

Severe Acute Respiratory Syndrome Coronavirus 3a Protein Is a Viral Structural Protein

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The present study showed the association of a severe acute respiratory syndrome coronavirus (SCoV) accessory protein, 3a, with plasma membrane and intracellular SCoV particles in infected cells. 3a protein appeared to undergo posttranslational modifications in infected cells and was incorporated into SCoV particles, establishing that 3a protein was a SCoV structural protein.

Severe acute respiratory syndrome (SARS) coronavirus (SCoV) is the etiological agent of a new emerging infectious disease, SARS, which originated in southern China in 2002 and spread to various areas of the world in the 2003 epidemic (5, 11, 12, 23, 29). Like all coronaviruses, the SCoV genome carries the most 5'-end gene 1, which encodes proteins for viral RNA synthesis; four viral structural protein genes, S, M, E, and N; and several (about eight) putative accessory genes (16, 25, 28). The biological functions of coronavirus accessory proteins are poorly characterized. In most cases, they are dispensable for virus replication in cell culture while some appear to contribute to viral pathogenesis (1–3, 21, 22, 30, 31). In mouse hepatitis virus, the accessory protein, I protein, is a viral structural protein (9). Among the putative SCoV accessory genes, the 3a gene product, 3a protein, was detected in SCoV-infected cells and in the lungs of SARS patients (32); the 3a gene is also called X1 (25) and U274 (27). Topology prediction of 3a protein based on the amino acid sequence suggests that 3a is a type III transmembrane protein with three transmembrane domains.

To examine 3a protein synthesis in SCoV-infected cells, accumulation of 3a protein in Caco2 cells that were infected with the Urbani strain of SCoV was examined by Western blot analysis with anti-3a antibody. Anti-3a antibody was prepared by injection of a purified glutathione *S*-transferase (GST)–3a fusion protein (amino acids 127 to 274 of 3a protein were fused with the C terminus of GST protein), expressed in *Escherichia coli*, into a rabbit and subsequent affinity purification of the serum by the GST-3a fusion protein. Several 3a-related signals were detected in SCoV-infected cells and not in mock-infected cells (Fig. 1). Among them, a 31-kDa protein, which corresponded to the predicted size (30.9 kDa) of 3a protein, was most abundant (Fig. 1, arrow). Translation of *in vitro*-synthesized, capped RNA transcripts carrying the 3a gene in rabbit reticulocyte lysate also resulted in the production of 31-kDa

protein (data not shown), suggesting that the major 31-kDa protein represented an unmodified 3a protein in infected cells. Besides the 31-kDa major band, several larger, faint bands ranging from 34 to 41.5 kDa were detected, suggesting posttranslational modification of the protein. The nature of 3a protein modification requires further studies. The presence of two smaller bands of approximately 25.5 and 26.5 kDa in infected cells suggested that some 3a protein molecules underwent specific proteolytic processing and/or that these two signals represented quasistable degradation products.

Analysis of the subcellular distribution of 3a protein by confocal microscopy with anti-3a antibody demonstrated the cytoplasmic localization of 3a protein in SCoV-infected cells (Fig. 2A). Strong signals were also detected at peripheral regions of the infected cells. No signal was detected in mock-infected cells (Fig. 2B).

Analysis of the subcellular localization of 3a protein in SCoV-infected Caco2 cells by immunoelectron microscopy with anti-3a antibody showed that 3a protein was localized in the cytoplasm as well as at the plasma membrane (Fig. 3A and B), whereas the nucleus of the infected cells (Fig. 3A) and uninfected cells (one cell at the upper left corner in Fig. 3A; also Fig. 3D) showed only background signals. More importantly, 3a protein was distributed in cytoplasmic (Fig. 3A and C) and plasma membrane (Fig. 3B, “V”) regions in which many virus particles had accumulated. Essentially the same subcellular localization of 3a protein was detected in infected Vero E6 cells (data not shown). To eliminate the possibility of nonspecific binding of anti-3a antibody to SCoV, we counted the number of intracellular virus particles labeled with 3a-specific immunogold signals in immunoelectron micrographs of SCoV-infected Caco2 cells stained with anti-3a antibody or the preimmune serum. Out of 200 virus particles counted randomly, 75 particles (37.5%) had the immunogold label in the cells stained with anti-3a antibody, while only 7 (3.5%) had signals in the cells stained with preimmune serum. The difference was statistically significant, as determined by the chi-square test ($P < 0.001$), establishing the association of 3a protein with SCoV. These data suggested that 3a protein could be incorporated into SCoV particles. The data obtained from

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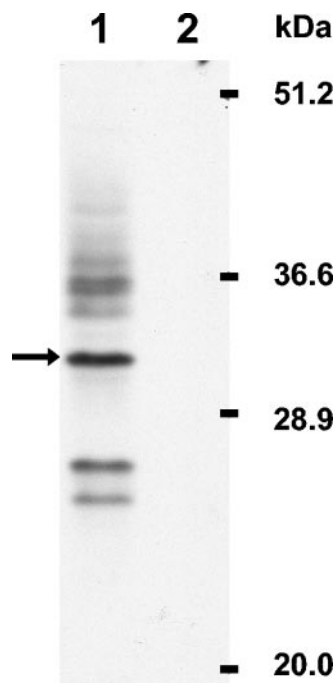


FIG. 1. Western blot analysis of 3a protein. Human colonic adenocarcinoma Caco2 cells were infected with SCoV at a multiplicity of infection of 0.01, and cells were solubilized with sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer (100 mM Tris-HCl [pH 6.8], 4% sodium dodecyl sulfate, 0.2% bromophenol blue, 20% glycerol, and 200 mM beta-mercaptoethanol) at 5 days p.i. Cell extracts were applied to a sodium dodecyl sulfate-15% polyacrylamide gel, and Western blot analysis was performed using anti-3a antibody. Lane 1, SCoV-infected Caco2 cells; lane 2, mock-infected Caco2 cells. Arrow; major 31-kDa 3a protein.

these microscopic analyses were consistent with the report of 3a protein association with cytoplasmic membrane in 3a protein-expressing cells (27) and a confocal microscopic study demonstrating that 3a protein was distributed over the cyto-

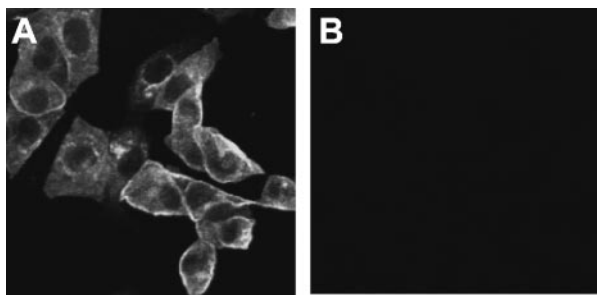
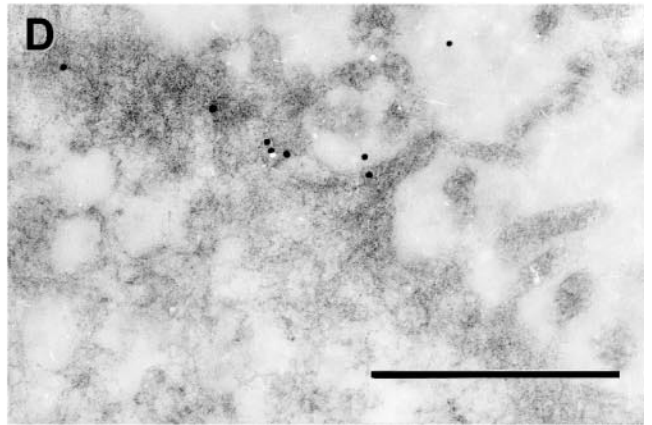
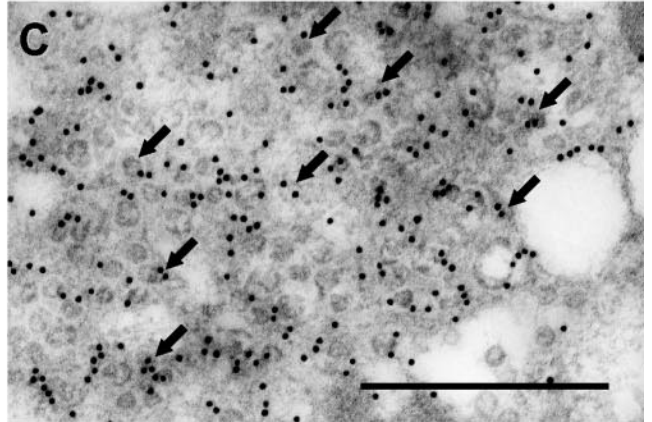
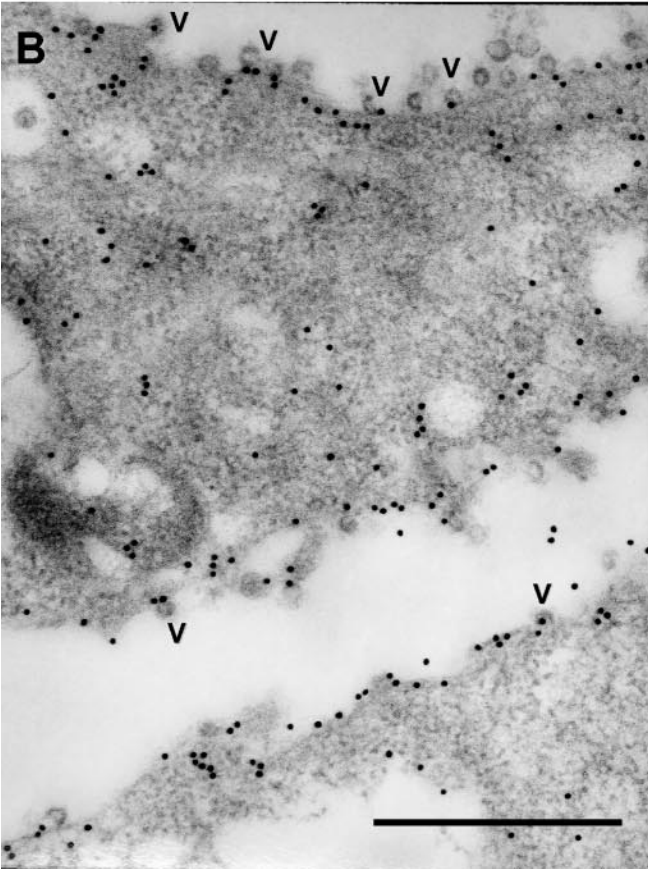
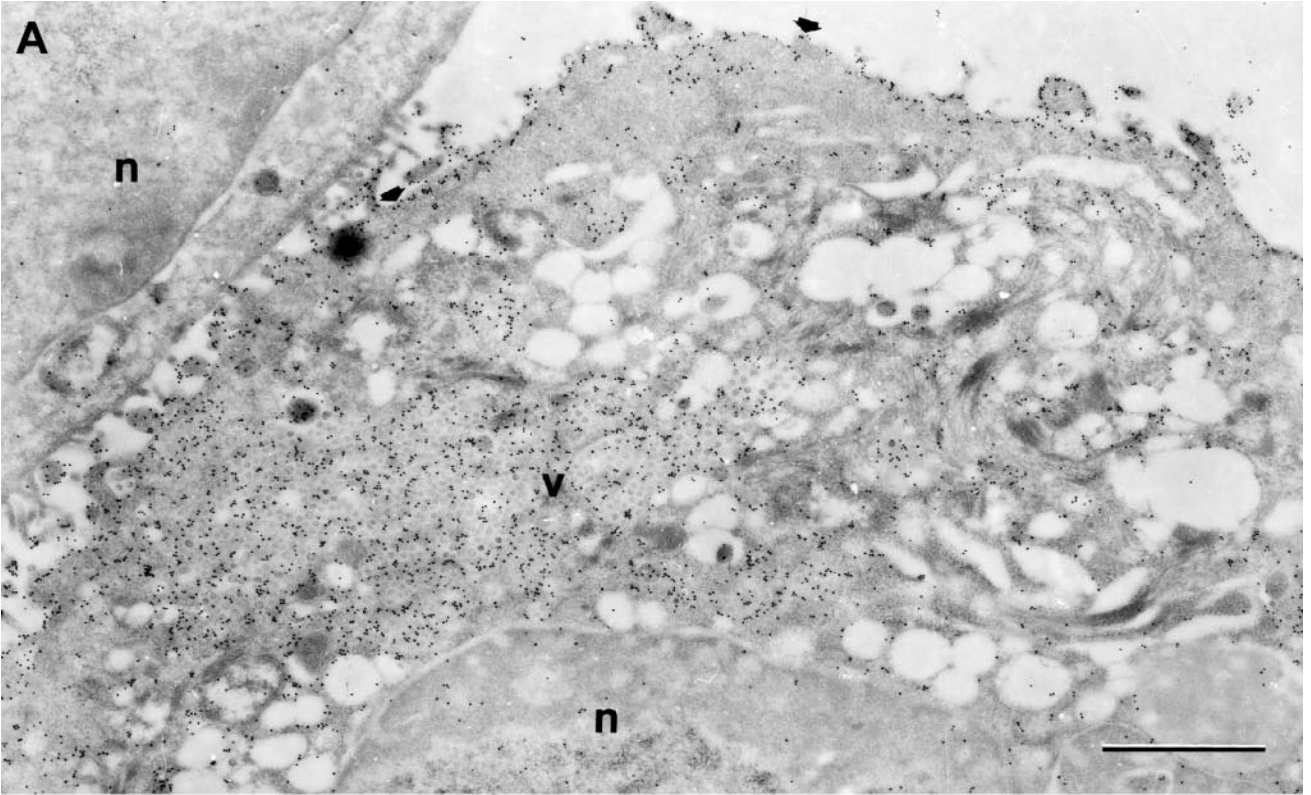


FIG. 2. Confocal microscopic analysis of 3a protein in SCoV-infected cells. Vero E6 cells growing in eight-well chamber slides (Lab-Tek, Naperville, Ill.) were infected with SCoV at a multiplicity of infection of 1 (A) or mock infected (B). At 24 h p.i., cultures were incubated overnight with 4% paraformaldehyde and then treated with 0.25% Triton X-100 for 15 min. Subsequently, cells were incubated with anti-3a antibody and goat anti-rabbit secondary antibody conjugated with Alexa Fluor 488 dye (Molecular Probes, Eugene, Oreg.). Cells were observed under the Zeiss LSM 510 UV META laser scanning confocal microscope in the University of Texas Medical Branch Infectious Disease and Toxicology Optical Imaging Core.

plasm and was partly concentrated in the Golgi apparatus of infected cells (32).

To test the possibility that 3a protein is a viral structural protein, the presence of 3a protein in the purified SCoV was examined. To minimize the possible copurification of intracellular proteins with the purified SCoV, we propagated SCoV in Caco2 cells, which did not show any visible cytopathic effects during SCoV replication (17). Prior to SCoV purification, culture supernatants from SCoV-infected Caco2 cells were irradiated frozen with 2×10^6 rads from a Gammacell ^{60}Co source (model 109A; JL Shepherd and Associates, San Fernando, Calif.). After confirmation of the complete inactivation of virus infectivity, the sample was clarified by centrifugation at $450 \times g$ for 15 min. SCoV particles were partially purified by two subsequent ultracentrifugations on a discontinuous sucrose gradient consisting of 60, 50, 30, and 20% sucrose with the use of a Beckman SW28 rotor (10, 19); the sample was first centrifuged at 28,000 rpm for 3 h, and the virus particles at the interface of 30 and 50% sucrose were further centrifuged at 28,000 rpm for 18 h. The virus particles at the interface of 30 and 50% sucrose were collected, diluted, and then further applied on a continuous sucrose gradient of 20 to 60% sucrose. The samples were centrifuged at 28,000 rpm for 18 h. Subsequently, 10 fractions were collected, and sucrose density in each fraction was measured. SCoV in each fraction was pelleted through a 20% sucrose cushion at 38,000 rpm for 2 h with a Beckman SW41 rotor. The pellet in each fraction was subjected to Western blot analysis with anti-N protein antibody (kindly provided by Xiao-Hua Li), anti-M protein antibody (Abgent, San Diego, Calif.), and anti-3a antibody (Fig. 4A). Analysis of N protein and M protein demonstrated the distribution of the purified SCoV in the continuous sucrose gradient. The strongest signals of N and M proteins were detected in fractions 6 (sucrose density, 1.185 g/ml) and 7 (sucrose density, 1.160 g/ml), suggesting that the buoyant density of SCoV was similar to that of mouse hepatitis virus (14, 15). In addition to N protein of approximately 50 kDa, a signal of ~ 40 kDa was also detected in the purified SCoV. The 40-kDa species of N protein, which was barely detected in infected cells, was probably produced from the 50-kDa N protein by proteolytic processing. N protein of transmissible gastroenteritis coronavirus is cleaved by activated caspases in infected cells (6), while the mechanism of proteolytic processing of SCoV N protein is unknown. Multiple M protein signals indicated the presence of various glycosylated forms of M protein in SCoV particles. Like N and M proteins, several 3a protein-related signals were detected in fractions from 5 to 8, with the highest signal in fractions 6 and 7. Although most of the intracellular 3a-related signals were detected in purified SCoV, a 25.5-kDa signal was not detected in the purified SCoV. Also two signals (asterisks), which were prominent in purified SCoV, were not detected in infected cells. These data strongly suggested that 3a protein was a viral structural protein.

To eliminate a possibility that the 3a protein detected in purified SCoV represented a copurified intracellular 3a protein contaminant that was released into the culture fluids or that was associated with cell debris, Western blot analysis was performed to examine whether the intracellular host protein, actin, and the SCoV gene 1 protein, nsp1 (24), which is believed to be a nonstructural protein, were also found in the



purified SCoV (Fig. 4B); detection of actin and nsp1 proteins in the purified SCoV sample would indicate that the virus purification procedure was not appropriate. Antiactin goat polyclonal immunoglobulin G (I-19; Santa Cruz Biotechnology, Santa Cruz, Calif.) was used to detect actin. Anti-nsp1 antibody was raised in rabbits by immunizing them with the synthetic peptide (N-RKNGNKGAGGHSYG-C). Analysis of intracellular proteins from uninfected Caco2 cells and infected Caco2 cells at 5 days postinfection (p.i.) showed the presence of nsp1 only in infected cell extracts and actin in both cell extracts. Both actin and nsp1 proteins were not detected in the purified SCoV. Consistent with the data shown in Fig. 4A, N and 3a proteins were detected in purified SCoV and SCoV-infected cells but not in uninfected cells. These data strongly indicated that 3a protein in the purified SCoV sample was not a copurified intracellular 3a protein contaminant.

Our electron microscopic analysis convincingly showed the association of 3a protein with intracellular SCoV, as well as SCoV particles at the plasma membrane (Fig. 3). Western blot analysis demonstrated the presence of 3a protein in the purified SCoV (Fig. 4A). The species of 3a protein detected in the purified virion was not identical to that observed in the infected cells, indicating that 3a protein found in the purified SCoV was not an intracellular 3a protein contaminant (Fig. 4). The absence of actin and SCoV nsp1 proteins in the purified SCoV sample further confirmed that our virus purification method was appropriate (Fig. 4B). Based on these data we concluded that the 3a protein was a viral structural protein. A recent study revealed that a highly purified transmissible gastroenteritis coronavirus preparation is possible by the use of an immunopurification method (7). However, we were unable to use this new method to purify SCoV due to the lack of an appropriate monoclonal antibody. Analysis of highly purified SCoV samples by the immunopurification method may be useful to study the stoichiometric amount of 3a protein in the purified SCoV relative to other structural proteins.

Assembly of coronavirus S protein, E protein, and nucleocapsid is mediated by binding of these molecules to M protein in infected cells (4, 8, 10, 13, 18, 20, 26). Coimmunoprecipitation studies demonstrated that expressed 3a protein interacts with coexpressed SCoV M, E, and S proteins (27). A recent study using a crude SCoV preparation indicated the presence of interaction between 3a protein and S protein (33). Although these interactions have not been experimentally demonstrated in infected cells, the presence of 3a protein in SCoV indicates that the incorporation of 3a protein into SCoV particles was mediated by the interaction with viral envelope proteins at the virus budding sites. Further studies are required to determine whether association of 3a protein with SCoV particles has some role in SCoV assembly.

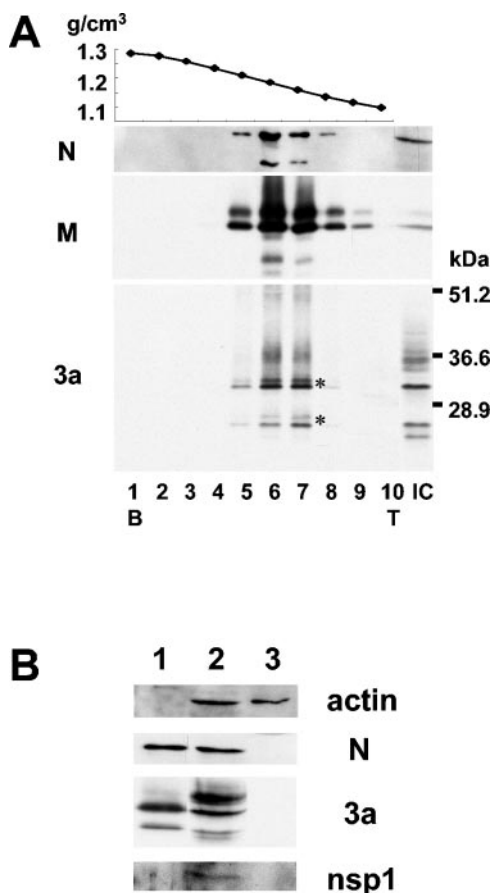


FIG. 4. Western blot analysis of N, M, and 3a proteins in purified SCoV. Caco2 cells were infected with SCoV at a multiplicity of infection of 1, and culture fluid was collected at 5 days p.i. Released SCoV was purified by sucrose gradient centrifugation as described in the text. (A) Ten fractions from a 20 to 60% sucrose gradient containing the virus particles were collected and numbered from bottom (B) to top (T) of the gradient. The top panel represents the density of each sucrose fraction. IC, intracellular proteins from SCoV-infected Caco2 cells. (B) Purified SCoV (lane 1), cell extracts from SCoV-infected Caco2 cells at 5 days p.i. (lane 2), and uninfected Caco2 cells at 5 days p.i. (lane 3) were analyzed for actin protein, N protein, 3a protein, and SCoV nsp1 protein.

3a protein carries a signal that may be important for rapid internalization of the protein from the plasma membrane (27). If 3a protein interacts with a host protein(s) in the plasma membrane, then there is a possibility that the complex of this putative host protein and 3a protein may be rapidly internalized. It will be interesting to test whether virion-associated 3a protein downregulates the expression of some of the mem-

FIG. 3. Immunogold labeling of 3a protein in SCoV-infected cells. Caco2 cells were infected with SCoV at a multiplicity of infection of 0.5, fixed at 48 h p.i., and embedded in LR White resin. Ultrathin sections of the cells were incubated with anti-3a antibody and goat anti-rabbit immunoglobulin G (heavy plus light) conjugated to 15-nm colloidal gold particles (Amersham Biosciences). (A) In a SCoV-infected cell the label is clearly associated with intracellular virus (v) and with the virions at the cell surface (arrows). An uninfected cell in the upper left corner is devoid of label. n, nucleus. Bar = 1 μ m. (B) 3a protein is associated with plasma membranes. V, SCoV particles associated with 3a protein. (C) Association of 3a protein with intracellular virus particles (arrows). (D) Portion of cytoplasm of a mock-infected Caco2 cell showing occasional staining of a few gold particles near the plasma membrane. Bars (B to D) = 0.5 μ m.

brane-associated host proteins to evade host immune responses and/or alter the cellular environment to one that is suitable for virus replication.

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