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Nuclear/nucleolar localization properties of C-terminal nucleocapsid protein of SARS coronavirus

Khalid Amine Timani, Qingjiao Liao, Linbai Ye*, Yingchun Zeng, Jing Liu, Yi Zheng, Li Ye, Xiaojun Yang, Kong Lingbao, Jingrong Gao, Ying Zhu

State Key Laboratory of Virology, College of Life Sciences, Wuhan University, Wuhan, Hubei Province 430072, PR China

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Abstract

A novel coronavirus (CoV) has recently been identified as the aetiological agent of severe acute respiratory syndrome (SARS). Nucleocapsid (N) proteins of the Coronaviridae family have no discernable homology, but they share a common nucleolar-cytoplasmic distribution pattern. There are three putative nuclear localization signal (NLS) motifs present in the N. To determine the role of these putative NLSs in the intracellular localization of the SARS–CoV N, we performed a confocal microscopy analysis using rabbit anti-N antisera. In this report, we show that the wild type N was distributed mainly in the cytoplasm. The N-terminal of the N, which contains the NLS1 (aa38–44), was localized to the nucleus. The C-terminus of the N, which contains both NLS2 (aa257–265) and NLS3 (aa369–390) was localized to the cytoplasm and the nucleolus. Results derived from analysis of various deletion mutations show that the region containing amino acids 226–289 is able to mediate nucleolar localization. The deletion of two hydrophobic regions that flanked the NLS3 recovered its activity and localized to the nucleus. Furthermore, deletion of leucine rich region (220-LALLLDRLNLRL) resulted in the accumulation of N to the cytoplasm and nucleolus, and when fusing this peptide to EGFP localization was cytoplasmic, suggesting that the N may act as a shuttle protein. Differences in nuclear/nucleolar localization properties of N from other members of coronavirus family suggest a unique function for N, which may play an important role in the pathogenesis of SARS.

Keywords: SARS–CoV; Nucleocapsid protein; Nuclear localization signal; Nucleolar localization; Cell cycle arrest

1. Introduction

Severe acute respiratory syndrome (SARS) is a life-threatening form of atypical pneumonia that emerged in Guangdong Province, China, resulting in 8098 probable cases and 774 deaths around the world. A number of laboratories worldwide have undertaken the identification of the causative agent (Drosten et al., 2003; Fouchier et al., 2003; Ksiazek et al., 2003; Peiris et al., 2003). The SARS–CoV genome is a single-stranded positive-sense RNA, classified within the order Nidovirales, family Coronaviridae, genus coronavirus of ∼29.7 kb and contains 14 open reading frames. The genome is composed of a stable region encoding functional replicase/transcriptase complex, a variable region representing four coding sequences for viral structural proteins (spike (S), envelope (E), membrane (M) and nucleocapsid (N)), and other putative nonstructural uncharacterized proteins. The gene order of SARS-CoV is similar to that of other coronaviruses; however, phylogenetic analyses and sequence comparisons indicate that this virus does not closely resemble any of the previously known human or animal coronaviruses (Marra et al., 2003; Rota et al., 2003; Ruus et al., 2003). Later study suggests that SARS-CoV represents an early split-off from coronavirus group 2 lineage relatively late in coronavirus evolution (Snijder et al., 2003) and this result has found support from other studies using alternative methods (Gorbalenya et al., 2004; Stavrinides and Guttman, 2004).
SARS–CoV N protein is 422 amino acids in length, basic in nature, which suggests it may assist in RNA binding, and can self-associate to form dimers (Sarjit et al., 2004b). Recently it has been reported to be phosphorylated in vitro (Zakhartchouk et al., 2005) although the physiological consequence of such covalent modification is yet to be determined. A recent report showed that phosphorylation of coronavirus infectious bronchitis virus (IBV) N protein determined the recognition of virus RNA (Chen et al., 2002). In addition, serine/arginine rich (SR; a176–204) motif, which present in SARS–CoV N, may be the site for the location of phospho-serines. Based on the available information, several functions have been postulated for the coronavirus N proteins throughout the virus life cycle (Laude and Masters, 1995), including viral packaging (Davies et al., 1981), viral core formation (He et al., 2004b; Risco et al., 1996) and signal transduction pathway (He et al., 2003) or part of host defense response invoked to counteract viral infection (Chang et al., 2004).

Sub-cellular localization of the viral protein is one of the commonly used approaches to characterize the involvement of the protein in the virus assembly. Although the SARS–CoV N protein harbors a putative nuclear localization signal (NLS) (Marra et al., 2003), the N protein is distributed predominantly in the cytoplasm of SARS–CoV-infected and N gene-transfected cells with a presence of weak fluorescence signals in the nucleus (Chang et al., 2004), therefore, it is not known whether this putative NLS is functional or not. Moreover, Qinfen and coworkers observed a special phenomenon during morphogenesis of SARS–CoV, where virus-like particles appeared in the nucleus of Vero E6 cells 48 h post-infection (Qinfen et al., 2004). On the other hand, an identical nucleolar-cytoplasmic localization pattern is observed for the N proteins of arteriviruses; porcine reproductive and respiratory syndrome virus (PRRSV) and mouse hepatitis virus (MHV) and IBV, respectively (Hiscox et al., 2002) and for N proteins of groups I, II and III reproductive and respiratory syndrome virus (PRRSV) and arterivirus (EAV) (Rowland et al., 1999; Tijdens et al., 2002) and for N proteins of groups I, II and III coronaviruses; transmissible gastroenteritis virus (TGEV), mouse hepatitis virus (MHV) and IBV, respectively (Hiscox et al., 2001; Wurm et al., 2001). Recently Rowland and Yoo have reported the nucleolar-cytoplasmic shuttling of PRRSV N protein through determining a possible nuclear export signal (NES) (Rowland and Yoo, 2003).

The nucleolus is highly specialized structure that participates in regulation of several host cell processes, including regulation of cell cycle, apoptosis and induction of antiviral responses (Rowland and Yoo, 2003). Targeting a protein to the nucleolus occurs through the diffusion of protein through the nucleoplasm and accumulation in the nucleolus. Localization to the nucleolus is mediated through nucleolar localization signal (NolS) that is in part an NLS, which functions by interacting with nucleolar components such as nucleolar proteins, rRNA or rDNA (Garcia-Bustos et al., 1991). Since many endogenous proteins that localize in the nucleus have been identified as regulators of the cell cycle (Chen et al., 2002; Garcia-Bustos et al., 1991) this beneficial control over the host cell cycle can either facilitate the early release of progeny virus or help the emerging virus to evade the host immune system for its persistent infection.

In this study, we examined the intracellular localization profile of N protein and a series of deleted mutants in SARS–CoV permissive cell lines and elucidated the possible role of N protein in modulation of the host cell cycle.

2. Materials and methods

2.1. Cells and virus infection

Vero and Vero E6 cells (obtained from China Centre for Type Culture Collection, CCTCC, Wuhan, China) were grown and maintained in Dulbecco’s modified Eagle’s medium (Gibco-BRL, USA) and minimal essential medium (Gibco) respectively, supplemented with 10% heat-inactivated FBS (Hyclone, Logan, Utah), 100 units/ml penicillin, and 100 μg/ml streptomycin, and incubated at 37°C in 5% CO2. Vero E6 cells were infected with SARS–CoV WHU strain at the MOI of 0.1, and were seeded after 24 h for both indirect immunofluorescence assay and Western blot analysis or after 48 h for RNA isolation.

2.2. Generation of polyclonal antiserum in rabbits

The RNA isolation, RT-PCR, construction of pGEMT-N plasmid contains an authentic copy of the N gene, sequencing (Genbank accession no. AY365036), subcloning of N gene into prokaryotic expression vector, expression and purification of N protein have been described previously (Timani et al., 2004). New Zealand rabbits were kept in conventional conditions and were handled in compliance with College of Life Sciences, Wuhan University (Wuhan, China) guidelines for animal care and use. Rabbit was immunized with 0.1–0.2 mg of purified recombinant N protein and was injected subcutaneously at multiple sites on its back. A booster injection was given 2 and 4 weeks later. Blood was drawn from the rabbit at 5 weeks following the immunization, the blood was allowed to clot at 4°C, antiserum was recovered by centrifugation at 5000 × g for 10 min at 4°C. A controlled serum was done by injecting normal saline at the same above conditions.

2.3. Plasmids construction and cell transfection

The pGEMT-N plasmid was used as the template to amplify coding region of N protein with different deletion mutations by PCR. The wild type (1269 nt) and deletion mutant fragments of the N gene were made as shown in (Fig. 3B). Each fragment was digested with BamHI and EcoRI, and ligated into BamHI and EcoRI digested pcDNA3 (Invitrogen), an eukaryotic expression vector, such that transcripts were under the control of the CMV immediate-early promoter. To generate deletion mutant (NΔ220–231) of N protein gene with internal deletion of aa220–231, two PCR...
manufacturer's instruction and used as the template to site directed mutagenesis kit (Stratagene) according to the
made as shown in (Fig. 6). Different parts of the N gene
fluorescent protein (EGFP)-tagged fusion proteins were
N226–422 construct. The nucleotide sequence and reading
fracton of pEGFP-N1 vector (Clontech) in-frame with the
Rfl and EcoRI sites of pEGFP-N1 vector (Clontech) in-frame with the
EGFP. The GN226–231 was obtained as above and cloned
into Rfl and EcoRI sites of pEGFP-N1. pCMV-flag-N
369–376 by using the same primers for GN226–422 construct. The nucleotide sequence and reading
frame of the constructs were verified by restricted digestion and
DNA sequencing. The primers sequence used in this
study are available from the authors upon request.

2.4. Establishments of stable nucleocapsid protein
expressing cell line

Vero E6 cells grown on 24-well tissue culture plates were transfected with 0.5 μg of pcDNA-N containing the full length
N gene or pcDNA3 as a control and after 48 h, transfected cells were selected with 800 μg/ml of G418 (Life
Technologies). Three weeks later, resistant colonies were iso-
lated and screened for N protein expression by RT-PCR and
Western blot analysis.

2.5. Western blot analysis

Total proteins were resolved by 10 or 15% SDS-PAGE
and transferred onto PVDF membranes (Bio-Rad, Hercules,
CA). The residual binding sites on the membranes were
blocked by incubation with 5% non-fat dry milk in TBS–T
buffer (20 mM Tris–HCl pH 7.6, and 137 mM NaCl con-
taining 0.02% Tween-20) for 2 h. Following the blocking,
membranes were subsequently probed with rabbit anti-N
polyclonal antibody (1:200 dilution) for 1 h at RT, then again washed
with cold PBS. Cells were either mounted in mounting
media or stained with propidium iodide (PI) to visualize nuclear DNA. Fluorescence images were viewed
under laser confocal scanning microscope (Leica Laser
Technik, Germany) with appropriate filters.

2.7. Flow cytometry and colony formation efficiency
assays

For cell cycle analysis, ∼60% confluent cells were grown
on six-well tissue culture plates. The following day cells were
transfected as described above. After 36 h, cells were washed
with PBS and removed from the plates by treatment with
trypsin-EDTA and re-suspended in 500 μl of ice cold PBS.
These cells were fixed with 70% ethanol. Cells were washed
twice with PBS after incubation at 4 °C for 30 min or stored at
−20 °C until performing the analysis. To remove the double-
stranded RNA, cells were further incubated with 1 μl RNase
(10 mg/ml) for 30 min at 37 °C, followed by staining with
20 μl PI (1 mg/ml) for 15 min. The ∼10,000 cells were ana-
lyzed in Coulter Epics XL flow cytometer (Backman Coulter,
USA) and the cell cycle analysis was done using the cell quest
program by manual setting regions for G0/G1, and G2/M.
For determination of colony formation efficiency assay,
the same number of cells (1 × 10^2) from stable transfected N
protein and new′ as a control were seeded on 35 mm diameter
dishes then cultured for 6–8 days until colonies could be
visualized. Colonies were washed with PBS and stained with
0.25% Coomassie Brilliant Blue (R-250) in 50% methanol
and 10% acetic acid.

3. Results

3.1. Characterization of anti-N protein antiserum

A polyclonal antibody directed against N protein was pro-
duced in order to determine its intercellular localization. We
generated anti-N rabbit antisera using an Escherichia coli
produced fusion protein (SARS-CoV N protein with 6XHis-
tag at the N-terminus) as antigen. The antigenicity of the
To examine the reactivity and specificity of the N protein antisera, Western blot analysis was performed. The results demonstrated that rabbit antisera notably reacted with N protein from SARS-CoV-infected cells. Intriguingly, a series of shorter bands were also observed (Fig. 1A, lane 1), most likely they are isomers of N protein due to Caspases degradation. The antisera also strongly reacted to a protein with an apparent molecular mass of ∼47 kDa (Fig. 1B lane 1). The cell lysate was prepared from Vero E6 cells constitutively expressing N protein after IPTG induction (described elsewhere; (Timani et al., 2004)) (lane 1), N protein was verified as recombinant N protein after immunoreactivity Western blot. The transferred PVDF membranes were probed with rabbit anti-N polyclonal antibody. The molecular mass of N protein in the blot was verified as ∼47 kDa. Approximate mobilities of protein markers are indicated on the left of (A).

Recombinant N protein was confirmed by immunoreactivity with SARS patients sera using ELISA assay, showed high sensitivity and specificity (Timani et al., 2004).

3.2. Intracellular localization of SARS–CoV N protein in infected and transfected cell lines

To identify the localization of N protein in SARS–CoV-infected Vero E6 cells where also other viral proteins are expressed, cells were fixed at 24 h post-infection and analyzed by indirect immunofluorescence assay using FITC-labeled secondary antibody directed to rabbit anti-N polyclonal antibody. The PI was used to visualize the nuclear DNA and the images were digitally superimposed to depict the distribution of N protein and nuclear DNA. Our results showed that N protein was localized predominantly in the cytoplasm in SARS-CoV-infected cells (Fig. 2A) whilst in a few cells a weak fluorescence label was also observed in the nucleus (Fig. 2B–D). In a group of infected cells (Fig. 2A), assuming that the amount of fluorescence from the FITC is proportional to the amount of N protein in the infected cells, in some cells a few bright fluorescence granules were found in the cytoplasm while in others a strong fluorescence were observed around the nuclear envelope, more likely the cells are at different infectious stages. No significant fluorescence was observed in pre-immune serum (Fig. 2A, inset panel). A similar observation was found in Vero E6 cells transfected with plasmid expressing full length N protein.

3.3. Subcellular localization of N protein alters upon N- and/or C-terminal deletion mutations

To determine if the N- or C-terminal truncated forms alter the localization pattern of the N protein, a different deletion mutants were constructed (Fig. 3B) taking into consideration the location of stretches of basic amino acids which are the principle components of NLS and the leucine rich region (LRR; see below). The amino acid sequence analysis of N protein was done by using software program PSORT II (Nakai and Kanehisa, 1992) and three classical putative NLS motifs were identified, labeled NLS1, NLS2 and NLS3 at positions 38–44, 257–265 and 369–390 amino acids, respectively (Fig. 3A). Furthermore, a Kyte–Doolittle hydrophilicity analysis of the protein sequence predicted that NLS motifs are in hydrophilic regions and should be easily accessible. An indirect immunofluorescence assay was used to analyze the intracellular localization of N protein in Vero E6 cells using anti-N polyclonal antibody. All the slides were further stained with PI to visualize the nuclear DNA. The differentially fluorescing images were gathered separately and then digitally superimposed (Fig. 4A–4H). Vero E6 cells transfected with mutant N176–422, which contains NLS1, NLS2 and SR rich motifs were showed a localization pattern similar to the wild type protein (Fig. 4A). Vero E6 cells transfected with mutant N335–422 (contains putative NLS2 and NLS3) was localized to the cytoplasm and nucleus accumulated mainly in the nucleus (bright fluorescence spot inside the nucleus; Fig. 4D). The deletion mutant N226–300 lacking both the NLS1 and NLS3 was also localized to the cytoplasm and the nucleoli (Fig. 4E). To further confirm this, deletion mutant N226–422/Delta1 showed a pattern of intranuclear localization similar to N226–422 and N226–300 (Fig. 4F). Furthermore, the deletion mutant N335–422, which contains

Fig. 1. Characterization of the rabbit anti-N polyclonal antibody. (A) Total cell lysates from SARS–CoV-infected Vero E6 (lane 1) and Vero E6 cells only as a control (lane 2) were resolved by 15% SDS-PAGE. (B) Total cell lysates from E. coli expressing recombinant N protein after IPTG induction (described elsewhere; (Timani et al., 2004)) (lane 1), N protein constitutively expressing Vero E6 cells (lane 2) and neo+ control (lane 3) were resolved by 10% SDS-PAGE. Both (A) and (B) were analyzed by Western blot. The transferred PVDF membranes were probed with rabbit anti-N polyclonal antibody. The molecular mass of N protein in the blot was verified as ∼47 kDa. Approximate mobilities of protein markers are indicated on the left of (A).
NLS3, the highest content of basic residues among the three NLS motifs, was unexpectedly present in the cytoplasm only (Fig. 4G). The deletion mutant N1–225 included the NLS1 and SR-rich motifs was localized to the cytoplasm and nucleus but the accumulation in the nucleolus could not be confirmed (Fig. 4B) because the FITC fluorescence was weak even after the experiment was repeated more than once and this could be due to the low antigenicity of the N-terminal of N protein in comparison with C-terminal (personal observation).

To confirm that this bright fluorescent spot was located in the nucleolus rather than being an artifact located in the nucleoplasm, monoclonal antibody specific to nucleolar protein B23 was used. The deletion mutants N226–422 (data not shown), N226–300 (Fig. 5) and N226–422/Δ1369–376 (data not shown) transfected cells were double labeled with FITC-labeled anti-N polyclonal antibody (Fig. 5A) and TRITC-labeled anti-B23 monoclonal antibody (Fig. 5B); confocal microscopy revealed the co-localization of N and B23 proteins (Fig. 5C) and the nucleoli appeared yellow. These results implied that the region containing amino acids 226–300, which includes putative NLS2 was responsible for the nucleolar localization of the C-terminal N protein.

3.4. Expression and intracellular localization of N-EGFP fusion proteins

Various deletion mutants of EGFP fusion protein genes were constructed (Fig. 6) to study the intracellular localization pattern in another cell line, Vero cells. To eliminate the possibility that positively charged proteins may relocate to the nucleus because of a post-fixation artifact (Lundberg and Johansson, 2002), the coverslips containing cells were overlaid on the slides then directly visualized under confocal microscopy. Our results showed that the wild type EGFP was diffusely localized to the cytoplasm, nucleoplasm and excluded from the nucleolus (Fig. 7A) which is consistent with passive diffusion. The fusion of N protein to EGFP (GN1–422) altered the distribution of EGFP, resulting in the accumulation of fluorescent protein in the cytoplasm and exclusion from the nucleus (Fig. 7B). The similar observation for the wild type EGFP-N was observed in GN176–422 mutant fragment whereas in few cells a weak fluorescence was also observed in the nucleus (Fig. 7G). The protein products of mutants GN226–422(Fig. 7H and I), GN226–300(Fig. 7J) and GN226–422/Δ369–376(Fig. 7M and N) were accumulated in the cytoplasm and nucleus and
Fig. 3. (A) The diagram identifies the location of putative NLSs and LRR domains within SARS–CoV N protein. Amino acid sequence analysis software program, PSORT II (Nakai and Kanehisa, 1992) was used to locate the classical NLS motifs. The numbers identified the amino acid position covered by each domain. The NLS was classified into “pat4”, “pat7” and “bipartite” motifs. For details, see the text. (B) Schematic representation of a summary of different deletion mutants of SARS–CoV N and their respective intracellular localization. Dash lines and numbers indicate the deleted amino acids and amino acids positions, respectively while bars represent translated amino acids. N: nucleus; C: cytoplasm. (+) and (−) designate for presence and absence of protein expression, respectively.

had high amounts of fluorescence intensity in the nucleolus. Further deletion from the C-terminal of GN226–300 to generate GN226–289 (Fig. 7K and L) fragment was still localized to the nucleolus. The Nucleoli, when viewed under phase-contrast microscope, are apparent as highly refractive bodies within the nucleus (Fig. 7C, F, I, L, N, Q and T). The GN335–422 fragment was localized in the cytoplasm only (Fig. 7O) which was similar to the localization pattern for N335–422. Interestingly, deletion of 24 amino acids from N-terminal and 33 amino acids from the C-terminal of GN336–422 to generate GN360–389 altered the distribution of EGFP, resulting in accumulation of fluorescent protein in the nucleus and its decrease in the cytoplasm (Fig. 7P and Q). This showed that the peptide region 360–389 contains an active NLS3. Furthermore, GN1–175 and GN1–225 mutant fragments contain NLS1 and NLS1 and SR rich motifs respectively, were localized in the nucleus but excluded from the nucleolus (Fig. 7P and Q). This suggested that N-terminal N protein may also contain an active NLS1 that directs an exogenous protein to the nucleus but not to the nucleolus. These results showed that the region aa226–289 of the N protein contains NoLS and thus its activity is independent from putative NLS1 and NLS3.

3.5. The leucine rich region may act as nuclear export signal

Why the wild type N and its mutant fragment N176–422 localize in the cytoplasm, although the presence of aa226–289 peptide region responsible for the nucleolar localization in its C-terminal? To address this question, we performed amino acid sequence analysis of the of N protein and showed that it contains LRR (220-LALLLLDRLNL-231; Fig. 3A) which satisfies the consensus requirements for the classical NES; X-R(2–4)-X-R2-X-R-X, where X is leucine, isoleucine or valine and R is any amino acid (Henderson and Eleftheriou, 2000; Hope, 1999). Furthermore, Kyte–Doolittle hydrophilicity analysis showed that this region has highest hydrophobicity. To determine if the N protein LRR sequence is functional, different deletion mutants were constructed, NΔ220–231 and GNA220–231. These mutants contain the full length N protein with an internal deletion of LRR (Figs. 3B and 6), cloned to pcDNA3 and pEGFP-N1, respectively. In addition, the peptide aa220–231 was placed at the C-terminal of EGFP to produce GN220–231. The Vero cells transfected with mutant plasmids were examined by indirect immunofluorescence using rabbit anti-N polyclonal antibody
or direct fluorescence for EGFP fusion mutant fragments. The NΔ220–231 localized in the cytoplasm and nucleus mainly in the nucleolus similar to the localization pattern of N226–300 (Fig. 4H). The localization pattern for GNΔ220–231 was similar to GN226–289, i.e., the localization resulted in accumulation of fluorescent protein in the cytoplasm and nucleus with high fluorescence intensity in the nucleolus (Fig. 7S and T). To determine if this LRR could export a heterogenous protein from the nucleus, a construct GN220–231 was used. This construct was localized predominantly in the cytoplasm (Fig. 7R) whilst in some cells, fluorescent granules were surrounding the nuclear envelope with very low fluorescence label in the nucleus (data not shown). These observations suggest that LRR may act as an NES and this also might explain the cytoplasmic localization of mutant proteins N176–422 (Fig. 4C) and GN176–422 (Fig. 7G). Another point to note is ~20% of transfected cells only showed nucleolar localization of C-terminal N protein; this may be due to other unknown factors favoring the protein to the cytoplasm.

The GN226–422 and N226–422 mutant fragments contain the half part of the LRR (220-LALLLL) at its N-terminal and the GN1–225 contains the other half (226-DRLNRL) at its C-terminal (Figs. 3B and 6), shown in red box), were localized to the cytoplasm and nucleus, thus the full peptide sequence of LRR is required for export of protein out from the nucleus.

Fig. 4. Intracellular localization of various deletion mutants of SARS-CoV N protein in the transfected cells. Vero E6 cells were transfected with different deleted mutants. After 24 h, the cells were fixed and analyzed by indirect immunofluorescence assay using rabbit anti-N polyclonal antibody (green). Additionally, cells were stained with PI to visualize the nuclear DNA (red) and were examined by confocal microscopy. The two colors were then merged and yellow color when it occurs is the region where green and red co-localized. (A)–(H) represents the merged panels of indicated deletion mutants. Arrow points to the cell nucleus or to the yellow region. Magnification 40×.
3.6. Stable cell line constitutively expressing N protein affects cell cycle

To study if the constitutively expressing N protein could affect the cell cycle, Vero E6 cells were stably transfected with N protein expression vector and the expression level of N protein and mRNA were detected by Western blot (Fig. 1 lane 2) and RT-PCR (data not shown), respectively. Then we determined the colony formation efficiency of N-expressing Vero E6 cells. The same numbers of cells were
Fig. 8. SARS–CoV N expression inhibits the colony formation efficiency. Vero E6 cells of constitutively expressing N protein and neor control cells (1 × 10^4) were seeded on 35 mm culture dishes and cultured until colonies were visually seen. Colonies were stained as described under “Section 2”. The assay was carried out in triplicate and the results were reproducible.

Table 1

| Neor (%) | N protein (%) |
|----------|---------------|
| G0/G1    | 67.9 ± 0.5    |
| S        | 18.0 ± 1.0    |
| G2/M     | 14.1 ± 1.1    |

* N protein-expressing Vero E6 cell line in a constitutive system was analyzed by flow cytometry. The values represent the mean ± SD of triplicate experiments of total cell population in each phase of cell cycle.

The results also revealed that LRR (220-LALLLD-RLNRL) may act as NES. Since, few cells showed a weak expression of wild type N protein in the nucleus (Fig. 2D), possible reasons could be that LRR exhibits a strong export signal favoring the N to the cytoplasm (for review, see reference (Rowland and Yao, 2003)). Furthermore, the location of LRR is just close to the N-terminal of NLS2, which might
strains of TGEV (Eleouet et al., 2000) and another report protein late in the infection has been observed with different et al. showed that SARS–CoV N induced apoptosis in COS-1 result in nuclear retention of N, demonstrating that pro-tein shuttles between cytoplasm and nucleus before playing way. Inactivation of this pathway with leptomycin B (LMB) N proteins utilize the CRM1-mediated nuclear export path-

tein in the pathogenesis of SARS and that N protein might have contributed in some way to the severity of the recent out-break.

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have affected its function; these findings suggest that N may act as shuttle protein. Moreover, additional experiments are needed to study the molecular mechanism of SARS-CoV N protein LRR. The PRRSV coronavirus and EVA arterivirus N proteins utilize the CRM1-mediated nuclear export path-way. Inactivation of this pathway with leptomycin B (LMB) resulted in nuclear retention of N, demonstrating that pro-tein shuttles between cytoplasm and nucleus before playing role in virus assembly (Rowland and Yoo, 2003; Tigms et al., 2002). When GNI–422 expressing cells were treated with 20 mg/ml of LMB for 4 h or for a prolonged period of time (overnight) or even at higher concentration (100 ng/ml) for 2 h, the localization of N protein was unaltered, i.e. predomi-nantly cytoplasmic (data not shown), possible reasons could be that N protein exported via exportin-independent pathway or LRR could act as masking signal rather than NES.

Why does the SARS–CoV N protein exhibits different and complex localization pattern is unknown and needs to be addressed in future. Recent reports demonstrated that SARS-CoV induced apoptosis in virus-infected cells (Mizutani et al., 2004a, 2004b; Yan et al., 2004), whilst Surjit et al. showed that SARS-CoV N induced apoptosis in COS-1 cells under stressed condition and activated Caspase-3 and -7 (Surjit et al., 2004a). The appearance of shorter form of N protein late in the infection has been observed with different strains of TGEV (Eleouet et al., 2000) and another report demonstrated the cleavage of recombinant N of SARS-CoV by Caspase-3 but not Caspase-6 in vitro and the pattern of peptide fragments distribution were similar to that in infected cells (Yang et al., 2004) which is consistent with our result (Fig. 1A, lane 1). Taken together, we hypothesized that at a late stage of infection, the activation of Caspases during the apoptosis of the infected cells by SARS-CoV leads to the cleavage of N protein at particular sites. Some of the cleavage products may contain an active NLS leading to its translo-ca tion to the nucleus and that may disrupt or usurp nuclear function whereas self-association of some N molecules (He et al., 2004a) may protect N from cleavage by Caspases and remain in the cytoplasm to participate in the virus assembly.

In our present study, further investigation of the effect of N protein on the cell growth was carried out. Vero E6 cells constitutively expressing N protein exhibited a pronounced retardation of cell growth by reduced colony formation effi-ciency concomitantly with a shortened S phase and a pro-longed G2/G0 phase of the cell cycle. Many viral proteins that have nuclear/nucleolar localization properties were reported to disrupt cell cycle, for instance, TGEV and IBV N pro-teins cause aberrant cell division by disrupting cytokinesis and this inhibits or delays cell growth (Chen et al., 2002; Wurm et al., 2001) and in HeLa cells, HIV-I Vpr which is a nuclear-cytoplasmic protein causes growth retardation and G1 arrest (Nishizawa et al., 1999). Why is the case of NNSA, N-terminally deleted form but not the full length localizes to the nucleus and the protein causes growth inhibition and cell cycle perturbations through G2 arrest (Arima et al., 2001; Song et al., 2000). Recently Chen and Makino demonstrated that MHV coronavirus infection in asynchronously growing cells led to the inhibition of host DNA synthesis and accumu-lation of cells in the G0/G1 phase and they subsequently attributed to a replicase protein (Chen and Makino, 2004; Chen et al., 2004). Future studies need to be designed to elucidate the role of N protein in regulation of cell cycle, e.g. interaction with nucleolar proteins and activation or sup-

pression cell cycle regulators. Thus, the localization to the nucleus/nucleolus implies that N protein may involve in the regulating of cell cycle possibly by delaying the cell cycle to promote intracellular conditions for virus assembly and sequestering ribosomes for translation of viral proteins or by participating in the modulation of nuclear functions, a strategy to optimize virus replication [see (Hiscos, 2003) for a review]. The identification of different nuclear and nucleo-
rar localization properties of N protein from other members of Coronaviridae family suggests a unique function for N protein and will enable us to investigate the role of N pro-
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