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Cellular Characterization of SARS Coronavirus Nucleocapsid

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ABSTRACT

The Severe and Acute Respiratory Syndrome coronavirus (SARS CoV) is a newly-emerged virus that caused an outbreak of atypical pneumonia in the winter of 2002-2003. Polyclonal antibodies raised against the nucleocapsid (N) of the SARS CoV showed the localization of N to the cytoplasm and the nucleolus in virus-infected and N-expressing Vero E6 cells. Like other coronavirus N proteins, the SARS N is probably a phosphoprotein. N protein expressed in mammalian cells is apparently able to "spread" to neighboring cells. For N to spread to neighboring cells, it must be exported out of the expressing cells. This is shown by the immunoprecipitation of N from the culture medium of a stable cell line expressing myc-N. Deletion studies showed that the 27 kD C-terminal domain of N (C1/2) is the minimal region of N that can spread to other cells. The nucleolar localization and spreading of N are artefacts of fixation, reminiscent of other protein-transduction domain (PTD)-containing proteins.

Keywords: SARS, coronavirus, nucleocapsid, nucleolus, transduction, fixation

INTRODUCTION

In the winter of 2002-2003, a new virus that caused SARS, an atypical pneumonia, emerged from the Southern part of China and infected people from at least 30 countries. At the end of the outbreak in July 2003, the consolidated number of patients and deaths due to SARS reported by the World Health Organization, was 8096 and 774 respectively. This outbreak not only incurred great social and medical costs to affected countries, it also had devastating effects on regional as well as global economies.

A novel coronavirus was established to be the causative agent for SARS (Drosten et al., 2003; Ksiazek et al., 2003), and was subsequently named SARS coronavirus. Its

genome of 29.6 kb revealed 14 open reading frames (orfs), encoding the replicase, spike, membrane, envelope and nucleocapsid (N), which are similar to other coronaviruses, and several other unique proteins (Marra et al., 2003; Rota et al., 2003).

This study describes the characterization of N, the nucleocapsid that encapsidates the viral RNA during viral particle assembly. The N proteins of other coronaviruses have been shown to be phosphoproteins (Stohlman et al., 1983; Yoo et al., 1992; Wootton et al., 2002) that have specific RNA-binding activity (Cologna et al., 2000; Nelson et al., 2000) and RNA replication activity (Chang and Brian, 1996). The SARS CoV N, like two other coronaviruses (Hiscox et al., 2001; Rowland, 2003), localizes to the nucleolus in fixed cells. The SARS CoV N is the major antigen recognized by convalescent antisera and can be used as a diagnostic marker for the detection of SARS antibodies (Krokhin et al., 2003; Tan et al., 2004). Several independent studies showed that the N proteins of other coronaviruses are relevant targets for T cell-mediated immune response (Wege et al., 1993; Collisson et al., 2002; Liu et al., 2001).

Here, we report a novel finding that the N protein of the SARS CoV is able to "spread" across cells. Several proteins such as the HIV TAT (Vives et al., 1997; Frankel and Pabo, 1998), *Drosophila* Antennapedia (Joliot et al., 1991), and herpes simplex virus structural protein VP22 (Elliott and O'Hare, 1997), have been described to be able to spread and also mediate the delivery of proteins fused to it across cell membranes. This process, termed "protein transduction", was thought to be energy-independent and receptor-independent. Recent studies, however, indicate that the translocation of proteins containing basic peptides or PTD's across cell membranes, was a fixation artefact (Lundberg and Johansson, 2002; Richard et al., 2003). The mechanism of protein transduction is suggested to be mediated by non-specific adhesion of PTD's, followed by endocytosis (Console et al., 2003; Lundberg et al., 2002; Potocky et al., 2003).

We showed that SARS N protein can be exported out of the cells and apparently cross cell membranes to neighboring cells. We showed that the nucleolar localization and spreading of N are artefacts of fixation, reminiscent of other PTD-containing proteins. The mechanism of export of N is still unknown, and will be an interesting subject for investigation. The possible export of N into the interstitial fluid could contribute to the immunogenicity of N, and makes N a potential and unique vaccine candidate for T cell immunity.

MATERIALS AND METHODS

Construction of Plasmids

The N orf was amplified from SARS CoV RNA by RT-PCR. RT was performed with an oligo-dT primer, and PCR was done with specific primers to amplify the N orf. To express untagged and tagged proteins in mammalian cells, DNA fragments were cloned into pXJ40 (Xiao et al., 1991) or pXJ40myc respectively. pXJ40GFP (Zhao et al., 2000) and pEGFP-N1 (Clontech) were used for expressing GFP-fusion proteins. For selecting stably transfected cell lines, myc-N was cloned into pXJ41, a derivative of pXJ40 that contains a neomycin-resistance gene.

Tissue culture

Vero E6, green monkey kidney fibroblasts, was maintained in standard Dubelcco's Minimal Eagle's medium supplemented with 10% fetal calf serum (HyClone Laboratories) and antibiotics, penicillin at 10 units/ml and streptomycin at 100 μg/ml (Sigma, U. S. A.).

To select for stable cell lines expressing myc-N, Vero E6 was transfected with pXJ41-myc-N overnight without selection. The next day, the transfected cells were trypsinized and plated at $1/10^{th}$, $1/50^{th}$ and $1/100^{th}$ density in medium containing 1 mg/ml G418 (GIBCO). After 7-10 days, isolated colonies were picked and transferred to individual wells in 96-well plates. G418-resistant clones were expanded further. Expression of myc-N was shown by Western blot (WB) and immunofluorescence (IF) analyses. A cell line that expresses a high level of myc-N in all the cells, myc-N#13, was selected for immunoprecipitating N from the medium (Figure 3A).

SARS CoV and IBV cultures in Vero E6 cells

Vero E6 were plated at near confluency and SARS CoV (strain 2774, Ruan et al., 2003) added at multiplicity of infection (MOI) of 0.1. When the cell monolayer showed the desired cytopathic effects (CPE), the cells were fixed for immunofluorescence (IF) or harvested to extract proteins.

IBV (strain P65c1) were used to infect Vero-3 cells (Shen et al., 2003) at MOI of 1. The supernatant was harvested when more than 80% of cells showed CPE. Cell debris was removed by centrifugation at 5,000 rpm (Beckman SS34) for 15 min. The supernatant containing viral particles were extracted for proteins for WB.

Phosphatase treatment

Transfected cells were lysed in lysis buffer (0.5% TX100, 0.5% NP40, 0.4 mM PMSF, 1 mM EDTA, 1 mM EGTA), and centrifuged to remove cell debris. Twenty μg of total protein from the supernatant was treated with 10 U active calf intestinal phosphatase (CIP, from Roche), at 30^oC for 30 min. As controls, 20 μg proteins were boiled in sample buffer directly, or treated with inactivated CIP, in the presence of 15 mM p-nitro-phenylphosphate, to inhibit cellular phosphatases. CIP was inactivated by incubating the enzyme at 80^oC for 10 min. After CIP treatment, the samples were boiled in sample buffer and any change in molecular weight due to dephosphorylation of proteins were visualized by WB analysis.

Immunoprecipitation (IP)

Transiently transfected cells did not express sufficient protein to be immunoprecipitated from the medium. To increase the expression, a stably transfected Vero E6 cell line in which 100% of the cells express myc-N, myc-N#13, was used. The cells were plated in a 6-cm plate at about 70% confluency for 18 h, after which and the medium and cells were collected. The medium was spun twice at 5000 rpm to remove cell debris, and 500 μl used for each IP. The cells were lysed in lysis buffer (PBS containing 0.5% TX100, 0.5% NP40, 1 mM EDTA, 1 mM EGTA, 0.4 mM PMSF), and the extracted proteins diluted to 1 μg/μl. For each IP, 100 μg of cellular proteins was used.

Protein A-G beads (Oncogene Research Products) were washed twice in IP buffer before use. For each IP, 10 μg packed volume of beads per 100 μl lysate or medium was used, with rabbit anti-N at 1:5000 dilution. The mixture was

incubated overnight at 4°C with constant rolling. The beads were washed 5 times in cold lysis buffer, and the bound proteins eluted in sample buffer by boiling for 3 min. For confirmation of results, anti-myc-conjugated agarose (Santa Cruz Biochemicals) was also used for IP. The presence of bound N protein was detected by WB analyses.

IF and WB analyses

Subconfluent Vero E6 cells were infected with SARS CoV at MOI 0.1 and grown on coverslips until cells showed a CPE of about 25%. The medium was discarded and the coverslips fixed in acetone for 20-30 min on ice. The acetone was then removed and the coverslips completely air-dried.

To observe spreading of N, cells were fixed and prepared for IF when they were confluent or subconfluent. Fixation for IF was done in 100% methanol at –20°C (Goh et al., 2001). Anti-myc antibodies (Santa Cruz Biochemicals), and fluorescein-conjugated secondary antibodies (Santa Cruz Biochemicals, U.S.A.) were applied at 1:100. An anti-N rabbit antiserum generated against a GST-NΔN120 (a.a. 121-422) was used at 1:2000 and mouse antisera raised against the same fusion protein were used at 1:200 for IF.

WB analyses were performed as described in (Khu et al., 2001). WBs were probed with anti-myc (Santa Cruz Biochemicals) at 1:1000-1:2000 dilution, anti-NΔN120 rabbit antiserum at 1:50 000 dilution and mouse anti-NΔN120 antisera used at 1:1000-1:2000 dilution.

RESULTS AND DISCUSSION

N is a phosphorylated protein

The N protein was expressed as a GST-fusion protein in bacteria and was found to be soluble, but upon elution from GSH-beads, a large portion of GST-N precipitated out. GST-NΔN120 (a.a. 121-422) was more soluble when eluted from GSH beads. Purified GST-NΔN120 (Tan et al., 2003) was injected into 2 rabbits and 5 mice to raise antibodies. All animals showed strong immune response to the antigen. Figure 1A depicts a WB with one mouse antiserum that shows no cross-reactivity to Vero E6 cell lysate, or to IBV viral particles harvested from infected culture medium (Figure 1A). A band of about 48 kD corresponding to the predicted size of unmodified N is seen in virus-infected cells early in infection (Figure 1A, 25% CPE). The molecular weight of N is higher in late infection (50-75% CPE), when expressed alone in Vero E6, and in the supernatant containing mature viral particles (Figure 1A). This upshift is probably due to phosphorylation, as was previously reported for other coronavirus N proteins (Stohlman et al., 1983; Yoo et al., 1992; Wootton et al., 2002). To confirm that the increase in molecular weight was due to phosphorylation, and to define the subdomain of N that is phosphorylated, Vero E6 cells were transfected with constructs expressing myc-tagged deletion mutants, N1, N2 and N3 (Figure 3B) and the cell lysates were untreated, or treated with inactivated or active CIP. Changes in the protein molecular sizes due to dephosphorylation were analyzed on WBs (Figure 1B). N1 and N3 showed no obvious change upon CIP treatment, indicating that these protein regions do not contain many phosphorylated amino acid residues. Interestingly, N2, when dephosphorylated showed a drastic decrease in size to that of the predicted unmodified protein. The amino acid sequence of this region contains 18 serine residues compared to 8 or 10 in N1 and N3 respectively. Most of

the possible phosphorylated sites in N are likely to be found in N2. Like their coronaviral N relatives, the SARS CoV N is also likely to be a phosphoprotein.

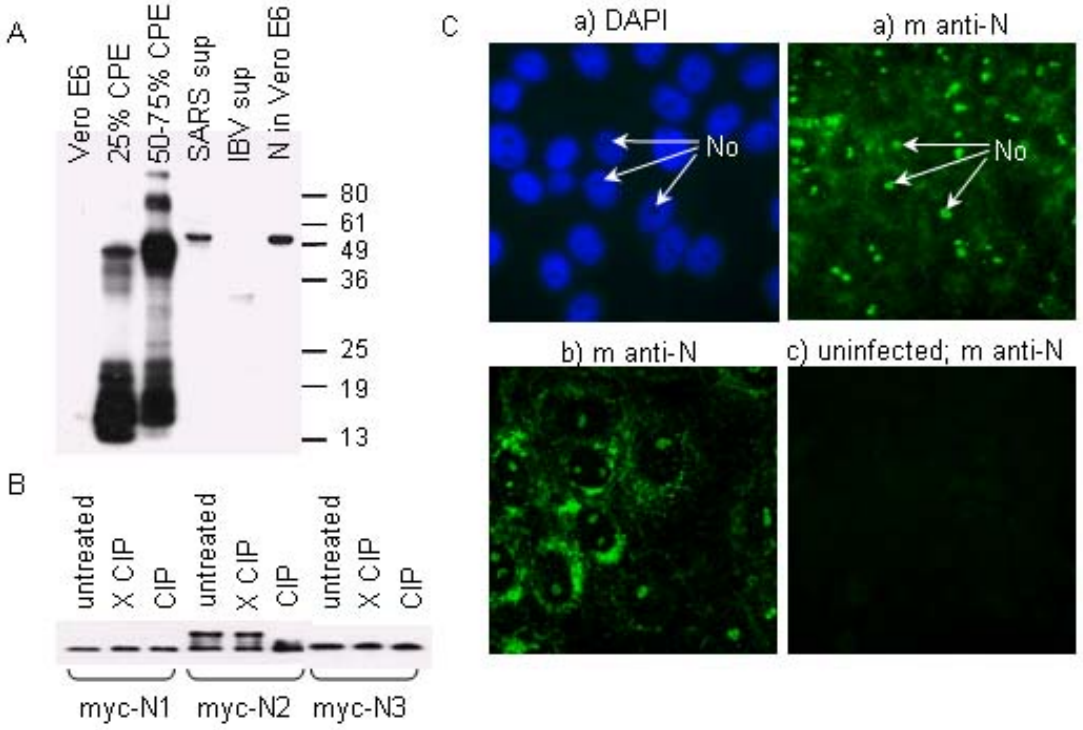

Figure 1: *N expression and localization in Vero E6 infected with the SARS virus, and in transfected cells.* A) 20 µg of protein from uninfected Vero E6 (Vero E6), Vero E6 infected with SARS CoV at about 25% CPE and 50-75% CPE, 20 µl medium from CPE 50-75% SARS viral culture (SARS sup), 20 µl medium from an IBV culture (IBV sup), 20 µg protein from Vero E6 transfected with pXJ40-N plasmid were boiled in sample buffer and separated on a 10% gel, and a WB of this gel probed with a mouse anti-N serum. B) Myc-tagged N1, N2 and N3 were expressed in Vero E6 cells and 20 µg each of untreated extracted protein (untreated), protein treated with inactivated CIP (X CIP) or active CIP (CIP) and their molecular sizes analyzed by WB analysis probed with mouse anti-N antibodies. C) Vero E6 cells infected with SARS CoV at 25% CPE were fixed in acetone and stained with mouse anti-N antibodies, followed by anti-mouse-FITC secondary antibodies. (a) The localization of N was visualized on a Zeiss Axioplan microscope and the image captured on an Axiocam digital camera. The DAPI stain shows areas unoccupied by nuclear DNA corresponding to nucleoli (No). More detailed IF was visualized on a Biorad Radiance 2000 Confocal microscope, showing exclusively nucleolar staining in some cells, while in others, both nucleolar and cytoplasmic staining (b). No background signal was seen in uninfected cells probed with mouse anti-N antibodies (c). All cells shown here were confluent or near confluent.

Another point to note is the presence of cleaved forms of N protein in virus-infected cells. Smaller fragments below 25 kD were detected with anti-N antibodies, indicating that these are proteolytic fragments of N (Figure 1A). N proteins of other coronaviruses have been shown to be cleaved by caspases (Eleouet et al., 1997), although no caspase cleavage motif is present in the SARS CoV N protein sequence (Krokhin et al., 2003). More detailed biochemical analysis is needed to characterize the phosphorylation and cleavage of N, and to understand the physiological importance of these protein modifications.

"Spreading" and nucleolar localization of SARS CoV N are artefacts of fixation

The localization of N was observed in SARS-CoV-infected Vero E6 cells by IF with mouse anti-N serum. Consistent with the localization of N of other coronaviruses, SARS-CoV N was also found in the cytoplasm and the nucleolus (Figure 1C) (Hiscox et al., 2001, Rowland et al., 2003). The nucleolar structures correspond to the areas unoccupied by nuclear DNA (Figure 1C, a). In many cells, only nucleolar localization was observed (Figure 1C, a), while in other cells, cytoplasmic staining was also seen (Figure 1C, b). N transiently expressed in Vero E6 cells was also localized to the cytoplasm and the nucleolus. The nucleolar localization of N is not due to the cross-reaction of anti-N antibodies to a nucleolar protein because uninfected cells (Figure1C, c) stained with rabbit or mouse anti-N antisera did not show any signal in the nucleolus.

In a time course experiment, Vero E6 were transfected with a construct expressing untagged N (Figure 2A). Six hours after transfection, a gradient of N-specific staining spreading from the transfected cells to surrounding cells was evident. This halo of N protein increases with increasing time of N expression, so that at 14 hours post-transfection, almost all cells in the monolayer culture have N in the nucleolus and cytoplasm. As a comparison, the N protein from IBV was transiently expressed in Vero E6 cells does not exhibit this spreading phenomenon.

A similar phenomenon described for proteins containing PTD's was recently shown to be an artefact of incomplete fixation, resulting in the dispersion of PTD proteins from endocytic vesicles to the cytoplasm, nucleus and other cellular structures (Lundberg and Johansson, 2002; Richard et al., 2003). To determine if N behaves like a PTD protein, a variety of fixation conditions were applied on Vero E6 cells transiently expressing myc-N. Generally, the nucleolar localization of N was observed in mild fixation conditions in methanol (2.5-7.5 min) at -20^{o}C and paraformaldehyde (5-10 min) at 4^{o}C. In more stringent fixation conditions N was only detected in the cytoplasm. Similarly, the spreading effect was not seen in cells fixed in methanol for more than 20 min, and in cells fixed in PFA even for very brief periods (2-5 min). A range of fixation conditions and localization data are shown in Table 1. This artefactual localization of N was confirmed by the observation of live Vero E6 cells expressing GFP-N (Figure 2B) or N-GFP (data not shown). GFP fusion proteins were only present in the cytoplasm and surrounding cells did not show any fluorescence. Upon methanol fixation, GFP-N (Figure 2C), GFP-N became localized to the nucleolus and cytoplasm of the transfected and surrounding cells, suggesting that the GFP moiety at either the C- or N-terminus did not impede the movement of N to the

nucleolus and into the surrounding cells upon fixation. Together, these data indicate that the SARS N behaves like a PTD-containing protein and that the nucleolar localization and spreading of N are artefacts of fixation.

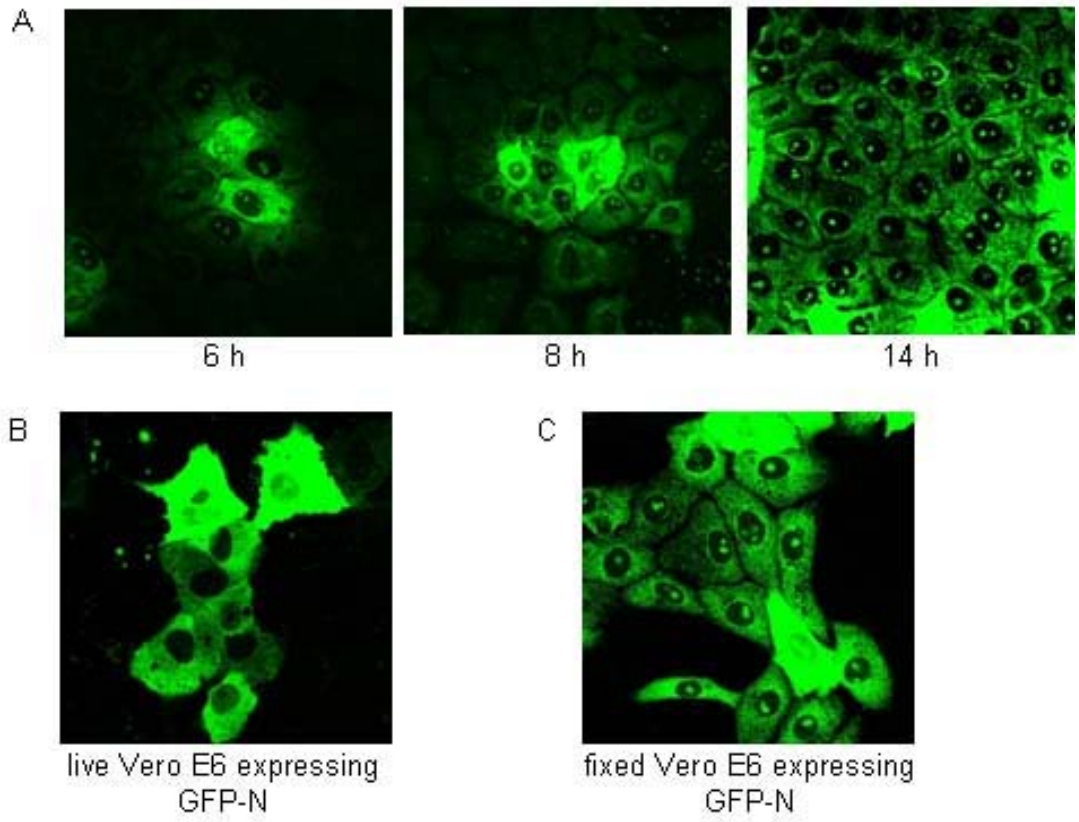

Figure 2: *N translocation and nucleolar localization are seen in fixed cells but not in live cells.* (A) Vero E6 cells were transfected with a construct expressing untagged N. Six, 8 and 14 hours post-transfection, and the cells processed for IF with anti-N antibodies. Increasing expression and increasing "spreading" of the N protein to the cytoplasm and nucleoli of neighboring cells was observed in the time-course experiment. These 3 images were taken at the same gain setting on the Confocal microscope. B) Vero E6 cells expressing GFP-N were observed without fixation. C) Upon fixation, GFP-N expressed in Vero E6 cells appears to spread to neighboring cells, and became localized to the nucleolus. Cells in C were visualized with a mouse anti-N polyclonal antibody and these images were taken on a Radiance 2000 Confocal microscope (Biorad). All cells shown here were confluent or near confluent.

A short peptide of basic residues (KKDKKKK) towards the C-terminus of N is probably not sufficient for membrane binding and translocation upon fixation, as N3 which contains this basic peptide and IBV N which has a similar sequence (KKEKKLKK), is not able to spread across cells.

Table 1: Fixation conditions and N localization

Fixative, temp	Fixation time (min)	Staining	Nucleolus	Spreading
Methanol, –20°C	2.5	+	+	+
	5	+	+	+
	7.5	+	+	+
	10	+	-	+
	15	+	-	+
Acetone, -20°C	5	+/-	+	+
	20	+	+	+
Acetone, 22°C	5	+	+	+
	20	+	+	+
PFA, 4°C	2.5	-	N.D.	N.D.
	5	-	N.D.	N.D.
	10	+/-	+/-	-
	20	+	-	-
PFA, 22°C	2.5	-	N.D.	N.D.
	5	+/-	+/-	-
	10	+	-	-
	20	+	-	-

Fixation conditions affect the nucleolar localization and spreading effect of N. Vero E6 cells expressing myc-N were fixed with in methanol, acetone or paraformaldehyde (PFA) and at various temperatures (temp) for the lengths of time indicated. The fixed cells were processed for IF and labeled with mouse anti-N antisera followed by anti-mouse IgG conjugated to FITC. The intensity of the IF, nucleolar localization and spreading effect observed under each condition were recorded. Where the cells were not stained (-), nucleolar localization and spreading could not be determined (N.D.). Weak and strong signals are indicated as (+/-) and (+) respectively.

Export of N into the medium

For N to "spread" to surrounding cells, it must be exported out of the expressing cells into the medium. To detect N in the medium, we used a stably transfected Vero E6 cell line in which all the cells express myc-tagged N. After allowing these cells to grow for 18 hours, the medium was collected and cellular proteins extracted. The cell lysate and medium were incubated with anti-N polyclonal antibodies and Protein A-G beads. Two bands were detected in cell lysates, and both were immunoprecipitated by anti-N antibodies (Figure 3A, lanes 1, 2). The lower of the two bands could correspond to the caspase-cleaved product or the unphosphorylated form, which is not always seen. Although myc-N could not be detected in the medium (Figure 3A, lane 4), the upper band was precipitated by the anti-N antibodies from the medium (Figure 3A, lane 5). The lower band was not immunoprecipitated, indicating that it was not exported into the medium. This observation also argues against the possibility that the presence of N in the medium was due to the release of N proteins from lysed cells. When no antibody was added, no protein was immunoprecipitated, indicating that N was specifically immunoprecipitated by the rabbit anti-N antibody (Figure 3A, lanes 3, 6). As a confirmation, anti-myc polyclonal

with Protein A-G beads, and anti-myc monoclonal antibody conjugated to agarose beads also immunoprecipitated myc-N from the culture medium.

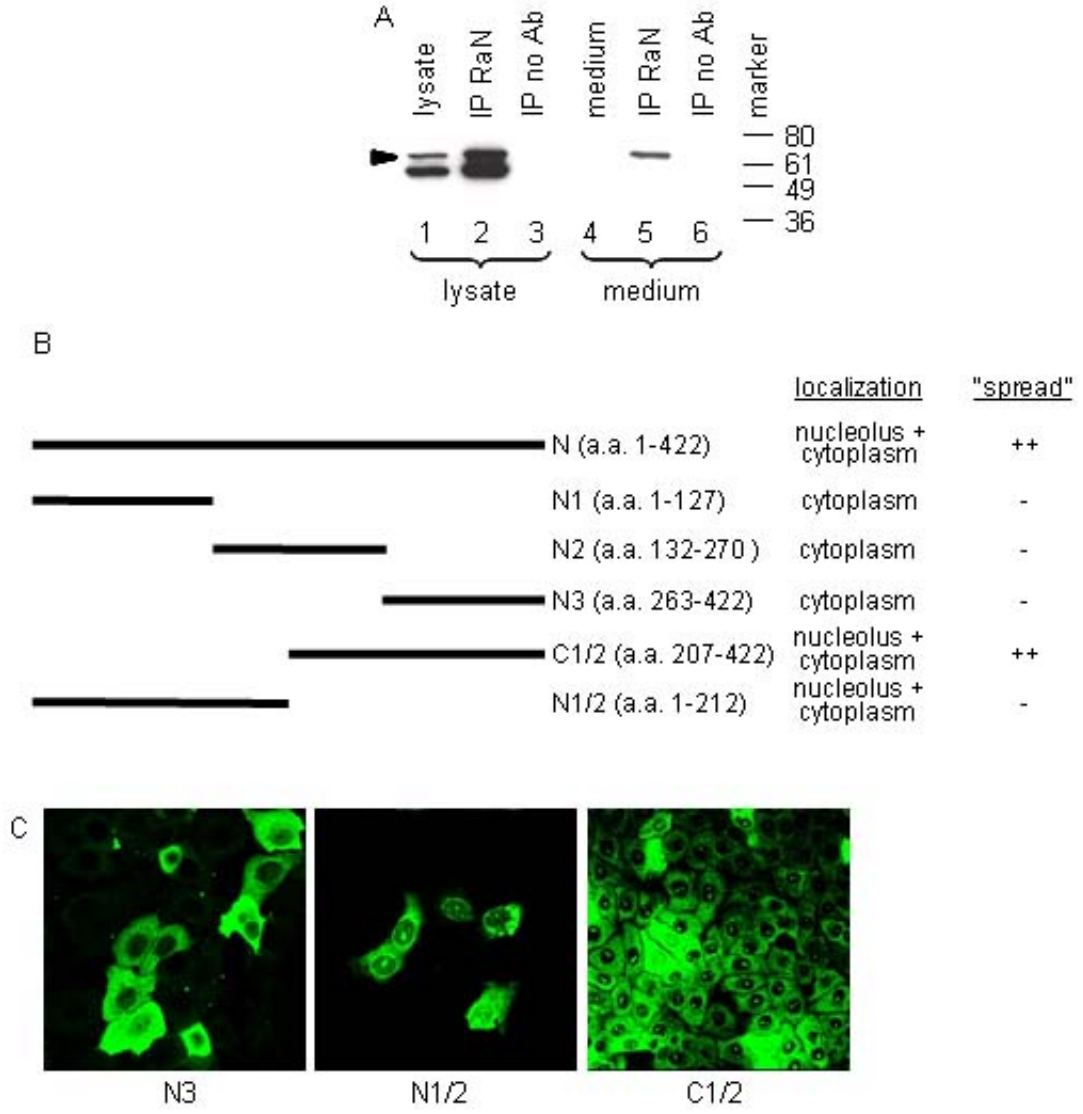

Figure 3: *Requirements for translocation of N to neighboring cells.* (A) A stably transfected cell line expressing myc-N was plated for 18 h, after which the medium was collected and the cells extracted for proteins. Total protein (100 µg at 1 µg/µl) and 500 ml medium were incubated with 20 ml packed volume of Protein A-G beads with (lanes 2, 5) or without (lanes 3, 6) rabbit anti-N antibody. Bound proteins were eluted and detected on a WB with a mouse anti-myc antibody. To detect for myc-N expressed in the cells or exported in the medium, 20 µg of total protein (lane 1) and 30 µl of medium (lane 4) were included on the WB. B) A schematic representation of deletion mutants of N, their cellular localization and their ability to spread to surrounding cells are summarized. C)

Cellular localization of the myc-tagged deletion mutants N1/2, N1, N2, N3 and C1/2 was observed in transiently transfected Vero E6 cells. N1, N2 and N3 show the same cytoplasmic localization pattern. N1/2 and C1/2 are localized to the cytoplasm and nucleolus, but only C1/2 can spread efficiently.

N is apparently exported into the medium, and this could explain how it can be subsequently taken up by cells that do not express N. The mechanism of export is not yet known, as N does not possess classical signal peptide or membrane anchoring sequences for targeting it to the ER to the exocytosis pathway. N may define a novel protein export pathway. The ability of SARS N to exit cells could explain how an internal viral protein is a major antigen that induces strong humoral response in all infected patients tested (Tan et al., 2004).

To define if there are sub-domain(s) of N that allows N to be exported and therefore spread, a series of deletions were made (Figure 3B). Fragments N1, N2 and N3, which correspond to about one-third of N each were localized to the cytoplasm but not in the nucleolus, and also failed to cross to neighboring cells (Figure 3B, C). C1/2 and N1/2, which correspond to the C-terminal and N-terminal halves of N were localized to the nucleolus and cytoplasm, but only C1/2 was able to spread well (Figure 3C). The minimal region that can translocate across cells is the C-terminal half of N, and smaller regions contained in N2 and N3 were incapable of crossing cell membranes. Based on these observations, C1/2 of about 27 kD therefore defines the minimal region that can be exported out of the cells into the medium, and somehow cross cell membranes of neighbouring cells.

In vitro uptake experiments (Figure 4) in which recombinant N was added to the cells suggest that cellular modification, such as phosphorylation of N may play a part in conferring the appropriate properties to allow full length N to spread across cells, as cells do not take up N expressed in bacteria (Figure 4C). However, recombinant N microinjected into cells can cross cell membranes and become localized to neighbouring cells upon fixation (Figure 4D). Cellular modification, possibly phosphorylation of N, allows full-length N to be exported and/or adhere to cell membranes. Deletion of the N-terminal 120 amino acids also allows N to be taken up from the medium (Figure 4C).

Interestingly, protein spreading such as that exhibited by the RNA-binding ORF3 protein of plant umbraviruses without the formation of mature viral particles, has been proposed to be a mechanism for rapid viral infection (Taliansky and Robinson, 2003). It has been suggested that the spreading of HSV tegument protein VP22 serves to prepare cells for subsequent infection of HSV virions.

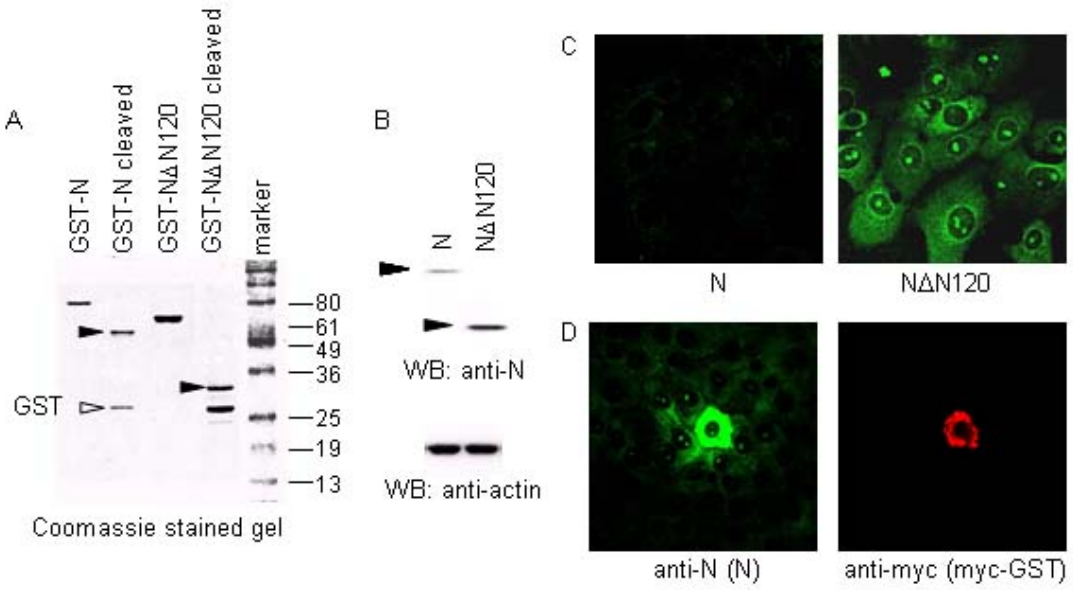

Figure 4: *Cells "internalize" recombinant NΔN120 but not full-length N.* A) 1.5-2 µg of cleaved and uncleaved recombinant GST-N and GST-NΔN120 were separated by gel electrophoresis and stained with Coomassie. B) Vero E6 cells were incubated with 5 mM recombinant N and NΔN120 for 15 h, lysed and 15-20 µg total protein loaded. WB was probed with rabbit anti-N antibody, and then reprobed with anti-actin antibody to show equal loading. C) IF of Vero E6 cells incubated with recombinant N and NΔN120. Recombinant N was not detected in cells while NΔN120 was present in the cytoplasm and nucleolus. D) Vero E6 cells were microinjected with recombinant N (5 mg/ml) together with a plasmid expressing myc-GST (0.1 mg/ml) using an Eppendorf Microinjector. The cells were incubated for 14 hours, then stained for N and myc-tagged GST with anti-N and anti-myc antibodies respectively. GST remained in the injected cell, while N was detected in neighboring cells.

In conclusion, we have shown that the SARS CoV N is a phosphoprotein that is localized to the cytoplasm, and only to the nucleolus under mild fixation conditions. It behaves like a PTD protein, in that it is able to "spread" to neighbouring cells, and is taken up *in vitro* by cells upon fixation. Although the spreading and *in vitro* uptake are artefacts of fixation, the exit of N must occur in order for N to spread to surrounding cells. This was shown by the IP of N from the culture medium. The mechanism of export is not known, but is dependent on the C-terminal half of N and probably cellular modification.

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