The Severe Acute Respiratory Syndrome Coronavirus Nucleocapsid Protein Is Phosphorylated and Localizes in the Cytoplasm by 14-3-3-Mediated Translocation

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The severe acute respiratory syndrome coronavirus (SARS-CoV) nucleocapsid (N) protein is one of the four structural proteins of the virus and is predicted to be a 46-kDa phosphoprotein. Our in silico analysis predicted N to be heavily phosphorylated at multiple residues. Experimentally, we have shown in this report that the N protein of the SARS-CoV gets serine-phosphorylated by multiple kinases, in both the cytoplasm and the nucleus. The phosphoprotein is stable and localizes in the cytoplasm and coprecipitates with the membrane fraction. Also, using specific inhibitors of phosphorylation and an in vitro phosphorylation assay, we show that the nucleocapsid protein is a substrate of cyclin-dependent kinase (CDK), glycogen synthase kinase, mitogen-activated protein kinase, and casein kinase II. Further, we show that the phosphorylated protein is translocated to the cytoplasm by binding to 14-3-3 (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein). 14-3-3 proteins are a family of highly conserved, ubiquitously expressed eukaryotic proteins that function primarily as adapters that modulate interactions between components of various cellular signaling and cell cycle regulatory pathways through phosphorylation-dependent protein-protein interactions. Coincidentally, the N protein was also found to downregulate the expression of the theta isoform of 14-3-3 (14-3-3θ), leading to the accumulation of phosphorylated N protein in the nucleus, in the absence of growth factors. Using short interfering RNA specific to 14-3-3θ we have inhibited its expression to show accumulation of phosphorylated N protein in the nucleus. Thus, the data presented here provide a possible mechanism for phosphorylation-dependent nucleocytoplasmic shuttling of the N protein. This 14-3-3-mediated transport of the phosphorylated N protein and its possible implications in interfering with the cellular machinery are discussed.

A novel coronavirus has been associated with a worldwide outbreak of atypical pneumonia referred to as severe acute respiratory syndrome coronavirus (SARS-CoV) (1–2). SARS-CoV has exhibited a high mortality rate in infected people and has spurred intense research efforts around the world to deal with the serious threat to mankind posed by this novel coronavirus. To date more than 8,000 people have been infected, 800 are dead, and mortality rates reaching over 40% in certain populations have been documented (5).

SARS-CoV is a positive-sense, single-stranded RNA virus with ~30,000 nucleotides, which are organized into approximately 14 open reading frames, taking into consideration only those exceeding 50 amino acids in translational capacity (12, 15). Like other known coronaviruses, SARS-CoV is an enveloped virus containing three outer structural proteins, the membrane, envelope, and spike proteins. The nucleocapsid (N) protein, together with the viral RNA genome, presumably forms a helical core located within the viral envelope. The SARS-CoV nucleocapsid protein contains 423 amino acids and has been predicted to be a phosphoprotein of 46 kDa (12) and a nucleic acid binding protein and also postulated to be involved in viral transcription and replication (6). Recently, our lab has shown that this protein is capable of self-association to form dimers (19) and can induce programmed cell death and actin reorganization in mammalian cells under stress (18).

In this report we have studied the ability of the N protein to be phosphorylated in COS-1 and human hepatoma (Huh-7) cells. We have been able to show that the majority of phosphorylation occurs at the serine residues and the phosphorylated N protein localizes predominantly in the cytoplasm and associates with the membrane. Further, we provide evidence for phosphorylation-dependent 14-3-3 binding which may enable N to be efficiently transported from the nucleus to the cytoplasm. Interestingly, we have also observed downregulation of 14-3-3θ transcript levels in N-expressing cells in the absence of growth factors, leading to an accumulation of the N protein in the nucleus. Finally, inhibition of 14-3-3θ expression using short interfering RNA (siRNA) specific for it resulted in significantly increased nuclear localization of the N protein. Hence, we postulate that under different cellular conditions, interplay between these two proteins may regulate the intracellular localization of the N protein.

MATERIALS AND METHODS

Plasmids and reagents. The pCDNA3.1 Myc N construct and pCDNA3.1 Myc only (mock) was used in all the experiments. The N gene cloning has been described earlier (18). The green fluorescent protein (GFP) siRNA was obtained...
from Vijay Kumar. The 14-3-3α siRNA expression plasmid was constructed by cloning the 14-3-3α siRNA oligomer bearing Apal and EcoRI overhangs into the pSilencer 1.0 U6 vector. The upper and lower oligomer sequences were designed based on the human 14-3-3α siRNA sequence (24). 3′-Silencer, M1A RNAi, 5′-CGAATCCGAGAAGGTCGCGCTCGGATAG TCCCTTTTTT-3′ and 5′-AAATTAAAAAGAAGATTGCGGCCATCGTC TCTTGTACCTTCCTGGAGATCCGGCC-3′, respectively. These oligo-

nucleotides were designed based on the human 14-3-3α siRNA sequence (24). 3′-Silencer, M1A RNAi, 5′-CGAATCCGAGAAGGTCGCGCTCGGATAG TCCCTTTTTT-3′ and 5′-AAATTAAAAAGAAGATTGCGGCCATCGTC TCTTGTACCTTCCTGGAGATCCGGCC-3′, respectively. These oligo-

Putative amino acid sequence required for recognition by the corresponding enzyme.

Respective amino acid sequence of N protein responsible for recognition by the corresponding enzyme. [ ], either/or substitution; ( ), possible presence of both amino acid residues.

Putative consensus amino acid sequence required for recognition by the corresponding enzyme.

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Histone H1 or maltose-binding protein samples were used directly after boiling for 5 min in 10 mM 4X SDS dye. Samples containing N protein were again immunoprecipitated using anti-Myc antibody, and proteins eluted by boiling in 2X SDS dye. Protein bands were resolved by 12% SDS-PAGE and detected by autoradiography.

RNA isolation, cDNA subtraction, and Northern analysis. RNA was isolated from transfected COS-1 cells using RNeasy kit (QIAGEN) as described by the manufacturer. cDNA subtraction was conducted as described in Singh et al. (17). Northern analysis was performed as described by Sambrook et al. (16).

RESULTS AND DISCUSSION

The nucleocapsid protein of SARS virus is a predicted phosphoprotein of 46 kDa. In silico analysis using the NET PHOS 2.0 program revealed multiple putative phosphorylation sites for glycogen synthase kinase, protein kinase A, casein kinase I, casein kinase II, and one site each for cyclin-dependent kinases (CDK), protein kinase B, mitogen-activated protein kinase, and Polo-like kinase (Table 1). This analysis indicates that the N protein gets phosphorylated at multiple residues.

Based on these predictions, we conducted initial experiments to test if N undergoes phosphorylation in vivo. Plasmids expressing C-terminally Myc-tagged N (pCDNA 3.1 Myc N) were transfected into COS-1 cells as described in Materials and Methods; 40 h posttransfection, cells were labeled with 350 μCi of [32P]orthophosphoric acid for 2 h, and immunoprecipitated using anti-Myc antibody. The immunoprecipitated proteins were resolved in 12% SDS-PAGE and detected by autoradiography. As shown in Fig. 1A, mock-immunoprecipitated (using preimmune serum) cells did not show any phosphate-labeled protein (Fig. 1A, lane 1) whereas N immunoprecipitated (using anti-Myc antibody) cells showed the N protein migrating at ~48 kDa (Fig. 1A, lane 2), which corresponds to the approximate molecular size of the protein.

In order to prove that the observed band corresponds to the phosphorylated N protein, aliquots of the sample were treated with [32P]orthophosphoric acid for 5 min in 10 μl 4X SDS dye. Samples containing N protein were again immunoprecipitated using anti-Myc antibody, and proteins eluted by boiling in 2X SDS dye. Protein bands were resolved by 12% SDS-PAGE and detected by autoradiography.
lysates with anti-Myc antibody and then Western blotting the samples with a phosphorylation-specific antibody. As shown in Fig. 1C, only the phosphoserine-specific antibody could detect the N protein. We were unable to detect any band corresponding to N using phosphothreonine and phosphotyrosine antibodies (data not shown). However, the possibility of low-level phosphorylation at these residues cannot be ruled out owing to limited sensitivity of the immunoblotting procedure.

In order to discriminate against possible cross-reactivity of the anti-Myc antibody and phosphorylation-specific antibodies, the cells had been labeled with {superscript}35S Promix and the same Western blot was air dried and exposed to X-ray film to detect the expression of N (Fig. 1C, lower panel). To show that phosphoserine antibody recognized phosphorylated residues only, aliquots of the sample were treated with λ phosphatase prior to immunoprecipitating with anti-Myc antibody (Fig. 1C, lane 3). The corresponding band in this control experiment was undetectable. Hence we conclude from these experiments that the majority of N protein is serine-phosphorylated.

Further, we checked the kinetics of the N protein phosphorylation by performing a pulse-chase assay of the protein labeled with {superscript}32Porthophosphate and compared this with the corresponding levels of {superscript}35S Promix-labeled N protein. COS-1 cells were pulse-labeled for 20 min with 500μCi of {superscript}32Porthophosphate or 250 μCi of {superscript}35S Promix, respectively, and then chased in complete medium for 30, 60, 90, or 120 min. Equal amounts of the protein were immunoprecipitated using anti-Myc antibody (Fig. 2). The lower panel shows that N was phosphorylated stably throughout the chase period in comparison to the total protein labeled with {superscript}35S Promix (upper panel). The graph shows relative band intensity of the phosphorylated and total N protein at different time points as derived from three independent sets of experiments. The data suggest that the N protein is phosphorylated immediately after synthesis and remains stably phosphorylated.

We further investigated whether phosphorylation regulated the spatial distribution of the N protein within the cell. The functional activity of many cellular proteins is known to be regulated by altered spatial distribution following posttranslational modification, e.g., many cell cycle regulatory proteins such as p27 and CDC6 are regulated by this mechanism (13). Nucleocapsid proteins in other coronaviruses have been shown to be distributed in both the nucleus and cytoplasm (26). In order to find out whether the N protein of SARS localizes in the nucleus, nuclear and cytoplasmic fractions were isolated from {superscript}35S Promix-labeled N-transfected COS-1 cells and immunoprecipitated using anti-Myc antibody. Surprisingly, N was found to be abundantly present in the cytoplasmic fraction (Fig. 3A, upper panel). Very little N protein was found in the nuclear fraction (lane 4).

To rule out any possibility of cross contamination between the cytoplasmic and nuclear fractions, an aliquot of the lysate was Western blotted with anti-calnexin antibody. Calnexin is known to localize in the cytoplasm and membrane. In this experiment, we detected a band only in the cytoplasmic fraction (Fig. 3B, upper panel) indicating that there is no cross contamination between nuclear and cytoplasmic fractions. To rule out the possibility of nuclear contamination of the cytoplasmic fraction, an aliquot of the lysate was Western blotted with phospho-c-Jun antibody, which showed exclusive nuclear distribution (Fig. 3B, lower panel).

The cellular localization pattern of the N protein was further verified by an indirect immunofluorescence assay using anti-Myc antibody, whereby the N protein was found to be in the cytoplasm (Fig. 3C, left panel images). Cells were stained with DAPI (blue color) which stains the nucleus and N protein was stained with Texas red (red color). In Fig. 3C, right panel images show superimposition of nuclear stain over the Texas red stain in the same field to show any possible colocalization. Similar distribution pattern was also observed when N protein was checked for colocalization with 14-3-3 (Fig. 6C, discussed later). We then performed a nuclear fractionation assay using {superscript}32Porthophosphate-labeled N-expressing cells to check the distribution of the phosphorylated N protein. Although we could detect significant amounts of N in the cytoplasmic fraction (Fig. 3A, lower panel, lane 2), we were unable to detect any in the nuclear fraction (Fig. 3A, lane 4).

The immunofluorescence assay showed a prominent staining of the N protein around the plasma membrane. Hence we checked by biochemical fractionation whether the N protein associated with cell membrane. Immunoprecipitation of the membrane fraction with anti-Myc antibody revealed association of a significant amount of the N protein with the membrane (Fig. 3D, upper panel). Similarly, phosphorylated N was also found to associate with the membrane (Fig. 3D, lower panel).

Having seen phospo-N localization predominantly in the cytosolic fraction, we further investigated whether the N protein was phosphorylated in the cytosol or whether N gets phos-
phorylated in the nucleus and subsequently exported into the cytoplasm. In order to check this hypothesis, we blocked nuclear protein import using wheat germ agglutinin and observed the phosphorylation of N. N protein was found to be effectively phosphorylated despite the presence of the inhibitor, indicating that a cytosolic kinase was responsible for phosphorylation of the N protein (Fig. 4A). However, the possibility exists that multiple kinases phosphorylate N both in the cytoplasm and nucleus.

Among the putative kinases predicted to phosphorylate N (Table 1), the cyclin-CDK complex is known to be active inside the nucleus (13) and some others can act in both the nucleus and cytoplasm. Hence the possibility exists that multiple kinases phosphorylate N both in the cytoplasm and nucleus.

FIG. 3. Subcellular localization of N protein. (A) pCDNA 3.1 (lane 1 and 3) or pCDNA3.1 N (lane 2 and 4)-transfected cells labeled with [32P]orthophosphate (lower panel) or 35S Promix (upper panel) were fractionated and immunoprecipitated using anti-Myc antibody. Lanes 1 and 2: cytoplasmic fraction; lanes 3 and 4: nuclear fraction. (B) Cytoplasmic (lane 1) and nuclear (lane 2) fractions from control cell lysates were Western blotted with anti-calnexin (upper panel) and phospho-c-Jun (lower panel) antibody. (C) Mock (upper panel) or pCDNA 3.1 N (lower panel)-transfected cells were probed with anti-Myc antibody followed by Texas red and DAPI staining and the corresponding fluorescence was visualized using an immunofluorescence microscope. The left panel shows image of N expression and right panel shows a merged image of nuclear staining (DAPI) over the Texas red stain in the same field. The arrow shows cytoplasmic localization of the N protein. (D) pCDNA 3.1 (lane 1) or pCDNA 3.1 N (lanes 2 and 3)-transfected cells were labeled with 35S Promix (upper panel) or [32P]orthophosphate (lower panel). The membrane (lane 2) and cytoplasmic (lane 3) fractions were immunoprecipitated with anti-Myc antibody. Radiolabeled proteins were detected by autoradiography.

Interestingly, a combination of inhibitors effectively blocked phosphorylation almost completely (Fig. 4B, lane 2), thus implying that in vivo N is phosphorylated at multiple sites by multiple kinases. Moreover, the data also reveal that not all putative phosphorylation sites are functional in vivo, since the phosphatidylinositol 3-kinase inhibitor LY294002 as well as the protein kinase A inhibitor (H89) were unable to show any significant inhibition of N protein phosphorylation. However, this might not be true during a natural viral infection if other viral proteins were engaged in modulating the cellular as well as viral protein networks causing these sites to get preferentially phosphorylated.

Besides LiCl (glicogen synthase kinase 3 inhibitor), U0126 (mitogen-activated protein kinase inhibitor), and DRB (casein kinase II inhibitor), an inhibitor that could block N protein phosphorylation to a lesser extent but significantly (~30% inhibition) was olomoucine, which is known to inhibit cyclin-dependent kinase activity by acting as a competitive inhibitor for the ATP binding domain of the enzyme (11). CDKs are active as enzymes only when bound to their respective cyclins and the turnover of cyclins is strictly regulated in a cell cycle-dependent manner (13). Hence, N protein might be modulating the activity of cellular substrates of the cyclin-CDK complex by binding to and getting phosphorylated by the latter.

To investigate if the N protein is a direct substrate of the
cyclin-CDK complex, we analyzed the ability of the cyclin-CDK complex to phosphorylate in vitro-expressed N by performing an in vitro phosphorylation assay. The N protein was expressed using a rabbit reticulocyte lysate-based coupled transcription-translation system (TNT kit, Promega, USA) and expression of the N protein was verified on 12% SDS-PAGE from a small fraction of the reaction and staining by Coomassie brilliant blue (Fig. 5A). In order to immunoprecipitate cyclins D and A during their maximum endogenous levels, COS-1 cells were synchronized at the G0 phase of the cell cycle by serum starvation for 34 h and then stimulated with 10% serum to initiate cell cycle progression. Cells were harvested at 2, 12, 18, and 22 h poststimulation and aliquots of the lysate were Western blotted with cyclin D and cyclin A, whose expression begins during the G1 and S phases, respectively.

As shown in Fig. 5B, upper panel, cyclin D was detected at all time points, whereas cyclin A was maximally detected at 18 and 22 h (Fig. 5B, middle panel). As a control to check equal loading of protein, the cyclin D blot was stripped and reprobed with antibody to CDK2, which is present during all phases of cell cycle progression. Cells were harvested at 2, 12, 18, and 22 h poststimulation and aliquots of the lysate were Western blotted with cyclin D and cyclin A, whose expression begins during the G1 and S phases, respectively.

As shown in Fig. 5B, upper panel, cyclin D was detected at all time points, whereas cyclin A was maximally detected at 18 and 22 h (Fig. 5B, middle panel). As a control to check equal loading of protein, the cyclin D blot was stripped and reprobed with antibody to CDK2, which is present during all phases of cell cycle progression. Cells were harvested at 2, 12, 18, and 22 h poststimulation, immunoprecipitated using cyclin A or cyclin D antibody, and used in the in vitro phosphorylation assay as described in Materials and Methods. As shown in Fig. 5C, immunoprecipitated cyclin A and cyclin D could effectively phosphorylate the N protein, where as no corresponding band was observed in mock-translated or preimmune serum-immunoprecipitated samples. Further, the band intensity was found to be significantly reduced when treated with λ phosphatase (Fig. 5C, lanes 5 and 10, N+λ). This confirmed that the N protein was indeed a substrate of the cyclin-CDK complex. Histone H1, which is a well-known substrate of the cyclin-CDK complex, was used as a positive control for this experiment (Fig. 5C, lane 11).

Similarly, we performed the in vitro phosphorylation assay of N protein to check whether it is a substrate of mitogen-activated protein kinase. For this, cells were seeded in 60-mm dish at 50% confluence and 48 h postseeding cells were immunoprecipitated using anti-ERK1/2 antibody and in vitro phosphorylation was done as described above. As shown in Fig. 5D, N was found to be a substrate of the mitogen-activated protein kinases ERK1 and 2 (Fig. 5C, lane 2). Mock-translated, λ phosphatase-treated, and preimmune serum samples were used as controls. Also an aliquot of the sample was immunodepleted of ERK1/2 and processed simultaneously for in vitro phosphorylation to show the specificity of enzyme (Fig. 5C, lane 3). Maltose-binding protein was used as a positive control substrate to check the mitogen-activated protein kinase activity (Fig. 5C, lane 6).

In order to prove the hypothesis that the N protein is phosphorylated in the nucleus and then translocated to the cytoplasm, we performed a nuclear fractionation assay of 35S-labeled N-expressing cells in the presence of different phosphorylation inhibitors and looked for the presence of N protein in the nuclear fraction. As expected, in the presence of olomoucine, a significant fraction of the N protein was found to localize in the nuclear fraction. Further, DRB-treated cells also showed a fraction of N protein in the nucleus. In contrast, LiCl- and U012-treated cells showed the majority of the protein in the cytoplasmic fraction, indicating that either glycogen synthase kinase 3 and mitogen-activated protein kinase phosphorylation is dispensable for nucleocytoplasmic shuttling of the N protein or essential for nuclear translocation and/or CDK and casein kinase II-mediated phosphorylation of the N.
Interestingly, cells treated with all four inhibitors together showed that most of the N protein was localized in the cytoplasmic fraction, which further supported the above hypothesis (Fig. 6A).

Since phosphorylated N protein localized exclusively in the cytoplasm, we reasoned that phosphorylated N protein was immediately translocated to the cytoplasm. Amino acid sequence analysis of N protein revealed a putative 14-3-3 binding motif between 186 and 191 amino acid residues from the N terminus. 14-3-3 proteins are known to bind serine/threonine-phosphorylated ligands and transport them to the cytoplasm (14) besides taking part in many other cellular activities (8, 20). Hence, we checked the ability of N protein to bind 14-3-3.

Mock-transfected or N-transfected cells were immunoprecipitated using the respective antibodies and Western blotted using 14-3-3/H9252 antibody that recognizes all the 14-3-3 isoforms (Fig. 6B, upper panel). N protein was found to coimmunoprecipitate with 14-3-3 (Fig. 6B, lane 3). Since 14-3-3 is known to interact with the cargo in a phosphorylation-dependent manner, and we had earlier seen that a cocktail of inhibitors could almost block the phosphorylation of N protein, we simultaneously coimmunoprecipitated N protein maintained with the
kinase inhibitor cocktail and checked for its ability to bind to 14-3-3.

As shown in Fig. 6B, lane 4, 14-3-3 binding was significantly reduced in the presence of inhibitors. As a control to check N expression in the above samples, the same had been labeled with [35S] Promix, hence the blot was air dried and exposed to X-ray film (Fig. 6B, middle panel). To ensure equal loading of the samples, an aliquot of the samples was Western blotted using anti-calnexin antibody (Fig. 6B, lower panel). Similarly, the N protein was found to associate with 14-3-3 in human hepatoma cells to show that the same was true in a SARS-CoV replication competent cell line (data not shown). Further, we conducted immunofluorescence studies to colocalize N protein with 14-3-3. As shown in Fig. 6C, upper panel, 14-3-3 staining (fluorescein isothiocyanate labeled) and Texas red-labeled anti-mouse (Myc-tagged N, middle panel) antibodies and fluorescence was visualized by using an immunofluorescence microscope. The lower panel shows a merged image of the upper and middle panels done by using the Adobe Photoshop 6.0 program. The arrows show colocalization between N and 14-3-3 protein.
FIG. 7. N protein down-regulates 14-3-3 expression and localizes to the nucleus in the absence of growth factors. (A) pCDNA 3.1 (lane 1 and 3) or pCDNA 3.1 N (lane 2 and 4)-transfected cells were maintained for 24 h in the indicated medium 24 h posttransfection and RNA was isolated; 15 μg of total RNA of each sample was used for Northern blotting. The upper panel shows the level of 14-3-3. The same blot was probed for the level of rRNA to check equal loading in each lane (lower panel). Quantification was done using NIH Image Program and band intensity was normalized with reference to rRNA, relative fold intensity was calculated and the graph was plotted. Data represent the mean of three independent experiments. (B) pCDNA 3.1 (lane 1 and 3) or pCDNA 3.1 N (lane 2 and 4)-transfected cells were maintained for 24 h in the indicated medium 24 h posttransfection and aliquots of total cell lysates were Western blotted with 14-3-3 (first panel) or 14-3-3r antibody (second panel). The same
protein can induce apoptosis in the absence of growth factors. In an attempt to identify the mechanism of apoptosis induction by the N protein, we were trying to identify the genes that are down-regulated in N-expressing cells in the above conditions using a PCR-based cDNA subtraction technique (17). From this experiment we observed that the level of 2 independent 1.3-kilobase cDNA was down-regulated in the absence of growth factors in N-expressing cells. Sequencing of these two clones identified them to code for the 14-3-3 protein. This observation was further verified by a Northern blot analysis (Fig. 7A, upper panel, lane 4). However, there was no effect on 14-3-3 levels in N-expressing cells in the presence of serum (lane 2).

The lower panel shows the rRNA level as a loading control. We further analyzed the levels of 14-3-3 protein and found it to be down-regulated in N-expressing cells in the absence of growth factors (Fig. 7B, first panel, lane 4). However, there was no effect on 14-3-3 protein levels in the presence of growth factors (lane 2). Next we checked whether the N protein mediated 14-3-3 down-regulation was specific for 0 isoform only. For this aliquots of the lysate were immunoblotted with the anti-14-3-3 antibody, which specifically detects 1 isoform of 14-3-3. As shown in Fig. 7B (second panel), the level of 14-3-3 was found to be unaltered in N-expressing cells despite the presence or absence of serum. The ERK1/2 level was used as a control to check equal loading (Fig. 7B, 3rd panel). Quantification of the relative band intensity from three different experiments showed approximately 40% inhibition of 14-3-3 expression in serum-starved N-expressing cells compared to mock-transfected cells in the same condition.

Since N expression down-regulated 14-3-3 protein levels in the absence of growth factors, we subsequently asked whether the N protein localized to the nucleus under such conditions. Nuclear fractionation studies using 35S Promix-labeled N-expressing cell lysate in the absence of serum revealed that ~80% of the protein localized to the nucleus (Fig. 6A, lanes 13 and 14). The immunoprecipitation data were further confirmed by an indirect immunofluorescence assay of N-expressing serum-starved cells, whereby the majority of the N protein was found to be present in the nuclear fraction (Fig. 7D).

Panel I shows mock- and panel III shows N-transfected cells; both stained with Texas red. Same samples were also stained with 4′,6′-diamidino-2-phenylindole (DAPI), which is a nucleus-specific dye. Panels II and IV shows superimposition of DAPI with Texas red, thus confirming nuclear localization of N protein in serum starved cells. This observation further indicates that cytoplasmic translocation of the N protein is mediated through 14-3-3 binding. Further, membrane association of the N protein was found to be lost in the absence of growth factors (Fig. 7C, compare lane 4 with lane 2). This may be attributed to the inability of the N protein to return to the cytoplasm in the absence of serum.

Since 14-3-3 interaction appeared to be crucial for cytoplasmic translocation of N protein and the levels of 14-3-3 were found to be specifically down-regulated by the N protein, we further examined the involvement of 14-3-3 in N protein translocation by using an siRNA approach to silence the expression of 14-3-3. Transfection of an siRNA expression plasmid bearing 14-3-3-specific sequence into COS-1 cells was found to down-regulate 14-3-3 protein levels by approximately 50% (Fig. 7E). The fact that the siRNA was specific for the 14-3-3 isoform was ensured by immunoblotting aliquots of the lysate with 14-3-3-specific antibody, the level of which remained unaltered (data not shown).

Finally, plasmids expressing the N protein and 14-3-3 siRNA or GFP siRNA (control siRNA) were cotransfected into COS-1 cells; 48 h posttransfection, an increased amount of N protein was found to be retained in the nuclear fraction in 14-3-3 siRNA-treated cells (Fig. 7F, lane 4) compared to GFP siRNA-treated cells (lane 2). This further confirms that 14-3-3 protein is involved in nucleocytoplasmic translocation of the N protein. However, it might be argued that the observed phenomenon is an indirect effect of inhibition of 14-3-3. Since 14-3-3 regulates nucleocytoplasmic shuttling of many cellular factors, inhibition of its expression may block the activities of many factors that might influence cytoplasmic translocation of the N protein. Thus, confirmation of a direct role of 14-3-3 in nucleocytoplasmic shuttling of the N protein warrants further investigation.

Nucleocapsid proteins of many coronaviruses have been shown to be phosphorylated (26). However, neither the mechanism nor the functional relevance of phosphorylation is known. Here, we report that SARS-CoV nucleocapsid protein is also phosphorylated by multiple kinases. The N phosphoprotein was found to be predominantly cytoplasmic, membrane associated, and relatively stable. Further, we provided evidence that N is a substrate of the cyclin-dependent kinase, glycogen synthase kinase 3, casein kinase II, and mitogen-activated protein kinase. This agrees with our in silico data which show that the N protein does bear signature motifs for
phosphorylation by these kinases. Moreover, the above data provide clues for the fact that the phosphorylated N protein shuttles from nucleus to cytoplasm by binding to 14-3-3.

Coincidentally, while following an entirely different approach to understand the apoptosis inducing property of N, we found that the latter was able to down-regulate the levels of 14-3-3. We postulate that nuclear localization of the N phosphoprotein may be interfering with the cellular machinery, thus leading to triggering of apoptosis. However, further experiments need to be done to disentangle the exact mechanism.

From a viral point of view, we cannot pinpoint the exact functional significance of phosphorylation of N at this time. However, N phosphorylation might be serving multiple purposes to fulfill biological processes, e.g., phosphorylation may be increasing the stability of N, it may be helping in self-association of the protein, which is an essential step for viral assembly, it may be acting as a regulatory switch for subcellular localization of the protein, or it may be strategically modulating the activity of its kinases by competitively binding to their substrate binding site, thus creating a more favorable atmosphere for viral replication. Detailed analysis of such possibilities in a model system will allow critical insights into the regulatory functions of phosphorylation of the nucleocapsid protein during the SARS virus life cycle.

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