Monitoring of S Protein Maturation in the Endoplasmic Reticulum by Calnexin Is Important for the Infectivity of Severe Acute Respiratory Syndrome Coronavirus

Masaya Fukushi,^{a,b,c,d} Yoshiyuki Yoshinaka,^e Yusuke Matsuoka,^a Seisuke Hatakeyama,^a Yukihito Ishizaka,^f Teruo Kirikae,^a Takehiko Sasazuki,^g and Tohru Miyoshi-Akiyama^a

Department of Infectious Diseases, Research Institute, National Center for Global Health and Medicine, Tokyo, Japan^a; Disease Control and Prevention Center, National Center for Global Health and Medicine, Tokyo, Japan^b; Deputy Director-General's Laboratory, Research Institute, National Center for Global Health and Medicine, Tokyo, Japan^c; Department of Virology, Institute of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan^d; Department of Molecular Virology, Bio-Response, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan^e; Department of Intractable Diseases, Research Institute, National Center for Global Health and Medicine, Tokyo, Japan^f; and National Center for Global Health and Medicine, Tokyo, Japan^g

Severe acute respiratory syndrome coronavirus (SARS-CoV) is the etiological agent of SARS, a fatal pulmonary disorder with no effective treatment. We found that SARS-CoV spike glycoprotein (S protein), a key molecule for viral entry, binds to calnexin, a molecular chaperone in the endoplasmic reticulum (ER), but not to calreticulin, a homolog of calnexin. Calnexin bound to most truncated mutants of S protein, and S protein bound to all mutants of calnexin. Pseudotyped virus carrying S protein (S-pseudovirus) produced by human cells that were treated with small interfering RNA (siRNA) for calnexin expression (calnexin siRNA-treated cells) showed significantly lower infectivity than S-pseudoviruses produced by untreated and control siRNA-treated cells. S-pseudovirus produced by calnexin siRNA-treated cells contained S protein modified with N-glycan side chains differently from other two S proteins and consisted of two kinds of viral particles: those of normal density with little S protein and those of high density with abundant S protein. Treatment with peptide-N-glycosidase F (PNGase F), which removes all types of N-glycan side chains from glycoproteins, eliminated the infectivity of S-pseudovirus. S-pseudovirus and SARS-CoV produced in the presence of α -glucosidase inhibitors, which disrupt the interaction between calnexin and its substrates, showed significantly lower infectivity than each virus produced in the absence of those compounds. In S-pseudovirus, the incorporation of S protein into viral particles was obviously inhibited. In SARS-CoV, viral production was obviously inhibited. These findings demonstrated that calnexin strictly monitors the maturation of S protein by its direct binding, resulting in conferring infectivity on SARS-CoV.

Severe acute respiratory syndrome (SARS), a pulmonary infectious disease with significant morbidity and mortality, became epidemic worldwide, especially in China, Southeast Asia, and Canada, in 2002 to 2003 (5, 8). Its etiological agent was shown to be SARS coronavirus (SARS-CoV) (11, 23, 37), a new type of enveloped virus belonging to the *Coronaviridae* family, group 2, and containing a 29.7-kb, single-stranded, positive-sense RNA genome (30, 40). The SARS-CoV spike, located on the viral surface, is composed of a trimer of S glycoprotein (S protein) and is important for viral entry into target cells (28, 43, 47). S protein is synthesized as a 1,255-amino-acid precursor polypeptide, with glycosylation at 23 amino acids occurring in the endoplasmic reticulum (ER) and Golgi apparatus (13, 30, 40, 46). S protein can be proteolytically cleaved into noncovalently associated regions, i.e., the N-terminal half (S1 domain), which contains the receptor-binding domain (RBD) (residues 318 to 510), and the C-terminal half (S2 domain), which is responsible for fusion activity (3, 14, 26). The RBD binds to host angiotensin-converting enzyme 2 (ACE2), a homolog of ACE and a receptor for SARS-CoV (24, 27, 38, 45, 46). The host lectin L-SIGN also acts as a receptor for SARS-CoV infection by binding to another site distinct from the RBD (17, 22).

Calnexin is a transmembrane protein that functions as a molecular chaperone in the ER (2). Calnexin, along with its homologs calreticulin and Erp57, transiently binds to newly synthesized polypeptides containing monoglucosylated N-glycan side chains. Calnexin prevents the aggregation and premature degradation of

substrate proteins, ensuring their correct folding before these proteins continue along their intracellular trafficking pathways (16, 20, 34). The α -glycosidase inhibitors N-nonyl-deoxynojirimycin (NN-DNJ), N-butyl-deoxynojirimycin (NB-DNJ), and castanospermine, which target α -glycosidases I and II in the ER, disrupt the interaction of calnexin with its substrate proteins by interrupting glycan chain processing of immature glycoproteins (12, 31, 35, 41, 49).

We previously suggested that the N-terminal part of the S2 domain (S2-N) of S protein was important for SARS-CoV infection (42). We therefore attempted to identify the cellular proteins that bind to S2-N and examined the effect of this interaction on SARS-CoV infection.

MATERIALS AND METHODS

Cell lines and viruses. Wild-type and calnexin gene-deficient primary mouse embryonic fibroblasts (MEFs) (10) were kindly provided by Maurizio Molinari of the Institute of Research in Biomedicine (Bellinzona, Switzerland). Human embryonic kidney fibroblast (HEK293T) and Vero

Received 18 May 2012 Accepted 12 August 2012

Published ahead of print 22 August 2012

Address correspondence to Masaya Fukushi, mfukushi@hiroshima-u.ac.jp.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JVI.01250-12

E6 (African green monkey kidney) cell lines were kindly provided by Tohoku University. MEFs and both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin, and 50 µg/ml streptomycin. SARS-CoV, Frankfurt01 strain, was maintained in Vero E6 cells. All experiments using SARS-CoV were performed in biosafety level 3 (BSL3) level facilities.

Plasmids. A plasmid carrying the human codon-optimized S protein gene of SARS-CoV (47) was kindly provided by G. J. Nabel of the National Institutes of Health (Bethesda, MD). DNA fragments carrying S2-N and S protein mutant genes were amplified by PCR and introduced into TAG-Zyme pQE2 (Qiagen) for expression in *Escherichia coli* and into pcDNA6/Myc/His A (Invitrogen) for expression in mammalian cells. The full-length human *ace2* gene (27), kindly provided by H. Choe of Harvard Medical School (Boston, MA), was introduced into pcDNA3.1(+) (Invitrogen). Human calnexin gene DNA (44), the kind gift of I. Wada of Fukushima Medical University (Fukushima, Japan), was introduced into pQE2 and pSecTag2 (Invitrogen). The identity of each plasmid was confirmed by DNA sequencing.

Antibodies and α -glucosidase inhibitors. Rabbit polyclonal antibodies to S protein, the nucleocapsid protein of SARS-CoV, and calnexin were prepared in-house. Briefly, rabbits were immunized with each recombinant protein emulsified in Freund's adjuvant (Difco), and their sera were clarified on protein G-Sepharose (GE Healthcare). Monoclonal antibody to S protein (clone 5H10) was prepared in-house in KM mice, which produce human antibodies (21, 32). In brief, the spleen cells from KM mice immunized with recombinant S2-N protein were fused with mouse myeloma cell SP2/O-Ag14 cells, and the resulting hybridomas were screened for their ability to bind to recombinant S2-N protein by conventional enzyme-linked immunosorbent assays (21). Monoclonal antibody to S protein (Chemicon) was used for flow cytometry analysis. Polyclonal (Stressgen Bioreagents) and monoclonal (BD Biosciences) antibodies against calnexin were purchased, as were anticalreticulin (Calbiochem), antiactin (BD Biosciences), anti-c-myc (Sigma-Aldrich), and anti-p24 (Chemicon) antibodies. The ER α -glucosidase inhibitors *N*-nonyl-deoxynojirimycin (NN-DNJ), *N*-butyl-deoxynojirimycin (NB-DNJ), and castanospermine were purchased from Sigma-Aldrich, Inc., and Wako Pure Chemical Industries, Ltd.

In vitro pull-down assays. Vero E6 cells detached from plates with trypsin-EDTA were suspended in 20 mM HEPES solution, sonicated, and separated into pellet and supernatant (cytoplasmic) fractions by ultracentrifugation at 40,000 rpm for 1 h at 4°C. The pellet was resuspended in 20 mM HEPES–1% Triton X-100–1% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate} and again sonicated, followed by one freeze-thaw cycle and ultracentrifugation as described above, with the resultant supernatant defined as the membrane fraction. The cytoplasmic and membrane fractions were separately added to recombinant S2-N on *N*-hydroxysuccinimide (NHS)-activated Sepharose beads (GE Healthcare) or to beads alone as a negative control. The beads were washed 4 times with 20 mM HEPES–1% Triton X-100–1% CHAPS and suspended in buffer containing sodium dodecyl sulfate (SDS). After boiling, the samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the gels were stained with Coomassie brilliant blue. Specific proteins that bound to S2-N were excised from the gel and subjected to proteome analysis for identification.

Immunoprecipitation assays. HEK293T cells were transfected with plasmids carrying S protein and S protein mutant genes and cultured for 2 days in the presence or absence of α -glucosidase inhibitors. The cells were harvested with Nonidet P-40 (NP-40) lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP-40) containing a protease inhibitor cocktail (Sigma-Aldrich). The cell lysates were clarified by centrifugation at 12,000 rpm for 30 min at 4°C. The lysates were incubated with antibody to S protein or calnexin for 4 h at 4°C with rotation, followed by incubation with precleared protein G-Sepharose for 3 h at 4°C with rotation. The beads were washed 4 times with NP-40 lysis buffer, mixed with 2× SDS-

PAGE loading buffer, and boiled for 5 min, and the immunoprecipitated proteins were analyzed by Western blotting.

Enzyme-linked immunosorbent assays (ELISA). Recombinant S2-N was immobilized on microtiter plates for 1 h at room temperature. After three washes with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T) to remove free S2-N, SuperBlock blocking buffer (Thermo Fisher Scientific Pierce) was added to the wells, and the plates were incubated for 30 min at room temperature to block nonspecific binding. Following the addition of recombinant calnexin at the indicated concentrations, the plates were incubated for 1 h at room temperature, washed with PBS-T, and incubated sequentially with rabbit anticalnexin antibody and anti-rabbit-horseradish peroxidase (GE Healthcare) for 1 h each at room temperature. The wells were washed with PBS-T and incubated with 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase enzyme immunoassay (EIA) substrate (Bio-Rad) for 10 min at room temperature. After the addition of stop solution, the absorbance in each well was measured at 450 nm and at 620 nm as a reference using an Infinite 200 plate reader (Tecan).

Immunocytochemistry. Wild-type and calnexin gene-deficient MEFs transfected with a plasmid carrying the S protein gene were fixed in cold methanol, washed three times with PBS-T, and incubated with antibodies to S protein and calnexin for 1 h at 37°C. After three washes with PBS-T, the cells were incubated with anti-mouse IgG conjugated to Alexa Fluor 488 (Invitrogen) and anti-rabbit IgG conjugated to Alexa Fluor 568 (Invitrogen) for 1 h at 37°C. After three washes with PBS-T, the cells were mounted and viewed using an Olympus BX51 microscope and software DP controller to capture images (Olympus).

siRNA. Stealth Select RNA interfering with calnexin expression (CANX-HSS188689; CACCAGAACUCAACCUGGAUCAGUU) and Stealth RNA interference (RNAi) negative-control duplex were purchased from Invitrogen. A small interfering RNA (siRNA) with GC content identical to that of the calnexin siRNA was used as a negative control. HEK293T cells were mixed with each siRNA and Lipofectamine RNAiMAX (Invitrogen) and seeded onto poly-D-lysine plates (BD Biosciences). After incubation for 2 days, the cells were transfected with plasmids for S-pseudovirus production. Six hours later, the cells were washed and new DMEM was added to remove free S-pseudovirus plasmids. After further incubation for 2 days, the media containing S-pseudovirus were collected and filtered with a 0.45-µm filter to remove cell debris.

S-pseudovirus infection assay. Pseudotyped lentivirus carrying S protein (S-pseudovirus) was prepared in the presence or absence of α -glucosidase inhibitors, as described previously (15, 33, 43), and quantitated using a Retro-Tek HIV-1 p24 ELISA kit (ZeptoMetrix) in accordance with the manufacturer's instructions. S-pseudoviruses were normalized relative to the quantity of p24 protein. HEK293T cells transiently expressing ACE2 were added to the wells of 24 well poly-D-lysine plates and incubated with S-pseudovirus for 1 h at 37°C. Free S-pseudovirus was removed, culture medium was added, and the cells were incubated for 2 days. The cells were lysed with PicaGene reporter lysis buffer (Toyo B-Net) and centrifuged to remove debris. The supernatants were mixed with PicaGene (Toyo B-Net) substrate, and the luciferase activity in the cell lysates was measured using an Infinite 200 plate reader. Cells producing S-pseudoviruses were used in Western blot analysis and immunoprecipitation assays.

Ultracentrifugation, density gradient analysis, and enzymatic treatment. S-pseudovirus was purified by ultracentrifugation at 30,000 rpm for 1 h at 4°C in an SW55Ti rotor (Beckman Coulter). Concentrated S-pseudovirus was used in Western blot and density gradient analyses. Concentrated S-pseudovirus was treated with peptide-N-glycosidase F (PNGase F) (New England BioLabs), which removes all types of N-glycan chains from glycoprotein, or with endo- β -N-acetylglucosaminidase H (endo H) (New England BioLabs), which removes only high-mannose structures and hybrid structures from glycoproteins, in accordance with the manufacturer's instructions. For density gradient analysis, the concentrated S-pseudovirus was gently placed on the top of a 20 to 60% sucrose gradient and ultracentrifuged at 60,000 rpm for 3 h at 4°C in a

VTi65.2 rotor (Beckman Coulter) with weak acceleration and deceleration. Each sample was gently harvested into 16 tubes, each of which was again ultracentrifuged at 30,000 rpm for 1 h at 4°C in a TLA45 rotor (Beckman Coulter) to assess the infectivity of S-pseudovirus present in each fraction.

Infectivities of S-pseudoviruses treated with and without PNGase F.

S-pseudovirus (10 ng of p24 protein) was incubated in DMEM–10% FCS with and without 1 μ l (500 units) of glycerol-free PNGase F (New England BioLabs) for 0, 3, 6, 12, and 24 h, as described previously (4, 9, 17, 29). The S-pseudoviruses were purified by ultracentrifugation at 40,000 rpm for 1 h at 4°C in an SW55Ti rotor and suspended in 2 $\times$ SDS-PAGE loading buffer for Western blot analysis with anti-S protein and anti-p24 antibodies. To assess their infectivities, the S-pseudoviruses were used to infect ACE2-expressing HEK293T cells on 24-well poly-D-lysine plates for 1 h at 37°C, followed by washing with phosphate-buffered saline without Ca^{2+} and Mg^{2+} [PBS(–)] to remove free S-pseudoviruses. The cells were cultured for 2 days, harvested, and used for luciferase assays.

ER isolation. To assess whether S protein is present in ER treated or not treated with α -glucosidase inhibitors, ER was isolated using an endoplasmic reticulum isolation kit (Sigma) in accordance with the manufacturer's instructions. Briefly, HEK293T cells transfected with a plasmid carrying the S protein gene were cultured for 2 days in DMEM–10%FCS containing α -glycosidase inhibitors. The cells were detached and washed with PBS(–) several times. After incubation in hypotonic extraction buffer, the cells were suspended in isotonic extraction buffer and homogenized using a 27-gauge needle. Each homogenate was clarified by centrifugation at 3,500 rpm for 10 min at 4°C and at 16,000 rpm for 15 min at 4°C. The clarified supernatant was centrifuged at 45,000 rpm for 1 h at 4°C, with the pellet used as an ER sample; its identity was determined by Western blot analysis with antibodies against the ER-specific proteins calreticulin and calnexin.

Flow cytometry. HEK293T cells were transfected with a plasmid carrying the S protein gene, washed with PBS(–), and cultured for 2 days with DMEM–10%FCS containing α -glycosidase inhibitors. The harvested cells were treated sequentially with mouse anti-S protein antibody and anti-mouse antibody conjugated to fluorescein isothiocyanate (FITC) and were subsequently analyzed with a Cytomics FC500 (Beckman Coulter).

SARS-CoV plaque-forming assays. Vero E6 cells were infected with SARS-CoV at a multiplicity of infection (MOI) of 0.5 for 30 min, washed with PBS(–), and incubated for 16 h in DMEM containing α -glucosidase inhibitors at the indicated concentrations. The culture media were harvested and used as virus stock solutions. To determine the infectivity of SARS-CoV, the virus was adsorbed onto a Vero E6 monolayer for 1 h at 37°C. The cells were subsequently overlaid with 1% methylcellulose-containing medium, incubated for 6 to 7 days at 37°C, and stained with 2.5% crystal violet solution in 30% ethanol and 1% ammonium oxalate. After drying, the numbers of virus plaques were counted. Vero E6 cell lysates were analyzed by Western blotting to assess the expression of viral proteins. To characterize SARS-CoV produced in the presence of NN-DNJ, polyethylene glycol 8000 was added to the virus stock solution to a final concentration of 7%, and the samples were centrifuged at 4,000 rpm for 30 min at 4°C. Each pellet was suspended in PBS(–), centrifuged at 15,000 rpm for 5 h at 4°C, and suspended in 2 $\times$ SDS-PAGE loading buffer for Western blot analysis.

RESULTS

Specific binding of S protein to calnexin. To identify the cellular molecules that bind to the N-terminal part of the S2 domain of SARS-CoV S protein (residues 620 to 900, referred to as S2-N [see Fig. 2C]), we performed *in vitro* pulldown assays using lysates of Vero E6 cells, which are susceptible to SARS-CoV infection. We found that S2-N specifically bound to several cellular proteins, including calnexin, keratin 8, cytokeratin, and actin 5 (Fig. 1A), with the calnexin band signal on SDS-PAGE being the strongest.

In immunoprecipitation assays, full-length S protein bound to endogenous calnexin (Fig. 1B and C), but not to its homolog calreticulin (Fig. 1D), in HEK293T cells. The association between S2-N and calnexin was direct and dose dependent (Fig. 1E). Surface plasmon response analysis also showed that their association was direct and dose dependent in our preliminary experiment. Full-length S protein colocalized with endogenous calnexin in wild type MEFs (Fig. 1F, upper panels), but S protein localization did not differ significantly in calnexin gene-deficient MEFs (Fig. 1F, lower panels). To determine the regions of S protein and calnexin responsible for their binding, we performed immunoprecipitation assays using truncated mutants of both proteins. Full-length calnexin bound to all truncated S proteins except for the C-terminal region of the S2 domain (Fig. 2A to C), whereas full-length S protein bound to all truncated forms of calnexin (Fig. 2D and E).

Decrease of S-pseudovirus infectivity by calnexin siRNA. To determine whether the binding of S protein to calnexin is important for viral infectivity, we attempted to generate pseudotyped virus carrying S protein (S-pseudovirus) in the absence of calnexin. Since the amount of S-pseudovirus produced by genetically calnexin-disrupted MEFs was insufficient for the infection assay (data not shown), we attempted to produce S-pseudovirus in cells treated with calnexin-specific siRNA. We found that calnexin siRNA effectively inhibited the expression of endogenous calnexin in HEK293T cells (Fig. 3A). S-pseudovirus produced in calnexin siRNA-treated cells was significantly less infectious than S-pseudoviruses from untreated and control siRNA-treated cells, depending on ACE2 expression (Fig. 3B). To determine the mechanism underlying this decrease in infectivity, S-pseudoviruses concentrated by ultracentrifugation were analyzed by electrophoresis. A single S protein band was observed when S-pseudoviruses were produced by untreated and control siRNA-treated cells (Fig. 3C, lanes –), whereas multiple S protein bands, with higher mobility, were observed when S-pseudovirus was produced by calnexin siRNA-treated cells. To determine whether these differences in the number and mobility of bands were due to protein truncation or sugar modification, concentrated S-pseudoviruses were treated with PNGase F, which removes all types of N-glycan chains from glycoproteins, and endo H, which removes only high-mannose structures and hybrid structures from glycoproteins. The mobilities of three S proteins treated with PNGase F were the same (Fig. 3C, upper panel, lanes +), indicating that the S polypeptide produced by calnexin siRNA-treated cells is not a truncated form. In addition, the intensity of the band signal of S protein produced by calnexin siRNA-treated cells after PNGase F treatment was obviously greater than those produced by untreated and control siRNA-treated cells, although the same amount of virus particles, relative to p24 protein (Fig. 3C, lower panel), was loaded for electrophoresis. It is suggested that a large amount of S protein was incorporated into S-pseudovirus produced by calnexin siRNA-treated cells compared to those produced by untreated and control siRNA-treated cells. Following endo H treatment, the mobility gaps of S protein from untreated and control siRNA-treated cells were smaller than those of S protein treated with PNGase F (Fig. 3C, upper and middle panels), suggesting that both S proteins have a larger number of complex N-glycan chains than hybrid and high-mannose N-glycan chains, consistent with previous results (39). In contrast, the band patterns of S protein from calnexin siRNA-treated cells after PNGase F or endo H treatments

FIG 1 Specific binding between S protein of SARS-CoV and calnexin. (A) *In vitro* pulldown assay with S2-N. Recombinant S2-N covalently conjugated to NHS-Sepharose (+) or NHS-Sepharose alone (-) was mixed separately with the cytoplasmic and membrane fractions of Vero E6 cells. Specific bands on SDS-PAGE were identified by proteome analysis. The arrowhead indicates calnexin. (B and C) Immunoprecipitation assays using HEK293T cell lysates containing full-length S protein and endogenous calnexin. +, cell lysates were mixed with antibody to S protein (B) or calnexin (C). -, protein G-Sepharose without antibody. (D) Immunoprecipitation assays using HEK293T cell lysates containing full-length S protein and endogenous calnexin. Cell lysates were mixed with antibody to S protein (+). -, protein G-Sepharose without antibody. (E) Direct dose-dependent association between recombinant S2-N and recombinant calnexin. Immobilized S2-N was incubated with the indicated concentrations of calnexin, and bound calnexin was measured by ELISA. Each assay was performed in triplicate; results are presented as means $\pm$ standard deviations (SD). (F) Localization of S protein and endogenous calnexin. Wild-type (upper panels) and calnexin gene-deficient (lower panels) MEFs transfected with the S protein gene were stained with antibodies to S protein (green) and calnexin (red). Magnification, $\times 600$.

were similar to each other, suggesting that S protein from calnexin siRNA-treated cells contains more hybrid and high-mannose N-glycan chains than S proteins from untreated and control siRNA-treated cells.

S-pseudoviruses were also analyzed by density gradient ultracentrifugation. We found that the S protein and p24 protein peaks were in the same fraction (fraction 11) in gradients of S-pseudoviruses from untreated and control siRNA-treated cells (Fig. 3D) but were located in different fractions (fractions 13 and 11, respectively) in gradients of S-pseudovirus from calnexin siRNA-treated cells (Fig. 3D). When normalized relative to p24 protein in each fraction (fractions 10 to 13), the S-pseudovirus from calnexin siRNA-treated cells was significantly less infectious than the other S-pseudoviruses (Fig. 3E).

Decrease of S-pseudovirus infectivity by PNGase F treatment. To determine whether the glycosylation of S protein affects the infectivity of S-pseudovirus, S-pseudoviruses were incubated with PNGase F for various times. In the absence of PNGase F, the mobility of S proteins remained constant for 24 h (Fig. 4A, lanes -), whereas in the presence of PNGase F, the mobility of S proteins increased (Fig. 4A, lanes +). The infectivities of both S-pseudoviruses treated with or without PNGase F gradually decreased in a time-dependent manner, but this reduction was significantly greater for PNGase F treatment than that for treatment without

PNGase F (Fig. 4B). The decline in S-pseudovirus infectivity was also dependent on the concentration of PNGase F (data not shown).

Decrease of S-pseudovirus infectivity by α -glucosidase inhibitors. To assess S-pseudovirus infectivity following disruption of the interaction between S protein and calnexin, S-pseudoviruses were generated in HEK293T cells in the presence of the α -glucosidase inhibitors NN-DNJ, NB-DNJ, and castanospermine, all of which inhibit the interactions of calnexin with its substrates in the ER by interrupting glycan chain processing of immature glycoproteins (12, 31, 35, 41, 49). We found that each α -glucosidase inhibitor significantly and dose dependently reduced the infectivity of S-pseudovirus (Fig. 5A). Analysis of concentrated S-pseudoviruses showed that each α -glucosidase inhibitor significantly and dose dependently reduced the incorporation of S protein into S-pseudoviruses (Fig. 5B). Although these inhibitors did not significantly alter S protein expression in the cells, the upper band of the S protein doublet was dose dependently reduced and the lower band was shifted upward (Fig. 5C). Immunoprecipitation assays using whole-cell lysates revealed that calnexin bound to the lower, but not to the upper, S protein band (Fig. 5D). Since the amounts of precipitated S protein were not reduced, S protein in the ER and on the cell surface was analyzed. We found that each α -glucosidase inhibitor dose dependently re-

FIG 2 Binding of mutants of S protein and calnexin. (A and B) Lysates of HEK293T cells transfected with plasmids carrying truncated S protein mutant genes were used for immunoprecipitation assays with anticalexin antibody. (C) Schematic depiction showing S protein and the results of the immunoprecipitation assays with truncated S protein mutants and endogenous calnexin. The S2-N region (residues 620 to 900) and the RBD (residues 318 to 510) are shown as gray and hatched bars, respectively. Black bars show the putative N-glycosylation sites in the S1 and S2 domains. TM, transmembrane region. +, S protein mutants bound to calnexin. –, the S2-C mutant did not bind to calnexin. (D) Lysates of HEK293T cells transfected with plasmids carrying full-length S protein and truncated calnexin mutant genes were used for immunoprecipitation assays with anti-S protein antibody. (E) Schematic depiction showing the results of the immunoprecipitation assays with truncated calnexin mutants and full-length S protein. +, calnexin mutants bound to full-length S protein.

duced the amount of S protein in the ER (Fig. 5E), with flow cytometry analysis showing that each inhibitor dose dependently decreased S protein expression at the cell surface (Fig. 5F).

Reduction of SARS-CoV infection by α -glucosidase inhibitors. The effects of α -glucosidase inhibitors on SARS-CoV infection were determined using plaque-forming assays. SARS-CoV generated from Vero E6 cells in the presence of NN-DNJ was significantly and dose dependently less infectious than virus generated in the absence of NN-DNJ (Fig. 6A and B). Although NB-DNJ and castanospermine did not significantly reduce the numbers of plaques, these plaques were smaller than those observed in the absence of these inhibitors (data not shown). Western blotting showed that NN-DNJ dramatically and dose dependently reduced the amounts of S protein and nucleoprotein (Fig. 6C), indicating a decrease of SARS-CoV production. We found in Western blot analysis using Vero E6 cell lysates that NN-DNJ dose dependently shifted the mobility of S protein upward, similar to the case for the S protein of S-pseudovirus produced by HEK293T cells in the presence of α -glucosidase inhibitors (Fig. 6D).

DISCUSSION

We have shown here that the binding of SARS-CoV S protein to the cellular molecular chaperone calnexin plays a critical role in SARS-CoV infection. Calnexin strictly managed the folding of glycosylated S protein in viral production, with the result that daughter viruses acquired the infectious ability.

The interaction between S protein and calnexin was analyzed here, because calnexin provided the strongest signal in our pull-down assays using S2-N. These pulldown assays, however, showed that S2-N also bound to other cellular proteins containing actin. Recently, palmitoylated calnexin, which forms a supercomplex with the ribosome-translocon complex, was reported to interact with actin (25), suggesting that S protein may indirectly interact with actin through calnexin. Calnexin bound to all truncated S proteins except for S2-C, consistent with a previous report that 23 putative N-linked glycosylation sites are scattered throughout S protein (34, 40, 48). Calnexin associates with monoglycosylated N-glycan chains on polypeptides/proteins. The presence of many glycosylation sites in S protein is also consistent with our Western blot results with cell lysates, showing that S protein is present as two major bands with smearing. This result is consistent with a previous report using a full glycan analysis (39).

We found in the present study that S-pseudovirus produced by calnexin knockdown cells was significantly less infectious than S-pseudoviruses produced by untreated and control siRNA-treated cells. The S protein in S-pseudovirus produced by calnexin knockdown cells differed in quality and quantity from the S proteins in the other two S-pseudoviruses produced by untreated and control siRNA-treated cells. In the absence of both PNGase F and endo H, the S protein from calnexin knockdown cells consisted of multiple bands with smearing, whereas the S proteins from untreated and control siRNA-treated cells were each single bands.

FIG 3 Infectivity of S-pseudovirus produced by HEK293T cells treated with calnexin siRNA. (A) Expression of endogenous calnexin in HEK293T cells treated with calnexin and control siRNAs or without any siRNA (untreated). (B) Luciferase assay of the infectivity of S-pseudoviruses. S-pseudoviruses (10 ng of p24 protein) produced by HEK293T cells treated with the indicated siRNAs were used to infect cells transfected with *ace2*-carrying (black bars) or empty (white bars) plasmids. Assays were performed in triplicate; results are presented as means $\pm$ SD. ***, $P < 0.001$. (C) S-pseudovirus (100 ng of p24 protein) was concentrated by ultracentrifugation and was incubated with (+) or without (–) the enzymes PNGase F and endo H, followed by Western blot analysis. (D) Sucrose density gradient analysis of concentrated S-pseudoviruses. Concentrated S-pseudovirus (180 ng of p24 protein) was layered onto a 20 to 60% sucrose gradient and ultracentrifuged. The sample was divided into 16 aliquots. Each aliquot was again ultracentrifuged and analyzed by Western blotting. The fraction numbers are indicated. (E) Each S-pseudovirus (2 ng of p24 protein) in fractions 10 to 13 infected ACE2-expressing cells (12×10^4 cells/well). Black bars, hatched bars, and white bars indicate the S-pseudovirus from untreated cells, control siRNA-treated cells, and calnexin siRNA-treated cells, respectively. The luciferase activity of each cell lysate was measured. Assays were performed in quadruplicate, and results are presented as means $\pm$ SD. ***, $P < 0.001$.

However, with PNGase F treatment, the band mobilities of those three S proteins were identical, indicating that the S protein from calnexin knockdown cells differed in posttranslational modification rather than truncation. In addition, following PNGase F treatment, the band signal of S protein from calnexin knockdown cells, relative to p24 protein, was stronger than those from untreated and control siRNA-treated cells, suggesting that excess amounts of S proteins were incorporated into the former virus particles. Furthermore, when the S-pseudoviruses produced by untreated and control siRNA-treated cells were digested by PNGase F or endo H, the band mobilities of these S proteins differed. This result suggested that the majority of sugar chains with which the S proteins are modified are complex N-glycan, which is consistent with a previous report (39). Sucrose density gradient analysis showed that S-pseudovirus produced by calnexin knockdown cells consisted of highly dense particles with abundant S protein and normally dense particles with little S protein. These results indicated that in the absence of calnexin, excessive amounts of improperly glycosylated S protein are incorporated into virus particles, resulting in the latter being more dense and noninfectious. Based on these results, it is speculated that a decrease of calnexin led to an impairment of surveillance capability

in the ER quality control, with the result that the S protein with improper glycosylation is not displaced from the ER. This is consistent with the observation that the secretion of immature glycoprotein is enhanced in calnexin-deficient *Saccharomyces cerevisiae* (1, 36). In the process of maturation of newly synthesized proteins, posttranslational modification with N-glycan is closely involved in protein folding, which is intrinsically difficult and error prone (18–20). Therefore, S protein from calnexin knockdown cells would be misfolded because calnexin is one of the central players in the ER quality control.

Removal of N-glycan chains from correctly matured S protein by PNGase F treatment diminished the infectivity of S-pseudovirus, indicating that the glycosylation of S protein is critical for the infectivity of S-pseudovirus not only during but also after the process of viral production. Since the RBD in S protein is required for binding to ACE2 in SARS-CoV infection (45), the PNGase F-induced decrease in S-pseudovirus infectivity may be due to conformational changes of the RBD itself or to masking of the RBD by conformational changes of the entire S protein resulting from the removal of N-glycan chains.

α -Glucosidase inhibitors disrupt the interactions between calnexin and its substrates in the ER by interrupting α -glucosidase I

FIG 4 Infectivities of S-pseudoviruses treated with or without PNGase F. (A) S-pseudovirus was incubated with (+) or without (-) PNGase F for the indicated periods of time and analyzed by Western blotting with antibodies to S protein and p24 protein. (B) S-pseudovirus (10 ng of p24 protein) incubated in the presence (triangles) or absence (circles) of PNGase F for the indicated periods of time infected ACE2-expressing cells (12×10^4 cells/well). The luciferase activity of each cell lysate was measured. Assays were performed in quadruplicate, and results are presented as means $\pm$ SD. ***, $P < 0.001$.

and II (12, 31, 35, 41, 49). However, we found here that calnexin immunoprecipitated with S protein in the presence of α -glucosidase inhibitors. As mentioned above, there are several N-glycosylation sites in S protein (40). Therefore, calnexin can bind to S

protein that possesses at least one monoglucosylated N-glycan despite of the presence of α -glucosidase inhibitors. α -Glucosidase inhibitors affected the amount of S protein in S-pseudovirus, in the ER, and on the cell surface but not in cell lysates. S protein, which shifted upward on electrophoresis, was precipitated with calnexin, suggesting that calnexin binds to S protein possessing monoglucosylated N-glycans and monitors S protein folding. This monitoring would remove improperly glycosylated and incorrectly folded S proteins, as reported previously (6). Therefore, the amounts of S protein in the ER and at the cell surface would be reduced in the presence of α -glucosidase inhibitors. SARS-CoV was significantly less infectious when produced in Vero E6 cells in the presence than in the absence of NN-DNJ, due primarily to a decrease in viral production. The upward shift of S protein in these cell lysates was similar to that of S-pseudovirus in α -glucosidase inhibitor-treated HEK293 cells, suggesting that the decrease in SARS-CoV infectivity may be due to the virus containing less S protein in addition to decreased viral production.

Although S-pseudoviruses produced by both calnexin siRNA- and α -glucosidase inhibitor-treated cells had lower infectivity, the mechanisms underlying these decreases differed. In the absence of calnexin, incorrectly glycosylated S protein, which is likely misfolded, remained in the ER, resulting in the incorporation of non-functional S protein into daughter virions. However, in the presence of α -glucosidase inhibitors, incorrectly glycosylated S protein is sorted by calnexin and sent for ER-associated degradation (ERAD), resulting in the production of daughter virions without S protein.

Protein glycosylation is performed in a step-by-step manner (20). Nascent polypeptide is cotranslationally transferred to the ER, along with the large oligosaccharide $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$, by oligosaccharyltransferase. ER α -glucosidases I and II, the targets

FIG 5 Infectivities of S-pseudoviruses produced by HEK293T cells treated with α -glucosidase inhibitors. (A) S-pseudovirus (10 ng of p24 protein) produced by HEK293T cells in the presence of the indicated concentrations ($\mu\text{g/ml}$) of NN-DNJ, NB-DNJ, and castanospermine infected ACE2-expressing cells (12×10^4 cells/well), and the luciferase activity of the cell lysate was measured. Assays were performed in triplicate, and results are presented as means $\pm$ SD. ***, $P < 0.001$. (B) S-pseudovirus produced in the presence of α -glucosidase inhibitors was concentrated by ultracentrifugation and analyzed by Western blotting. (C) S protein in cells producing S-pseudovirus in the presence of each α -glucosidase inhibitor was analyzed by Western blotting. (D) Binding of S protein and calnexin in the presence of each α -glucosidase inhibitor was examined by immunoprecipitation assays with anticalnexin antibody. (E) S protein in the ER in the presence of each α -glucosidase inhibitor was analyzed by Western blotting. (F) S protein expression at the cell surface in the presence of each concentration of α -glucosidase inhibitor was analyzed by flow cytometry. The number 1, 2, and 3 in the concentration column represent 0, 1, and 10 $\mu\text{g/ml}$, respectively, of NN-DNJ and 0, 10, and 100 $\mu\text{g/ml}$, respectively, of both NB-DNJ and castanospermine.

FIG 6 Effect of NN-DNJ on SARS-CoV infection. (A) Plaque-forming assay using SARS-CoV produced in Vero E6 cells in the presence of the indicated concentrations of NN-DNJ. The cells were overlaid with methylcellulose-containing medium, incubated for 6 to 7 days, and stained with crystal violet solution. Plaques formed by 10^6 -fold dilutions of each virus are shown. (B) Number of SARS-CoV plaques produced in the presence of 3 and 30 µg/ml NN-DNJ relative to those produced in the absence of NN-DNJ. Assays were performed in triplicate; results are presented as means $\pm$ SD. ***, $P < 0.001$. (C) Western blot analysis of the amounts of S protein and nucleocapsid in concentrated SARS-CoV produced in the presence of the indicated concentrations of NN-DNJ. (D) Western blot analysis of Vero E6 cell lysates in the presence of the indicated concentrations of NN-DNJ.

of the α -glucosidase inhibitors, remove terminal glucose residues stepwise from N-glycan chains attached to nascent glycoproteins. Disappearance of the upper band in the S protein doublet and upward shifting of the lower band were dependent on the concentration of α -glucosidase inhibitors. The decreased incorporation of S protein into virus particles was also dependent on the concentration of α -glucosidase inhibitors. These findings suggested that the upper band in the S protein doublet may be mature S protein with infectious ability, whereas the lower band may be an intermediate form of S protein. Moreover, in the presence of α -glucosidase inhibitors, the amounts of bi- and triglycosylated S protein should increase, reducing its mobility and showing an upward shift, similar to the mobility shifts of hepatitis C virus gp E1 and E2 (7). α -Glucosidase inhibitors have been found to disrupt infection by several enveloped viruses, including dengue virus, human hepatitis C virus, and mouse hepatitis virus (12). The present study

showed that α -glucosidase inhibitors also decreased infection by SARS-CoV. Viral entry into target cells is one of the most important steps in virus propagation. Therefore, the association between S protein and calnexin may be a new target in the treatment of SARS. Future studies of α -glucosidase inhibitors or their derivatives may lead to the development of anti-SARS-CoV drugs.

ACKNOWLEDGMENTS

We thank Gary J. Nabel, Hyeryun Choe, and Ikuo Wada for providing plasmids carrying the spike, ACE2, and calnexin genes, respectively, and Maurizio Molinari for providing wild-type and calnexin gene-deficient MEFs. We also thank Makoto Yamashita and Masahiro Fujii for valuable advice and Sanshiro Hanada, Kenji Yamamoto, and Takemasa Sakaguchi for general help.

T.S. was supported by grants from the National Institute of Biomedical Innovation (grant 04-02) and the Japan Science and Technology Agency (JST).

REFERENCES

1. Arima H, Kinoshita T, Ibrahim HR, Azakami H, Kato A. 1998. Enhanced secretion of hydrophobic peptide fused lysozyme by the introduction of N-glycosylation signal and the disruption of calnexin gene in *Saccharomyces cerevisiae*. *FEBS Lett.* 440:89–92.
2. Bedard K, Szabo E, Michalak M, Opas M. 2005. Cellular functions of endoplasmic reticulum chaperones calreticulin, calnexin, and ERp57. *Int. Rev. Cytol.* 245:91–121.
3. Bergeron E, et al. 2005. Implication of proprotein convertases in the processing and spread of severe acute respiratory syndrome coronavirus. *Biochem. Biophys. Res. Commun.* 326:554–563.
4. Beyene A, Basu A, Meyer K, Ray R. 2004. Influence of N-linked glycans on intracellular transport of hepatitis C virus E1 chimeric glycoprotein and its role in pseudotype virus infectivity. *Virology* 324:273–285.
5. Cameron MJ, Bermejo-Martin JF, Danesh A, Muller MP, Kelvin DJ. 2008. Human immunopathogenesis of severe acute respiratory syndrome (SARS). *Virus Res.* 133:13–19.
6. Caramelo JJ, Parodi AJ. 2008. Getting in and out from calnexin/calreticulin cycles. *J. Biol. Chem.* 283:10221–10225.
7. Chapel C, et al. 2006. Antiviral effect of alpha-glucosidase inhibitors on viral morphogenesis and binding properties of hepatitis C virus-like particles. *J. Gen. Virol.* 87:861–871.
8. Chen J, Subbarao K. 2007. The immunobiology of SARS. *Annu. Rev. Immunol.* 25:443–472.
9. Chu VC, Whittaker GR. 2004. Influenza virus entry and infection require host cell N-linked glycoprotein. *Proc. Natl. Acad. Sci. U. S. A.* 101:18153–18158.
10. Denzel A, et al. 2002. Early postnatal death and motor disorders in mice congenitally deficient in calnexin expression. *Mol. Cell. Biol.* 22:7398–7404.
11. Drosten C, et al. 2003. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N. Engl. J. Med.* 348:1967–1976.
12. Dwek RA, Butters TD, Platt FM, Zitzmann N. 2002. Targeting glycosylation as a therapeutic approach. *Nat. Rev. Drug Discov.* 1:65–75.
13. Eickmann M, et al. 2003. Phylogeny of the SARS coronavirus. *Science* 302:1504–1505.
14. Follis KE, York J, Nunberg JH. 2006. Furin cleavage of the SARS coronavirus spike glycoprotein enhances cell-cell fusion but does not affect virion entry. *Virology* 350:358–369.
15. Haga S, et al. 2008. Modulation of TNF- α -converting enzyme by the spike protein of SARS-CoV and ACE2 induces TNF- α production and facilitates viral entry. *Proc. Natl. Acad. Sci. U. S. A.* 105:7809–7814.
16. Hammond C, Helenius A. 1994. Quality control in the secretory pathway: retention of a misfolded viral membrane glycoprotein involves cycling between the ER, intermediate compartment, and Golgi apparatus. *J. Cell Biol.* 126:41–52.
17. Han DP, Lohani M, Cho MW. 2007. Specific asparagine-linked glycosylation sites are critical for DC-SIGN- and L-SIGN-mediated severe acute respiratory syndrome coronavirus entry. *J. Virol.* 81:12029–12039.
18. Hebert DN, Molinari M. 2007. In and out of the ER: protein folding, quality control, degradation, and related human diseases. *Physiol. Rev.* 87:1377–1408.

19. Helenius A. 1994. How N-linked oligosaccharides affect glycoprotein folding in the endoplasmic reticulum. *Mol. Biol. Cell* 5:253–265.
20. Helenius A, Aebi M. 2004. Roles of N-linked glycans in the endoplasmic reticulum. *Annu. Rev. Biochem.* 73:1019–1049.
21. Ishida I, et al. 2002. Production of human monoclonal and polyclonal antibodies in TransChromo animals. *Cloning Stem Cells* 4:91–102.
22. Jeffers SA, et al. 2004. CD209L (L-SIGN) is a receptor for severe acute respiratory syndrome coronavirus. *Proc. Natl. Acad. Sci. U. S. A.* 101: 15748–15753.
23. Ksiazek TG, et al. 2003. A novel coronavirus associated with severe acute respiratory syndrome. *N. Engl. J. Med.* 348:1953–1966.
24. Kuba K, et al. 2005. A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus-induced lung injury. *Nat. Med.* 11:875–879.
25. Lakkaraju AK, et al. 2012. Palmitoylated calnexin is a key component of the ribosome-translocon complex. *EMBO J.* 31:1823–1835.
26. Li F, Li W, Farzan M, Harrison SC. 2005. Structure of SARS coronavirus spike receptor-binding domain complexed with receptor. *Science* 309: 1864–1868.
27. Li W, et al. 2003. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* 426:450–454.
28. Liu S, et al. 2004. Interaction between heptad repeat 1 and 2 regions in spike protein of SARS-associated coronavirus: implications for virus fusogenic mechanism and identification of fusion inhibitors. *Lancet* 363: 938–947.
29. Maginnis MS, Haley SA, Gee GV, Atwood WJ. 2010. Role of N-linked glycosylation of the 5-HT2A receptor in JC virus infection. *J. Virol.* 84: 9677–9684.
30. Marra MA, et al. 2003. The Genome sequence of the SARS-associated coronavirus. *Science* 300:1399–1404.
31. Mehta A, Zitzmann N, Rudd PM, Block TM, Dwek RA. 1998. Alpha-glucosidase inhibitors as potential broad based anti-viral agents. *FEBS Lett.* 430:17–22.
32. Miyoshi-Akiyama T, et al. 2011. Fully human monoclonal antibody directed to proteolytic cleavage site in severe acute respiratory syndrome (SARS) coronavirus S protein neutralizes the virus in a rhesus macaque SARS model. *J. Infect. Dis.* 203:1574–1581.
33. Moore MJ, et al. 2004. Retroviruses pseudotyped with the severe acute respiratory syndrome coronavirus spike protein efficiently infect cells expressing angiotensin-converting enzyme 2. *J. Virol.* 78:10628–10635.
34. Moremen KW, Tiemeyer M, Nairn AV. 2012. Vertebrate protein glycosylation: diversity, synthesis and function. *Nat. Rev. Mol. Cell Biol.* 13: 448–462.
35. Oda Y, Hosokawa N, Wada I, Nagata K. 2003. EDEM as an acceptor of terminally misfolded glycoproteins released from calnexin. *Science* 299: 1394–1397.
36. Parlati F, Dominguez M, Bergeron JJ, Thomas DY. 1995. *Saccharomyces cerevisiae* CNE1 encodes an endoplasmic reticulum (ER) membrane protein with sequence similarity to calnexin and calreticulin and functions as a constituent of the ER quality control apparatus. *J. Biol. Chem.* 270: 244–253.
37. Peiris JS, et al. 2003. Coronavirus as a possible cause of severe acute respiratory syndrome. *Lancet* 361:1319–1325.
38. Prabakaran P, Xiao X, Dimitrov DS. 2004. A model of the ACE2 structure and function as a SARS-CoV receptor. *Biochem. Biophys. Res. Commun.* 314:235–241.
39. Ritchie G, et al. 2010. Identification of N-linked carbohydrates from severe acute respiratory syndrome (SARS) spike glycoprotein. *Virology* 399:257–269.
40. Rota PA, et al. 2003. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. *Science* 300:1394–1399.
41. Sayce AC, Miller JL, Zitzmann N. 2010. Targeting a host process as an antiviral approach against dengue virus. *Trends Microbiol.* 18:323–330.
42. Shichijo S, et al. 2004. Assessment of synthetic peptides of severe acute respiratory syndrome coronavirus recognized by long-lasting immunity. *Tissue Antigens* 64:600–607.
43. Simmons G, et al. 2004. Characterization of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) spike glycoprotein-mediated viral entry. *Proc. Natl. Acad. Sci. U. S. A.* 101:4240–4245.
44. Wada I, et al. 1991. SSR alpha and associated calnexin are major calcium binding proteins of the endoplasmic reticulum membrane. *J. Biol. Chem.* 266:19599–19610.
45. Wong SK, Li W, Moore MJ, Choe H, Farzan M. 2004. A 193-amino acid fragment of the SARS coronavirus S protein efficiently binds angiotensin-converting enzyme 2. *J. Biol. Chem.* 279:3197–3201.
46. Xiao X, Chakraborti S, Dimitrov AS, Gramatikoff K, Dimitrov DS. 2003. The SARS-CoV S glycoprotein: expression and functional characterization. *Biochem. Biophys. Res. Commun.* 312:1159–1164.
47. Yang ZY, et al. 2004. pH-dependent entry of severe acute respiratory syndrome coronavirus is mediated by the spike glycoprotein and enhanced by dendritic cell transfer through DC-SIGN. *J. Virol.* 78:5642–5650.
48. Zhou Y, et al. 2010. A single asparagine-linked glycosylation site of the severe acute respiratory syndrome coronavirus spike glycoprotein facilitates inhibition by mannose-binding lectin through multiple mechanisms. *J. Virol.* 84:8753–8764.
49. Zitzmann N, et al. 1999. Imino sugars inhibit the formation and secretion of bovine viral diarrhea virus, a pestivirus model of hepatitis C virus: implications for the development of broad spectrum anti-hepatitis virus agents. *Proc. Natl. Acad. Sci. U. S. A.* 96:11878–11882.