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The Nucleocapsid Protein of Severe Acute Respiratory Syndrome Coronavirus Inhibits Cell Cytokinesis and Proliferation by Interacting with Translation Elongation Factor 1α[▽]

Bing Zhou,† Junli Liu,† Qiuna Wang, Xuan Liu, Xiaorong Li, Ping Li, Qingjun Ma, and Cheng Cao*

State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Biotechnology, Beijing 100071, China

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Severe acute respiratory syndrome coronavirus (SARS-CoV) is the etiological agent of SARS, an emerging disease characterized by atypical pneumonia. Using a yeast two-hybrid screen with the nucleocapsid (N) protein of SARS-CoV as a bait, the C terminus (amino acids 251 to 422) of the N protein was found to interact with human elongation factor 1-alpha (EF1α), an essential component of the translational machinery with an important role in cytokinesis, promoting the bundling of filamentous actin (F-actin). In vitro and in vivo interaction was then confirmed by immuno-coprecipitation, far-Western blotting, and surface plasmon resonance. It was demonstrated that the N protein of SARS-CoV induces aggregation of EF1α, inhibiting protein translation and cytokinesis by blocking F-actin bundling. Proliferation of human peripheral blood lymphocytes and other human cell lines was significantly inhibited by the infection of recombinant retrovirus expressing SARS-CoV N protein.

Coronaviruses (CoVs) are etiological agents of a number of respiratory and enteric diseases in humans and animals. The CoVs are a diverse group of enveloped viruses whose single-stranded positive-sense RNA genomes are among the largest of the RNA viruses, about 30,000 nucleotides. Among the three groups of CoVs, human CoVs (HCoVs) are found in both group 1 (HCoV-229E) and group 2 (HCoV-OC43) and are often associated with mild respiratory illnesses, including the common cold (21, 28).

Severe acute respiratory syndrome (SARS), which is caused by SARS-CoV infection, was first reported in Guangdong Province, China, in 2002 (11, 26, 36, 47). There were 8,000 cases reported from 30 countries, with a 5 to 8% mortality rate through May 2003 (http://www.who.int/csr/sars/country/table2003_09_23/en/print.html). SARS-CoV is an enveloped virus with a single strand, positive-sense RNA genome containing 11 major open reading frames. These open reading frames encode the replicase polyprotein, the spike protein, the membrane protein, the small envelope protein, and the nucleocapsid (N) protein. DNA sequence analysis suggests that SARS-CoV is distinct from all the other CoVs (32, 37). While the viral pathogenesis and vaccine development are being aggressively pursued, it is unclear why the virus infection is so severe, leading to respiratory failure with often fatal consequences, compared to the relatively minor pathogenesis from other HCoV infections.

CoV N proteins are typically 350 to 450 amino acids (aa) in length, extensively serine phosphorylated, highly basic, and associated with viral RNA to form a long, flexible, helical ribonucleoprotein (31). In addition to the structural role of N protein, additional functions include viral RNA packaging, viral RNA transcription, translation, and virus budding (1, 28, 41). The N protein of SARS-CoV contains 422 aa residues, sharing only 20 to 30% homology with the N proteins of other CoVs (32, 37). The N-terminal region (from aa 49 to 178) of the SARS-CoV N protein may contain the RNA binding domain, and the C terminus (from aa 213 to 422) may be responsible for self-association of the protein (23, 40, 46). The N protein is one of the most abundant structural proteins during SARS-CoV infection and is highly phosphorylated. It can also induce strong humoral and cellular immune responses, making it a potential vaccine candidate (25).

Human elongation factor 1 α (EF1α) is a major translation factor in mammalian cells. In its GTP-bound form, EF1α escorts aminoacyl-tRNA to ribosomes. Once associated with the ribosome, EF1α hydrolyzes GTP, dissociates from the aminoacyl-tRNA, and leaves the ribosome (33). EF1α is not only a major translation factor but also one of the most important multifunctional proteins, having roles in the quality surveillance of newly synthesized proteins (22), in ubiquitin-dependent degradation (7, 16), and in facilitating apoptosis (29). EF1α also has unconventional functions related to its association with the cytoskeleton. As the second-most abundant eukaryotic protein after actin (13), EF1α interacts with filamentous actin (F-actin) and promotes F-actin bundling (17, 27, 45), and it is considered an essential component for the formation of a contractile ring during cytokinesis (34, 35).

EF1α has been observed to bind to several viral proteins. The NS5A protein of bovine viral diarrhea virus interacts with EF1α, which may play a role in bovine viral diarrhea virus replication (24). Human immunodeficiency virus type 1 Gag polyprotein, which has key functions at nearly all stages of the viral life cycle, interacts with EF1α through tRNA, impairing translation in vitro and releasing viral RNA from polysomes, thereby permitting the RNA to be packaged into nascent virions (8).

* Corresponding author. Mailing address: Beijing Institute of Biotechnology, P.O. Box 130(8), 27 Taiping Rd., Beijing 100850, China. Phone: 86 10 6815 5151. Fax: 86 10 63853882. E-mail: caoc@nic.bmi.ac.cn.

† B.Z. and J.L. contributed equally to this work.

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In this report, we demonstrate that N protein of SARS-CoV associates with EF1α directly and induces EF1α aggregation. Because protein translation and cytokinesis were blocked by the expression of N protein, cell proliferation was inhibited.

MATERIALS AND METHODS

Yeast two-hybrid screen. Yeast two-hybrid screening was performed by using the Gal4-based Matchmaker Two-Hybrid System 3 kit according to the manufacturer's protocol (Clontech). Briefly, the plasmid pGBKT7-N was constructed encoding the full-length N gene fused in frame with the GAL4 DNA binding domain (bait) by inserting the PCR-generated fragment into the EcoRI and BamHI sites. pGBKT7-N was used to transform the *Saccharomyces cerevisiae* strain AH109 by the lithium acetate procedure. The strain containing pGBKT7-N was further transformed with a fetal liver cDNA library cloned in fusion with the GAL4 activation domain using the pACT2 vector (Clontech). Transformants expressing both the bait and interacting prey proteins were selected on synthetic dropout (SD) medium lacking Leu, Trp, and His (SD-Leu$^-$ Trp$^-$ His$^-$) and incubated at 30°C for 8 to 10 days and then replated on SD-Leu$^-$ Trp$^-$ His$^-$ Ade$^-$ medium containing X-α-Gal (5-bromo-4-chloro-3-indolyl-α-galactopyranoside) to verify the activation of all the reporter genes (ADE2, HIS3, and LacZ). The plasmids in positive blue colonies were isolated by transformation into *Escherichia coli*. The interaction was then confirmed in yeast. A prey vector containing simian virus 40 large T-antigen (pGADT7-T) and a bait vector containing murine p53 (pGBKT7-53) were used as a positive control (Clontech). pGADT7-T and a bait vector containing human lamin C (pGBKT7-Lam) were used as a negative control (Clontech).

Cell culture. 293T, HeLa, and MCF-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. K562 leukemia cells were grown in RPMI 1640 medium (Invitrogen) with the same supplements. Human lymphocytes were grown in lymphocyte medium (Takala) with supplementation of 500 units/ml interleukin-2, 50 ng/ml anti-CD3 antibody (CIMAB S.A., Havana, Cuba), and 5% heat-inactivated FBS.

Transfection. Mammalian cells (2×10^6 per 28-cm^2 dish) were grown in 60-mm cell culture dishes and transfected with 4 μg of plasmid DNA and 10 μl of Lipofectamine 2000 (Invitrogen) in Opti-MEM (Invitrogen) for 6 h, which was then replaced with normal growth medium (DMEM–10% FBS).

Vectors and epitope tagging of proteins. Procedures for recombinant DNA techniques were either standard (39), or the process was performed according to the manufacturer's instructions. The N gene (GenBank accession number AY274119) was amplified by reverse transcription-PCR from the SARS-CoV RNA of patient serum samples (upstream primer, CGGAATTCCATATGTCT GATAATGGACCCCAA; downstream primer, CGGGATCCTTATGCCTGA GTTGAATCAGC). The amplified product was then purified with a MiniElute PCR Purification kit (Qiagen). The purified product was subsequently digested with NdeI and BamHI and then ligated with the pET22b vector (Novagen) digested with the same restriction enzymes. The N gene was further cloned into BamHI and EcoRI sites of pcDNA3-based Flag vector (Invitrogen), pQCXIP (Clontech), and pGEX-4T-2 (Amersham/Pharmacia) and into the BglII and EcoRI sites of pEGFPC1 (Clontech). The EF1α gene was amplified from the human fetal liver cDNA library (Clontech) by using primers P1 (CGCGGATC CATGGGAAAGGAAAAGACTCA) and P2 (GGAATTCATTTAGCCTTCT GAGCT) and was cloned into the pcDNA3-based Flag vector in BamHI and EcoRI sites, as mentioned above. To generate a Myc-tagged clone, the EF1α gene was cloned into the EcoRI and BglII sites of pMyc-CMV (where CMV is cytomegalovirus) (Clontech). To determine the specific domain of the N protein that interacts with EF1α, several truncated N gene fragments were cloned in frame into the BglII and EcoR1 sites of pEGFPC1 (Clontech).

Immunoprecipitation and immunoblot analysis. Lysates from 2×10^6 cells were prepared in lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol [DTT], 10 mM sodium fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A) containing 1% Nonidet P-40. Soluble proteins were subjected to immunoprecipitation with anti-Flag M2 agarose (Sigma). The adsorbates were washed with lysis buffer and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto an Immobilon-P transfer membrane (Millipore) in 39 mM glycine, 48 mM Tris base, 0.01% SDS, and 20% methanol for 1 h at room temperature. The membrane was blocked with 5% milk–phosphate-buffered saline (PBS)–0.1% Tween 20 for 1.5 h at room temperature. Primary antibody was diluted in PBS–0.1% Tween 20 and incubated with the membrane for 1 h at room temperature. Secondary antibody incubation was performed in blocking solution for 1 h at room temperature. An aliquot of the total lysate (5%, vol/vol) was included as a control. Immunoblot analysis was performed with horseradish peroxidase (HRP)-conjugated anti-Flag (Sigma), anti-EF1α (UpState Biotechnology), anti-β-actin (Sigma), anti-green fluorescent protein (GFP) (Clontech), HRP-conjugated anti-Myc (Santa Cruz Biotechnology), anti-heat shock protein 70 (HSP70) (Sigma), and goat anti-mouse immunoglobulin G (IgG) (Amersham/Pharmacia) antibodies. The antigen-antibody complexes were visualized by chemiluminescence (PerkinElmer Life Sciences).

Protein binding assays. Glutathione *S*-transferase (GST) fusion proteins were generated by expression in pGEX-4T-2-based vectors (Amersham/Pharmacia) in *E. coli* BL21(DE3) (Novagen). The lysates from 2×10^6 cells were incubated for 2 h at 4°C with 2 μg of purified GST or GST fusion proteins bound to glutathione-conjugated agarose beads (Amersham/Pharmacia). The adsorbates were washed with lysis buffer and then subjected to SDS-PAGE and immunoblot analysis. An aliquot of the total lysate (5%, vol/vol) was included as a loading control on the SDS-PAGE gel.

In direct binding assays (Far Western), anti-Flag immunoprecipitates prepared from 2×10^6 293T cells transfected with Flag-EF1α were separated by SDS-PAGE and then blotted onto polyvinylidene difluoride (PVDF) membranes. Membranes were subsequently incubated with purified GST-N fusion proteins for 2 h at room temperature. The GST fusion proteins binding to PVDF membranes were probed with anti-GST antibody (Santa Cruz Biotechnology) and revealed by chemiluminescence.

Surface plasmon resonance. Recombinant N protein of SARS-CoV or 229E-CoV was coupled to a CM5 carboxymethyl dextran sensor chip (Amersham/Pharmacia). Binding assays were performed using a Biacore 2000 instrument (BIAcore AB, Uppsala, Sweden). Purified EF1α diluted in HBS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20) was applied to the chip at a flow rate of 10 ml/min at 35°C. BIAevaluation software, version 3.0, was used to assess binding kinetics.

In vitro translation assay. Recombinant N protein or GST was incubated with 10 μl of rabbit reticulocyte lysate (RRL; Promega), and after 1 h, 200 ng of firefly luciferase-encoding mRNA and amino acids were added to the mixture and incubated for another 1.5 h at 30°C, followed by a luciferase activity assay. In addition, a 1 μM concentration of purified EF1α protein was added to the preincubation mixtures of 10 μl of RRL and 0.4 μM N protein and subjected to an in vitro translation system with 200 ng of luciferase mRNA. Luciferase activity was measured with a TD-20/20 spectrophotometer (Promega). To further assess the effect of N protein on translation, 10^6 293T cells were washed by methionine-free medium and lysed by freezing and thawing, and the supernatants were treated with 2 μg/ml α-amanitin (Sigma) for 1 h at 37°C to block transcription. The supernatants were then incubated with or without 1 μM SARS-CoV N protein for 1 h before the methionine-free amino acid mixture supplemented with 1 μCi of [^{35}S]methionine (Amersham/Pharmacia) and 1 mM Mg^{2+} was added and incubated for another 1.5 h. Protein in the lysate was precipitated by 13% trichloroacetic acid (TCA) and subjected to liquid scintillation counting for quantitation.

Fluorescence microscopy. Cells were rapidly rinsed three times with PBS (10 mM sodium phosphate, pH 7.4, 150 mM NaCl) and incubated with 10 μg/ml Hoechst 33342 stain (Sigma) for 30 min. Cells were then fixed for 30 min at room temperature in 4% formaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature, and rinsed three times in PBS. Filamentous actin was stained by incubating samples with Texas Red-phalloidin (1:40) (Invitrogen) for 30 min at room temperature. Fluorescence images were acquired using a Zeiss LSM 510 Meta confocal imaging system.

Cell cycle synchronization and flow cytometry. A total of 2×10^5 293T cells were synchronized at G_0/G_1 with 400 μM mimosine (Sigma) treatment for 22 h, released by washing with DMEM, and then incubated for the period indicated in the figures. Cells were collected, washed with PBS three times, and fixed in 70% alcohol at −20°C overnight. The fixed cells were centrifuged, washed with PBS three times, digested with 20 μg/ml RNase for 30 min at room temperature, and then stained with 100 μg/ml propidium iodide in the dark for 30 min. Propidium iodide intensity was analyzed by flow cytometry (Beckman Coulter). Data from 10,000 cells were collected.

Cell proliferation assay. Cell proliferation was monitored by counting viable cells with a Cell Counting Kit-8 (Dojindo, Japan). At the time points indicated in the figures, cells were plated in 96-well plates, 10 μl of cholecystokinin octapeptide was added to each well, and the plate was incubated for 4 h at 37°C. The absorbance at 490 nm was measured with a Bio-Rad 680 microplate reader.

Preparation of retrovirus expressing N protein. 293T cells were plated on a 60-mm plate at 60 to 80% confluence (1×10^6 to 2×10^6 cells/60-mm plate) 12 h before transfection. Cells were cotransfected with 5 μg of the N protein-express-

ing retroviral vectors pQCXIP-N, pCG-VSV-G (where VSV is vesicular stomatitis virus), and pCG-gag-pol. Supernatant was harvested 48 h after transfection and then filtered through a 0.45-μm-pore-size filter (Millipore). Retrovirus was concentrated by polyethylene glycol precipitation (4). Viral titers were estimated by transfecting a known number of NIH/3T3 cells with different concentrations of retrovirus supernatant volumes according to recommended procedures (9). K562 cells and human lymphocytes were infected by the virus at a multiplicity of infection of 50 in the presence of 8 μg/ml polybrene (Sigma).

Purification of SARS-CoV N protein. pET22b-N was transformed into the expression host strain, BL21(DE3). The protein was expressed after induction with 0.2 mM isopropyl-β-D-thiogalactopyranoside. Cells were harvested by centrifugation, resuspended in buffer A (25 mM sodium phosphate [pH 8.0], 1 mM EDTA, 1 mM DTT), and sonicated. Soluble N protein in the lysate was purified by ion exchange chromatography with SP-Sepharose Fast Flow (25 mM sodium phosphate [pH 8.0], 1 mM EDTA, 1 mM DTT, 0.35 to 0.5 M NaCl) (Amersham/Pharmacia) and then by Superdex 200 gel filtration (Amersham/Pharmacia).

Purification of human EF1α. A total of 2×10^8 293T cells were harvested by centrifugation, resuspended in buffer A (25 mM sodium phosphate [pH 7.4], 1 mM EDTA, 1 mM DTT) and sonicated. Endogenous EF1α protein was purified by ion exchange chromatography with SP-Sepharose Fast Flow (Amersham/Pharmacia) (25 mM sodium phosphate [pH 7.4], 1 mM EDTA, 1 mM DTT, 0.55 to 0.6 M NaCl) and then further purified by gel filtration with Superdex 200 (Amersham/Pharmacia). The purified proteins were analyzed by SDS-PAGE and Western blotting.

RESULTS

N protein of SARS-CoV associates with EF1α. During the development of a DNA vaccine against SARS-CoV, we found that cytokinesis was blocked by the expression of the N protein of SARS-CoV in several cell lines. In addition, we also noted pathogenesis at the site of the intramuscular injection of mice following administration of an N protein expression plasmid. To address the molecular mechanisms of these phenomena, we evaluated potential protein-protein associations by yeast two-hybrid screening of the N protein of SARS-CoV with human proteins. The *S. cerevisiae* AH109 strain harboring pGBKT7-N and pACT2-EF1α(291-463) grew well on SD-Leu$^-$ Trp$^-$ His$^-$ Ade$^-$–X-α-Gal plates and yielded blue colonies.(Fig. 1A, left). As controls, transformants containing pGADT7-T and pGBKT7-p53 grew well with blue colonies because the large T-antigen of simian virus 40 interacts with p53, but the transformants containing pGBKT7-N or pGADT7-T and pGBKT7-Lam grew poorly in the same plate (Fig. 1A, lower right). These data suggest that a C-terminal domain of the human translation EF1α (aa 291 to 463) interacts with the N protein.

To delineate the molecular interaction between the N protein of SARS-CoV and EF1α, 293T cells were cotransfected with plasmids expressing Flag-tagged EF1α and GFP-tagged N protein. Cell lysates were immunoprecipitated with anti-Flag and probed with anti-GFP on immunoblots. An association between Flag-EF1α and GFP-N, but not GFP, was observed. Moreover, GFP-N does not bind Flag-conjugated beads in the absence of EF1α (Fig. 1B). The interaction between N protein and endogenous human EF1α was also revealed by immunoprecipitation of the Flag-N cell lysates with anti-Flag and subsequent immunoblotting with anti-EF1α. Again, endogenous EF1α does not bind Flag-conjugated beads in the absence of N protein (Fig. 1C). Furthermore, the C-terminal fragments (aa 208 to 422 and 251 to 422) of the N protein, but not the N-terminal part (aa 1 to 168 or 1 to 207), associate with EF1α (Fig. 1D). Taken together, these data indicate that a C-terminal region of the N protein of SARS-CoV interacts with human EF1α.

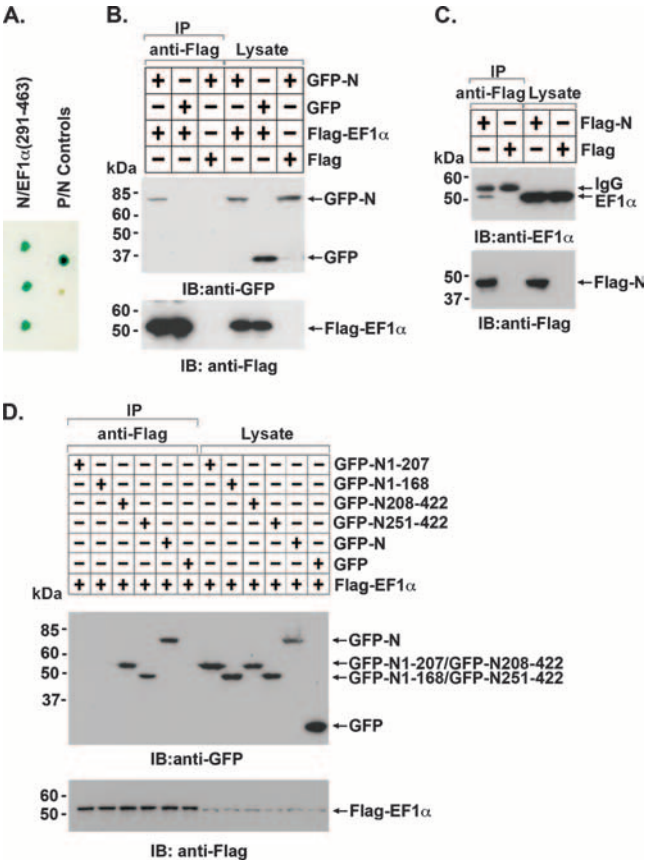

FIG. 1. N protein of SARS-CoV associates with EF1α. (A) Interaction of N protein with EF1α was revealed by a yeast two-hybrid system. Three transformants containing pGBKT7-N and pACT2-EF1α(291-463) (left panel) together with both positive (upper right) and negative (lower right) controls were inoculated on SD-Leu$^-$ Trp$^-$ His$^-$ Ade$^-$–X-α-Gal plates. Transformants containing pGADT7-T and pGBKT7-p53 were used as a positive control, and transformants containing pGADT7-T and a pGBKT7-Lam were employed as a negative control. (B) Lysates from 293T cells transfected with Flag-EF1α or Flag and GFP-N or GFP plasmids were subjected to immunoprecipitation with anti-Flag antibody and analyzed by immunoblotting with anti-GFP or anti-Flag antibody. (C) Lysates from 293T cells transfected with Flag-N or Flag plasmid were subjected to immunoprecipitation with anti-Flag and analyzed by immunoblotting with anti-EF1α antibody. (D) Lysates from 293T cells transfected with GFP-N or the indicated GFP-N truncation forms and Flag-EF1α were subjected to immunoprecipitation with anti-Flag and subsequently analyzed by immunoblotting with anti-GFP or anti-Flag antibody. IP, immunoprecipitation; IB, immunoblotting; P/N, positive/negative.

N protein of SARS-CoV binds EF1α directly. To rule out any indirect binding mediated by other components in the cell lysate, we tested for direct protein interactions. Lysates of 293T cells or 293T cells expressing Flag-EF1α were incubated with GST-N or GST proteins conjugated to agarose beads. Analysis of the adsorbates by immunoblotting with anti-EF1α or anti-Flag showed that the N protein interacts with EF1α (Fig. 2A). Also, anti-Flag immunoprecipitates prepared from cells expressing Flag-EF1α were subjected to SDS-PAGE and then transferred to a PVDF membrane. After incubation with

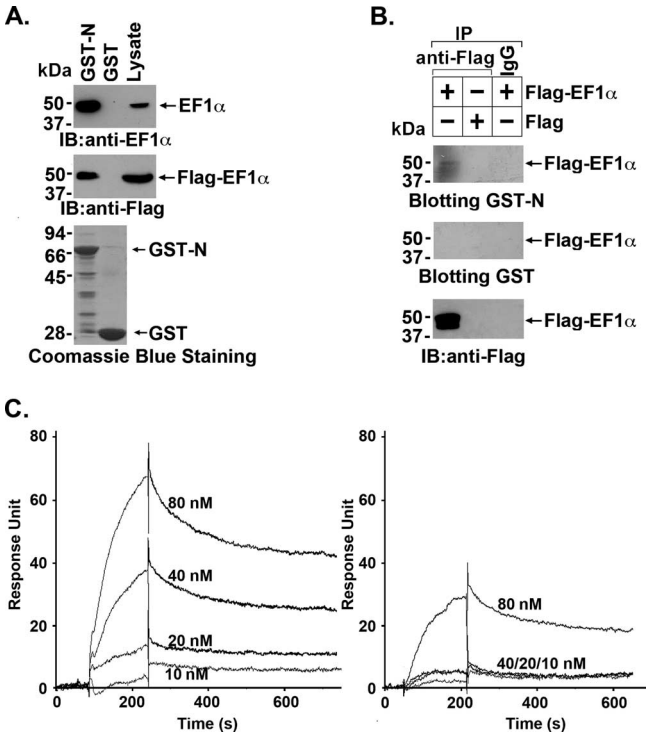

FIG. 2. SARS-CoV N protein binds directly with EF1α. (A) Lysates from 293T cells were incubated with GST or a GST-N fusion protein for 2 h. The adsorbates were analyzed by immunoblotting with anti-EF1α. Loading of the GST proteins was assessed by Coomassie blue staining. Lysates of 293T cells expressing Flag-EF1α were subjected to the same assay (middle panel). (B) Anti-Flag or IgG immunoprecipitates prepared from cells expressing Flag-EF1α or vectors were blotted onto a PVDF membrane. The PVDF membrane was incubated with GST-N (upper panel) or GST (middle panel) for 2 h, washed, and then incubated with HRP-conjugated anti-GST or anti-Flag antibody for 2 h; the antigen-antibody complexes were subsequently visualized by chemiluminescence. (C) The kinetics of EF1α with N protein of SARS-CoV (left) or 229E-CoV (right) was assessed by surface plasmon resonance. IP, immunoprecipitation; IB, immunoblotting.

soluble GST-N or GST proteins, the membrane was next treated with an anti-GST antibody. The results showed that EF1α binds to the N protein of SARS-CoV but not to GST as a control (Fig. 2B). Moreover, GST-N does not bind to IgG (Fig. 2B). Further, we assessed the direct interaction by surface plasmon resonance, and the results showed that EF1α binds to the N protein of SARS-CoV with much higher affinity than to the N protein of a much more weakly pathogenic 229E-CoV (3) (Fig. 2C). These data collectively demonstrate that N protein interacts with EF1α directly.

N protein of SARS-CoV induces aggregation of EF1α. Given that the N protein C-terminal region is involved in self-association, we therefore investigated whether N protein induces aggregation of EF1α. 293T cells were cotransfected with Flag-EF1α and GFP-N or GFP plasmids. Analysis of the anti-Flag immunoprecipitates by nonreducing SDS-PAGE and Coomassie blue staining showed that several proteins coprecipitated with EF1α in the presence of GFP-N but not GFP (Fig. 3A). The bands were excised, trypsin digested, and subjected to peptide fingerprint analysis by mass spectrometry. Most of the proteins coprecipitating with Flag-EF1α in the presence of GFP-N were found to be EF1α, indicating the presence of an aggregated form of EF1α. Interestingly, HSP70, which predominantly acts as a chaperone and preferentially binds to denatured or aggregated proteins, coprecipitated with Flag-EF1α in the presence of GFP-N (Fig. 3A). To substantiate the findings, 293T cells were transfected with Flag-N, Flag carrying aa 1 to 207 of the N protein [Flag-N(1-207)], Flag-N(208-422), or Flag plasmids. Analysis of the lysates by nonreducing SDS-PAGE and Western blotting revealed that aggregation of EF1α occurred in the cells expressing Flag-N or Flag-N(208-422) that bind to EF1α (Fig. 3B). Moreover, 293T cells were cotransfected with Myc-EF1α, Flag-EF1α, and GFP-N or, as a control, with GFP plasmids. Myc-EF1α was found in Flag-EF1α immunoprecipitates only when GFP-N was coexpressed (Fig. 3C). Further, purified EF1α was incubated with purified recombinant N protein or an equal amount of bovine serum albumin in PBS buffer at 4°C for 6 h and then subjected to ultracentrifugation. As shown in Fig. 3D, EF1α was detectable in the precipitates only in the presence of recombinant N protein. Importantly, more HSP70 was found in Flag-EF1α immunoprecipitates when N protein of SARS-CoV was coexpressed in the cells (Fig. 3E). These results collectively indicate that the N protein of SARS-CoV induces aggregation of EF1α.

N protein inhibits protein translation. In concert with the findings that N protein of SARS-CoV induces aggregation of EF1α, a 50% inhibition of a marker protein (luciferase) in in vitro translation was observed by the addition of recombinant N protein to a final concentration of 0.2 μM, while 99% inhibition was obtained at 0.6 μM. As a control, little change was found when GST protein was added (Fig. 4A). Then we examined whether exogenous EF1α would overcome the translation inhibition caused by N protein. The results from the luciferase assay showed that translation inhibition caused by N protein was significantly relieved by the addition of 1 μM EF1α (Fig. 4B). To confirm that the translation inhibition of N protein was not due to the interaction between N protein and luciferase mRNA, 10- and 50-fold amounts of mRNA were added to the in vitro translation system, and little if any effect was found on the translation efficiency (Fig. 4B, right two columns). In addition to the effects on specific reporter proteins, we also evaluated the effect of N protein on total protein synthesis. 293T cell lysates were incubated with 2 μg/ml α-amanitin (Sigma) for 1 h at 37°C to block transcription. The lysates were then incubated with or without 1 μM SARS-CoV N protein for 1 h before a methionine-free amino acid mixture supplemented with 1 μCi of [^{35}S]methionine (Amersham/Pharmacia) and 1 mM Mg^{2+} was added, and incubation continued another 1.5 h. Protein in the lysate was then precipitated by 13% TCA, and the radioactivity was assayed by liquid scintillation counting. As shown in Fig. 4C, a 60% inhibition of protein synthesis was observed by N protein (Fig. 4C). To further assess the effect of the N protein on translation in vivo, 293T cells were cotransfected with a luciferase reporter plasmid and Flag-N or vector. Luciferase activity in the cells expressing Flag-N was also substantially inhibited compared to the cells transfected with vector (Fig. 4D), while no obvious change in the luciferase mRNA level was noted (data not shown). To determine the total protein translation inhibition by N protein in cells, 293T cells were transfected with Flag-N

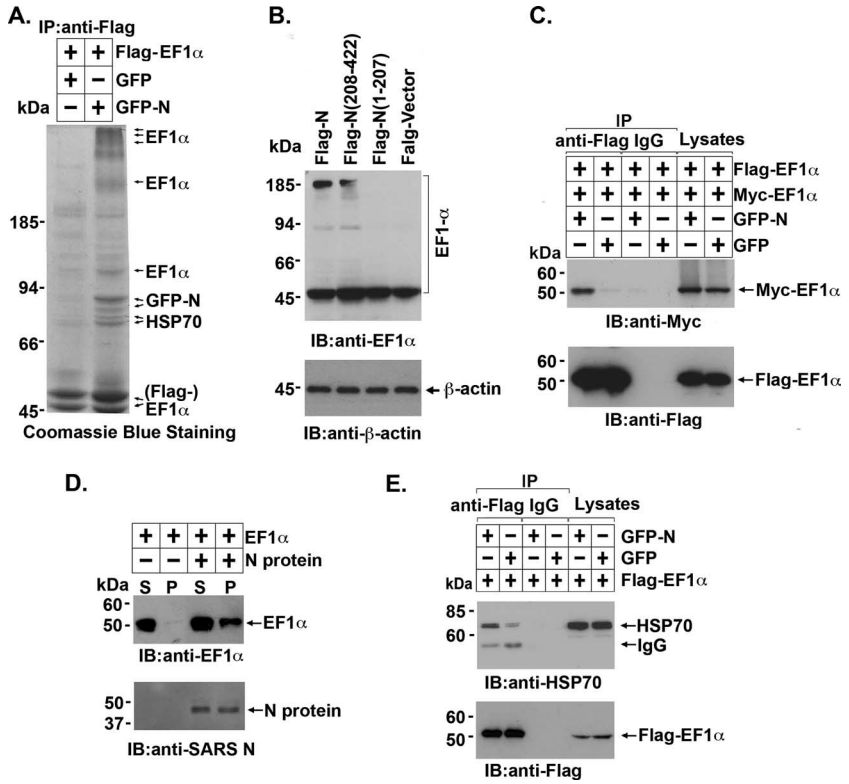

FIG. 3. N protein of SARS-CoV induces aggregation of EF1α. (A) Lysates of 293T cells expressing Flag-EF1α and GFP-N or GFP were subjected to immunoprecipitation with anti-Flag, fractionated by nonreducing SDS-PAGE without boiling, and stained with Coomassie blue. The indicated bands were excised, digested by trypsin, and subjected to a peptide fingerprint assay by matrix-assisted laser desorption ionization mass spectrometry. (B) Lysates from 293T cells transfected with Flag-N, Flag-N(208-422), Flag-N(1-207), or Flag vector were subjected to SDS-PAGE on a nonreducing gel and analyzed by immunoblotting with anti-EF1α or anti-β-actin. (C) Lysates from 293T cells transfected with Flag-EF1α and Myc-EF1α and GFP-N or GFP vector were subjected to immunoprecipitation with anti-Flag; immunoprecipitates were then analyzed by reducing SDS-PAGE and immunoblotting with anti-Myc or anti-Flag antibody. (D) EF1α (1 μM) was incubated with N protein (1 μM) or bovine serum albumin (1 μM) for 6 h at 4°C and then centrifuged at 130,000 × *g* for 1 h. Pellets (P) were analyzed by immunoblotting with anti-EF1α. The preultracentrifugation mixture was loaded as a control(s). (E) Lysates of 293T cells expressing Flag-EF1α and GFP-N or GFP were subjected to immunoprecipitation with anti-Flag, and immunoprecipitates were subsequently analyzed by immunoblotting with HSP70 antibody. IP, immunoprecipitation; IB, immunoblotting.

or Flag plasmids. After 48 h, cells were incubated with DMEM containing [^{35}S]methionine for 1 h in the presence of 2 μg/ml α-amanitin; protein synthesis was inhibited by about 60% (Fig. 4E). Therefore, protein translation was significantly inhibited by the N protein of SARS-CoV in vitro as well as in the cells.

N protein inhibits cytokinesis. A total of 70 to 90% of cellular EF1α is bound to F-actin (12) and promotes F-actin bundling (27, 45). To assess whether the aggregation of EF1α by the N protein of SARS-CoV affects F-actin bundles and cytokinesis, HeLa cells or 293T cells were transfected with the GFP-N plasmid and stained with Texas Red-X–phalloidin, which binds to F-actin selectively, at the time points indicated in the figure. As shown in Fig. 5A and B (upper panel), significantly fewer F-actin bundles were observed in the cells expressing high levels of GFP-N (Fig. 5B, stars) or Flag-N (data not shown), compared to the GFP or untransfected cells. In addition, we found that GFP-N or Flag-N fusion proteins were distributed mainly in the cytoplasm and that cytokinesis was inhibited significantly 48 h after transfection (Fig. 5A and B, 48 h); nearly 100% of N protein cells appeared to be multinucleated cells 72 h after transfection (Fig. 5B and C). As expected, few F-actin bundles accumulated in the N protein-containing cells (Fig. 5B, 48 or 72 h after infection). Similar results were also obtained with MCF-7 cells (data not shown). In concert with the findings that aa 251 to 422 of N [N(251-422)] bind directly with EF1α, expression of GFP-N(208-422) or GFP-N(251-422) but not GFP-N(1-207) or GFP led to the formation of multinucleated cells (Fig. 5C, lower four panels). Further, when peripheral blood lymphocytes (PBLs) were infected with retrovirus expressing SARS-CoV N protein, a high percentage of multinucleated cells was also observed (Fig. 5D), while little if any effect was observed in the cells that were infected with the control retrovirus not expressing the N protein. Moreover, the N protein of 229E-CoV interacted with EF1α at a significantly lower affinity (Fig. 2C) and induced the formation of multinucleate cells only when the transfected cells were cultured for more than 5 days in the presence of G418 for selecting N protein-expressing cells (data not shown). Further, when cells expressing GFP-N and DsRed-N were mixed and cultured for 36 h, no multinucleated cells expressing both GFP-N and DsRed-N were observed although green or red multinucleated cells were formed (data not shown). This experiment indicates that the multinucleate cells were not

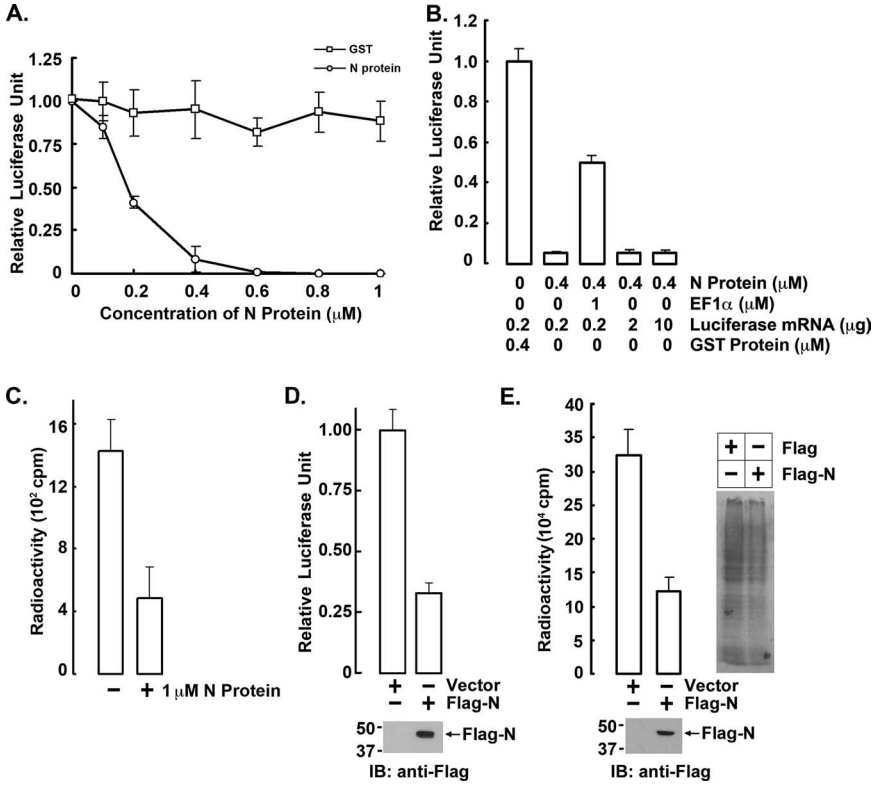

FIG. 4. Protein translation was inhibited by N protein of SARS-CoV. (A) N protein of SARS-CoV or GST was incubated with RRL for 1 h; luciferase-encoding mRNA was then added to the reaction mixture with an amino acid mixture and then incubated for another 1.5 h. The luciferase activity was measured, and the mean and standard deviations of three independent experiments were determined. (B) N protein or GST protein, EF1α protein, and luciferase mRNA at the indicated concentrations were added to the RRL reaction mixture for a translation reaction, followed by a luciferase activity assay. (C) 293T cells were washed five times with PBS, and 1 μM N protein of SARS-CoV was added to the cell lysates and then incubated with amino acids supplemented with [^{35}S]methionine in the presence of the transcription inhibitor α-amanitin. The protein produced was precipitated with 13% TCA and subjected to liquid scintillation counting. (D) Lysates of 293T cells transfected with luciferase and Flag-N or Flag vector were subject to a luciferase assay and Western blotting with anti-Flag. (E) 293T cells transfected with Flag-N or Flag vector were incubated with DMEM supplemented with 10 μCi/ml [^{35}S]methionine for 1 h. The proteins in the lysates of the above cells were precipitated with 13% TCA and subjected to liquid scintillation counting for quantification (upper left panel). The same amounts of the lysates were also subjected to SDS-PAGE and autoradiography (upper right panel). IB, immunoblotting.

formed by cell fusion. All of the data suggest that N protein inhibits cell kinesis by inhibiting F-actin bundling mediated by EF1α.

N protein of SARS-CoV inhibits cell proliferation. To assess the effect of N protein on cell proliferation, 293T cells transfected with Flag-N or Flag plasmids were synchronized at G_1/S by a mimosine blockade. Following release from G_1/S, a slightly slower transition through S phase to G_2/M was observed in the cells expressing the N protein (Fig. 6A); 293T cells grew more slowly when N protein was expressed (Fig. 6B). Importantly, proliferation of human PBLs or human K562 cells was also inhibited by infection of recombinant retrovirus vector containing the SARS-CoV N protein expression cassette compared to the cells infected with retrovirus without N protein expression (Fig. 6C and D). These data collectively demonstrate that N protein of SARS-CoV inhibits cell proliferation, mainly by inhibition of cell kinesis.

DISCUSSION

CoV N proteins commonly localize in the nucleolus (44). For CoVs other than SARS-CoV, the N proteins interact with fibrillarin and nucleolin, which are two of the major components of the nucleolus (5, 44). In the described mechanism, N protein may induce multinucleated cells by interfering with rRNA processing and ribosome biogenesis (5). There are eight putative nuclear localization signal (NLS) motifs in the SARS-CoV N protein with six in the domain of aa 369 to 390 (38). Unlike N protein of other CoVs, SARS-CoV N was mostly distributed in the cytoplasm, supporting previous results in Vero cells infected with SARS-CoV or in transfections with a plasmid expressing the N gene (38). NLS recognition may sterically block the interaction between aa 369 to 390 of N protein and a cytoplasmic protein (38). Our data suggest that EF1α, an abundant cytoplasmic protein, may block the nuclear translocation of the N protein by masking its NLS motifs through binding to residues 251 to 422 of SARS-CoV N protein, where most NLS motifs are embedded. These data also imply that multinucleated cells were not induced through interaction with fibrillarin or nucleolin by the N protein of SARS-CoV.

Cytokinesis takes place through the contraction of a contractile ring that is mainly composed of F-actin bundles (15,

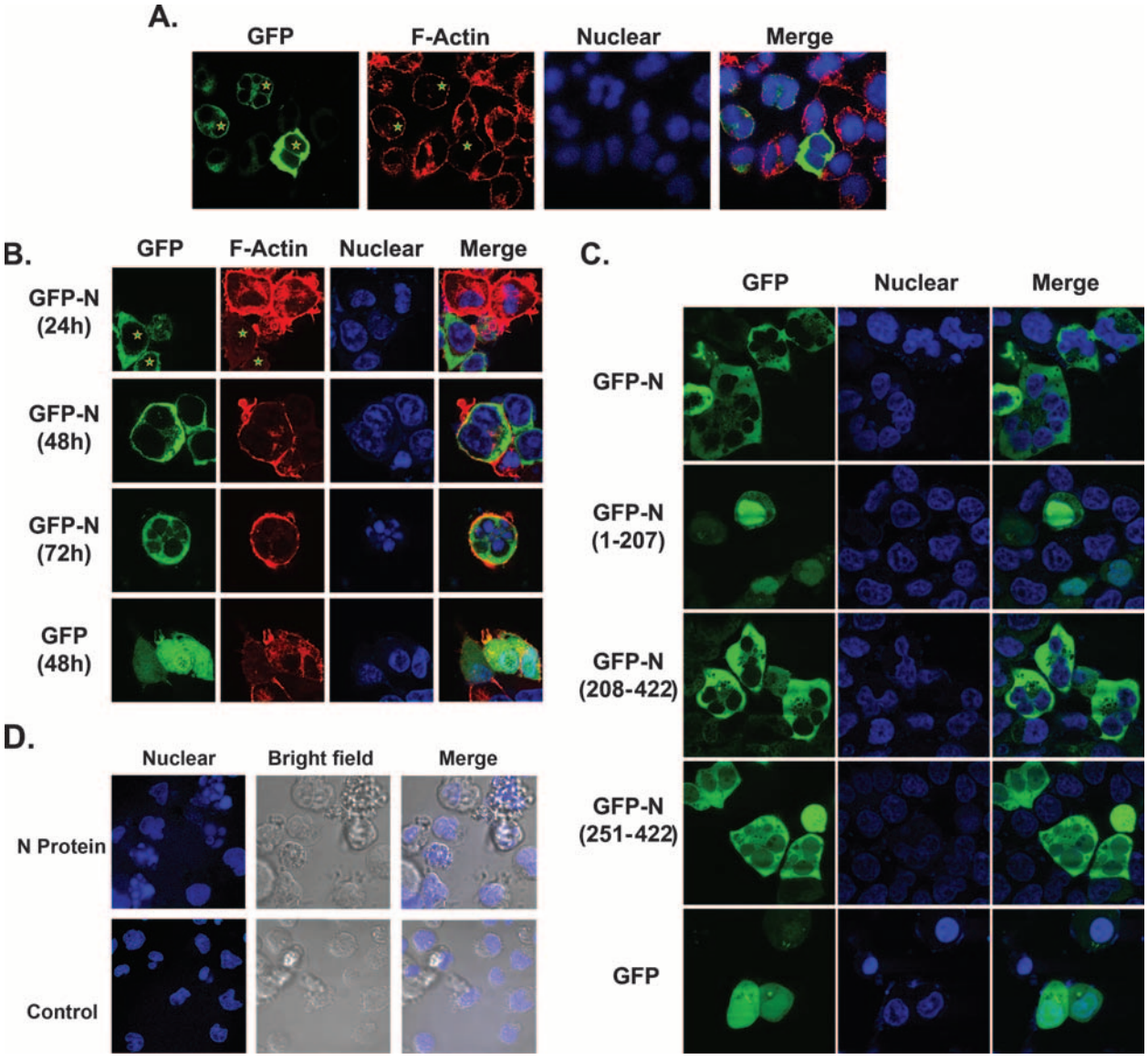

FIG. 5. N protein of SARS-CoV inhibits cytokinesis. HeLa (A) or 293T (B) cells were transiently transfected with GFP-N or GFP plasmids. At 48 h posttransfection (or at the time points indicated), cells were fixed, and F-actin was visualized with a laser confocal microscope after staining with fluorescently labeled phalloidin. (C) 293T cells were transfected with GFP-N, GFP-N(1-207), GFP-N(208-422), GFP-N(251-422), and GFP plasmids; after 72 h, cells were stained by Hoechst 33342 and analyzed by confocal microscopy. (D) PBLs were infected with retroviruses either expressing N protein or not. At 72 h posttransfection, cells were stained by Hoechst 33342 and analyzed by confocal microscopy.

43). An increasing body of evidence suggests that, in addition to its role in peptide elongation, EF1α may have additional functions. One of these noncanonical functions is a role for EF1α in the regulation of cytoskeletal dynamics. The first clear indication of the interaction between the cytoskeleton and EF1α was that one of the actin binding proteins (ABP-50), which was isolated from *Dictyostelium discoideum*, was identified as EF1α, and this protein was further shown to bundle F-actin during chemotaxis (45). Since then, the actin bundling activity of EF1α has been extensively demonstrated. It was also found that *Tetrahymena* EF1α was localized in the division furrow (instead of diffusely distributed in the cytoplasm) and involved in the formation of F-actin bundles in the contractile ring during cytokinesis (35). Approximately 70 to 90% of total cell EF1α, one of the most abundant proteins in eukaryotic cells, is bound to F-actin (12). In CoV-infected cells, N protein is the most abundant viral protein (20). A high-affinity interaction between N protein and EF1α may block EF1α-mediated F-actin bundling and the formation of the contractile ring, which leads to the formation of multinucleate cells by inhibiting cytokinesis. Our findings further support this hypothesis because F-actin bundles were not observed in the cells in which N protein was expressed, and only the EF1α-interacting domain of N protein induced multinucleation. In concert with these observations, it is noteworthy that N protein of HCoV-229E has a significantly lower affinity with EF1α than N protein of SARS-CoV and induces multinucleate cells much more slowly in a substantially smaller fraction of the transfected cells.

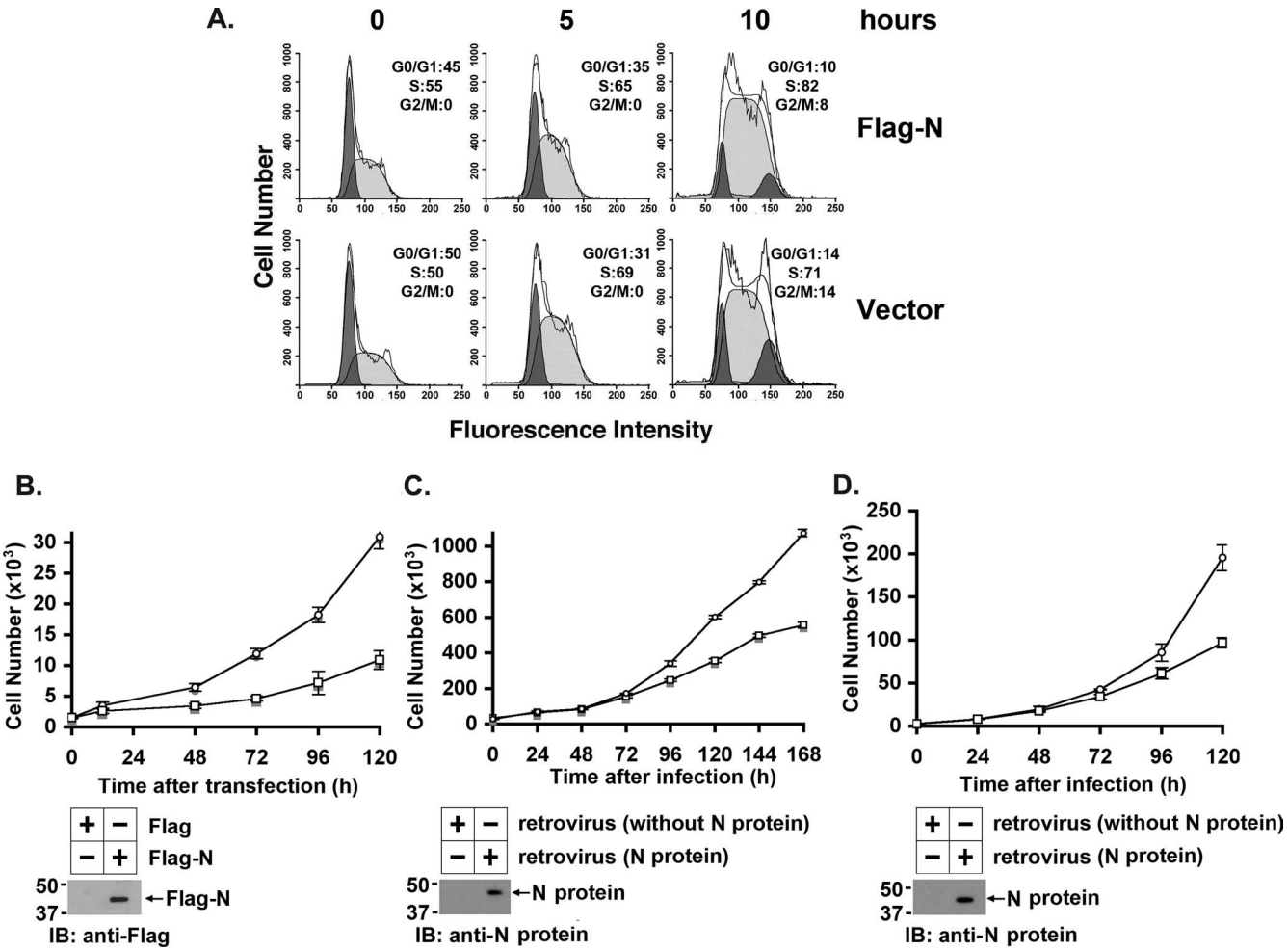

FIG. 6. N protein of SARS-CoV inhibits cell proliferation. (A) 293T cells transfected with Flag-N or Flag were synchronized with mimosine. Cells were harvested at the indicated time points after the withdrawal of mimosine and subjected to cell cycle determinations. (B) 293T cells were transfected with Flag-N (□) or Flag plasmids (○). Cell numbers were counted every 24 h after transfection. Lysates of the above cells were analyzed by Western blotting with anti-Flag. (C and D) PBLs (C) or human K562 cells (D) were infected with retrovirus expressing N protein (□) or vector (○) at a multiplicity of infection of 50, and cell numbers were counted every 24 h. Each data point represents the mean ± standard deviation of three independent measurements. Lysates of the above cells were analyzed by Western blotting with anti-N.

EFs in protein synthesis may be the target of interference by several pathogenic mechanisms. For example, diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A inhibit protein synthesis by ADP-ribosylation of eukaryotic EF2 (42); human immunodeficiency virus type 1 Gag polyprotein was shown to interact with EF1α and impair translation in vitro (8), and the Lit protease in *E. coli* cleaves EF-Tu and shuts down translation in response to specific binding of the Gol peptide of bacteriophage T4 binding to EF-Tu (2, 14). Inhibition of protein translation by N protein can be reversed by the addition of purified EF1α but not by 10- to 50-fold more mRNA. These data suggest that N protein inhibits protein translation by interacting with EF1α and not by enzymatic modification or binding to mRNA.

Unlike responses in other viral infections, the $CD3^+$, $CD4^+$, and $CD8^+$ lymphocytes in SARS-infected patients are dramatically reduced in patients with critical SARS, and these cell populations are gradually returned to normal as the patients' symptoms improve (10, 19, 30). Thus, a T-lymphocyte subgroup might play a key role in the process of SARS-CoV infection. Since actively dividing lymphocytes are one of the major targeted cell populations of SARS-CoV (18), SARS-CoV can destroy lymphocytes directly and interfere with the functions of the immune system. In this work, we found that expression of N protein in cells led to the inhibition of cytokinesis and proliferation of PBLs. Consistent with our observation, multinucleate syncytia of macrophages or epithelial cells have been observed in late-phase SARS-CoV but not in other CoV-infected patients (6) As the most abundant viral protein during SARS infection, N protein may lead to the slower proliferation of PBLs during SARS infection as a result of the molecular interactions described here. It will be important to perform experiments with infectious virus as follow-on studies; however, access to a biosafety level 3 facility for performing SARS-CoV work is currently not available in China. The experiments described in this study are not able to prove that N protein induces cytokinesis in the cells infected by SARS-CoV. However, the present observation that SARS-CoV N protein

inhibits cell proliferation by interacting with EF1α provides a clue for further research on the pathogenic mechanisms of SARS-CoV.

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