4b). This result suggests that M–N protein interaction is required not only for VLP formation, but also for genome packaging. Given our finding that the M and N proteins interact with each other (Fig. 2a), we surmise that the GFP-PS-bound N protein was packaged into particles by interacting with the M protein and then released into the culture medium.

RNA was detected by RT-PCR, but not by Northern blotting. The putative PS region used in our experiment may be insufficient for efficient packaging of the SARS-CoV genome, or other factors may be required. The SARS-CoV 3a protein, which is thought to be a structural component of the virion, interacts with the 5′-untranslated region of the SARS-CoV genome (33). Therefore, more efficient packaging of the RNA might occur if the 3a protein is also expressed.

We have demonstrated that direct interaction between N and M SARS-CoV proteins is critically important for the formation of VLP and for packaging of the genome into these particles. VLP and ELISA systems should allow more detailed information to be obtained on SARS-CoV particle formation at the molecular level.

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