ISSN 1672-9145 Acta Biochimica et Biophysica Sinica 2004, **36**(11): 754–758 **CN** 31-1940/Q

Expression, Purification and Sublocalization of SARS-CoV Nucleocapsid Protein in Insect Cells

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Abstract The causative agent of severe acute respiratory syndrome (SARS) is a previously unidentified coronavirus, SARS-CoV. The nucleocapsid (N) protein of SARS-CoV is a major viral protein recognized by acute and early convalescent sera from SARS patients. To facilitate the studies on the function and structure of the N protein, this report describe the expression and purification of recombinant SARS-CoV N protein using the baculovirus expression system. Recombinant hexa-histidine-tagged N protein with a molecular mass of 47 kD was produced in insect cells. Recombinant N protein was purified to near homogeneity by Ni^{2+}-NTA affinity chromatography. In addition, we examined the subcellular localization of the N protein by confocal microscopy in *Trichoplusia ni* BT1 Tn 5B1-4 cells infected with recombinant baculovirus. The N protein was found localized in the cytoplasm as well as in the nucleolus. The purified recombinant N protein can be used in further functional study of SARS-CoV.

Key words severe acute respiratory syndrome (SARS); SARS coronavirus; nucleocapsid protein; baculovirus; insect cells

In March 2003, a novel member of coronavirus, the severe acute respiratory syndrome coronavirus (SARS-CoV), was identified as the causative agent of SARS [1]. SARS-CoV is an enveloped virus containing a single-stranded, positive-sense RNA genome of about 29 kb nucleotides in length that encodes four viral structural proteins including the spike (S) glycoprotein, the matrix (M) protein, the small envelope (E) protein and the nucleocapsid (N) protein [2].

Similar to those of animal coronavirus, the N protein of SARS-CoV appears the major immunogenic antigen. It is abundantly produced during viral infection and is readily recognized by acute and early convalescent sera from SARS patients [3–12]. Clinical diagnose asserted the sensitivity and the specificity of SARS IgG serologic test [4–6]. Some antigenic peptides of the SARS-CoV N protein can be recognized by T-cells on the surface of infected cells [7], suggesting an intriguing role of the N protein in the generation of primary humoral immune response against SARS-CoV infection. A recent study showed DNA vaccines containing the N protein expression cassettes were capable of generating strong N-specific humoral and cellular immune responses and might potentially be useful in the control of infection by SARS-CoV [8,9]. These data suggest that the N protein is a suitable candidate for early diagnostic applications and developing SARS-CoV vaccines [10–12].

Previous study showed that association with the nucleolus is a common feature of the N protein of coronaviruses [13]. The N protein of the transmissible gastroenteritis virus (TGEV, group I), mouse hepatitis virus (MHV, group II), and infectious bronchitis virus (IBV, group III) all localize in the cytoplasm as well as a structure in the nucleus proposed to be the nucleolus [14–16]. However, Chang *et al*. [17] recently reported that the N protein of SARS-CoV was only present in the cytoplasm.

Received: August 16, 2004 Accepted: October 8, 2004
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In this paper, we report the successful expression and purification of the N protein of SARS-CoV with the baculovirus system and affinity chromatography. Furthermore, using confocal microscopy, we investigated the intracellular localization of the N protein in insect cells and found that the N protein was present in both the cytoplasm and a structure in the nucleus.

Materials and Methods

Reagents

Rabbit anti-SARS-CoV N protein polyclonal antiserum was generated by our lab. Mouse anti-6×His monoclonal antibody, peroxidase-conjugated (HRP) goat anti-mouse IgG, and alkaline phosphatase (AP) conjugated goat anti-rabbit IgG were purchased from Gibco BRL. Ni^{2+}-NTA Sepharose 6B fast flow was from Sigma.

Plasmid construction

The complete coding sequence for the N protein (BJ01 strain, GenBank accession No. AY536760) was amplified by PCR. The amplified product was digested with *Eco*RI and *Bam*HI, and then inserted into pBluescript II SK (+) vector (Stratagene) to create pBluescript-N. pBluescript-N was then cut with *Eco*RI and *Not*I, and the resulting fragment was inserted into the *Eco*RI and *Not*I sites of pFastBacI (Invitrogen) to form pFastBac-N, in frame to the C-terminal of 6×His tag. The inserted N fragment in pFastBac-N was sequenced.

Cell line

Trichoplusia ni BT1 Tn 5B1-4 (Tn 5B1-4) cells were maintained as monolayer cultures at 27 °C in TNM-FH insect medium (Gibco BRL) supplemented with 10% fetal calf serum.

Production of recombinant baculovirus

Bac-to-Bac system (Gibco BRL) was used to generate recombinant bacmid carrying the N coding sequence. *Escherichia coli* Max efficiency DH10Bac cells were transformed by pFastBac-N and plated onto LB agar plates containing 50 μg/ml kanamycin, 8 μg/ml gentamicin, 10 μg/ml tetracycline, 40 μg/ml IPTG, and 100 μg/ml Bluegal. Recombinant bacmids were isolated from the transformed bacterial cells and used to transfect Tn 5B1-4 cells with Lipofectin reagent (Invitrogen Life Technologies) following the manufacturer's instructions (Bac-to-Bac Baculovirus Expression System Instruction Manual). Recombinant baculoviruses were harvested from cell culture medium 72 h post-transfection and amplified to yield high-titer virus stocks.

Optimization of the expression of the recombinant protein

Tn 5B1-4 cells in 35 mm dishes were infected with recombinant viruses at a multiplicity of infection (MOI) of 5 to 10. Insect cells were harvested 24, 48, and 72 h post-infection and protein expression was analyzed by SDS-PAGE.

Purification of recombinant N protein

The harvested cells were broken by sonication. After centrifugation, the supernatant was applied to Ni^{2+}-NTA resin equilibrated with a binding buffer containing 5 mM imidazole, 500 mM NaCl, 1 mM PMSF and 20 mM Tris-HCl (pH 8.0) at room temperature. The column was then washed with a 10-column volume wash buffer containing 50 mM imidazole, 500 mM NaCl, 1 mM PMSF and 20 mM Tris-HCl (pH 8.0). The recombinant polyhistidine-tagged protein was finally eluted with elution buffer containing 1 M imidazole, 500 mM NaCl, 1 mM PMSF, and 20 mM Tris-HCl (pH 8.0). The purified protein was analyzed by SDS-PAGE.

Polyacrylamide gel electrophoresis and Western blot analysis

Protein samples were analyzed by SDS-PAGE according to the Laemmli's method. After electrophoresis, proteins were transferred to nitrocellulose membrane with Tris-glycine transfer buffer at 350 V for 1 h. The blot was incubated with rabbit anti-N polyclonal antiserum (1:5000) or mouse anti-6×His monoclonal antibody (1:1000). The AP conjugated goat anti-rabbit IgG antibody (1:5000) and the HRP conjugated goat anti-mouse IgG (1:3000) were used as the secondary antibody, respectively.

Immunofluorescence detection

Tn 5B1-4 cells were grown on glass coverslips treated with 2% glutin solution and infected by recombinant baculoviruses. Cells were fixed with 3.7% formaldehyde 24 h post-infection. Cells were sequentially incubated for 1 h at 37 °C with rabbit anti-N protein polyclonal antiserum (1:3000, provided by our lab), washed three times for 10 min each with excess phosphate-buffered saline (PBS), incubated with Texas Red-conjugated goat anti-rabbit IgG antibody (1:1000, Rockland), and washed three times for 10 min each with excess PBS. Fixed cells were separately stained with DAPI to visualize nuclear DNA.

Fluorescence microscopy was carried out with a Leica confocal microscope equipped with appropriate filter sets.

Results

Expression of the recombinant N protein

Recombinant baculovirus was obtained after transfection of Tn 5B1-4 insect cells with bacmid DNA containing the cDNA for the SARS-CoV N protein. Tn 5B1-4 cells were then infected with recombinant baculovirus and the expression of the N protein was optimized by varying the MOI and incubation time. The maximal expression was achieved at MOI of 5 and 48 h post-infection. Recombinant N protein with a molecular weight of about 47 kD was detected by SDS-PAGE [Fig. 1(A)] and confirmed by Western blot analysis (Fig. 2). The size of the recombinant protein approximates to the predicted molecular weight of the N fusion protein containing a C-terminal 6×His tag.

Purification of recombinant N protein

Recombinant N protein was rapidly purified to near homogeneity by a simple one-step Ni^{2+}-affinity purification procedure. Protein purity was analyzed and confirmed by SDS-PAGE [Fig. 1(B)]. A total of 1 mg of purified polyhistidine-tagged N protein was obtained from 1.6×10^7 cells.

Intracellular localization of the N protein

The subcellular location of the N protein was analyzed by confocal microscopy. The N protein expressed in Tn 5B1-4 cells was distributed throughout the cytoplasm alone [Fig. 3(A)] or both the cytoplasm and a structure in the nucleus [Fig. 3(D)]. To determine whether the structure in the nucleus was nucleolus and the N protein was loca-

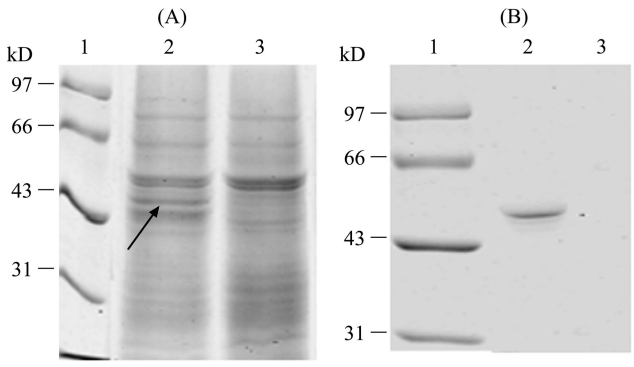

Fig. 1 SDS-PAGE analysis of recombinant N protein

(A) 1, molecular weight marker; 2, sample from Tn 5B1-4 cells infected with recombinant baculoviruses; 3, sample from Tn-5b1-4 cells without infected with recombinant baculoviruses. The arrow indicates the position of the recombinant N protein. (B) 1, molecular weight marker; 2, sample of purified N protein from insect cells infected with recombinant baculoviruses.

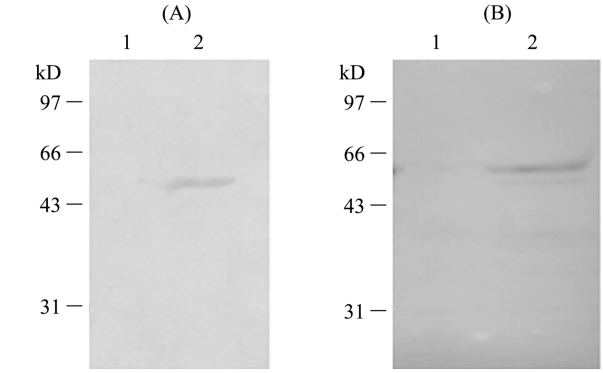

Fig. 2 Western blot analysis of recombinant N protein

(A) Western blot analysis of recombinant N protein performed using anti-6×His antibody. 1, sample from Tn 5B1-4 cells without infected with recombinant baculoviruses; 2, sample from Tn 5B1-4 cells infected with recombinant baculoviruses. (B) Western blot analysis of recombinant N protein performed using anti-N polyclonal antiserum. 1, sample from Tn 5B1-4 cells without infected with recombinant baculoviruses; 2, sample from Tn 5B1-4 cells infected with recombinant baculoviruses.

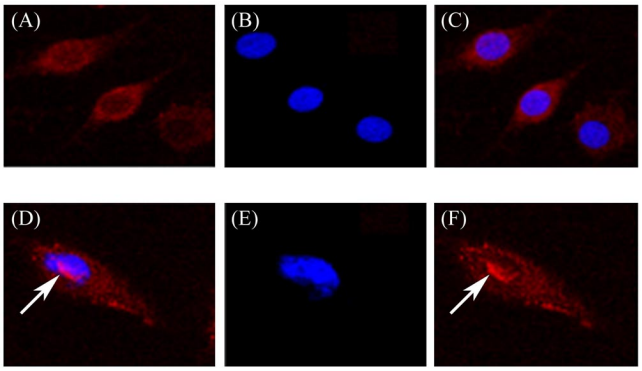

Fig. 3 Immunofluorescence detection of Tn 5B1-4 cells infected with recombinant baculoviruses

Tn 5B1-4 cells infected with recombinant baculoviruses were incubated for 24 h, fixed, and analyzed by indirect immunofluorescence detection using rabbit anti-SARS-CoV N protein antisera and Texas Red-conjugation goat anti-rabbit IgG antibodies. Additionally, cells were stained with DAPI to visualize the nuclear DNA. For images A, B, D and E, the differentially fluorescing N protein (A, D) and nuclear DNA images (B, E) were gathered separately from the same 0.5 μm optical section by using a confocal microscope and appropriate filters. Two images were digitally superimposed (A and B; D and E) to depict the distribution of the SARS-CoV N protein and nuclear DNA (C and F). The arrows indicated the position of nucleolus.

lized to this structure, cells were stained with DAPI, and the nuclear DNA visualized and nucleoli appeared no blue fluorescence [Fig. 3(B,E)]. Fluorescent images were obtained from the same 0.5-μm optical section by confocal microscopy, and digitally superimposed to localize the distribution of the N protein and nuclear DNA [Fig. 3(C, F)]. Nucleolus was identified as a distinct region within the nucleus in images from both optical section, and localization of the SARS-CoV N protein to the nucleolus was confirmed. However, in major of the cells infected by baculoviruses, no recombinant SARS-CoV N protein was detected in the nucleus [Fig. 3(C)]. A possible reason for the observation that fewer baculoviruses-infected cells showed localization of the recombinant N protein to the nucleolus is the number of cells undergoing mitosis and nucleoli are absent from mitotic cells.

Discussion

The present paper describes the expression, purification and sublocalization of recombinant SARS-CoV N protein using the baculovirus expression system. Compared with the prokaryotic expression system, the baculovirus-insect cell expression system is a convenient and versatile eukaryotic system for heterologous gene expression. It can provide correct folding of recombinant protein and other important post-translational modifications similarly to that of mammalian cells. These properties may play an important role in exerting biology functions [22]. The N protein of other coronaviruses are involved in the formation of viral capsid, the transcriptional regulation of viral genome, and the packaging of viral RNA. Present report showed that the N protein of SARS-CoV selectively activated the AP-1 pathway [18], induced apoptosis in COS-1 cells in the absence of growth factors [24] and interaction with membrane protein of the SARS-CoV [25]. Analysis of the structure and function of the SARS-CoV N protein will be beneficial for the understanding of the life cycle and pathogenicity of SARS-CoV. The purified recombinant N protein will be a valuable tool in the further functional study of SARS-CoV.

The clinical diagnosis of the infection by SARS-CoV is important to detect and treat SARS, which is a new and poorly understood disease. However, the serologic diagnosis remains an indispensable means for confirming viral infection status. The study of Paul *et al*. [4] indicated that an infected cell-base indirect immunoflurescence test for anti-SARS IgG antibody was sensitive and specific. ELISA based on the purified recombinant SARS-CoV N protein from bacteria expression system has high sensitivity and specificity [1]. These studies indicate that recombinant N protein holds a significant value for SARS diagnosis. Some researches show that when a recombinant protein is produced for antibody determination, the influence of the expression system is a major variable since it may affect the sensitivity and specificity of the serologic assay [20, 21]. The purified protein in this study can be used to compare with the prokaryotic recombinant N protein in the antigenic activity to optimize diagnostic methods.

In baculovirus expression system, recombinant protein is processed, modified, and targeted to its appropriate subcellular location, where usually reflects its original location [22]. The SARS-CoV N protein contains a putative nuclear localization signal (KKDKKKK, amino acid 370–376). We used recombinant baculovirus to infect Tn cells to study the localization of the SARS-CoV N protein. The protein was shown to localize either to the cytoplasm alone or to the cytoplasm and a structure in the nucleus. A recent study showed that in Vero E6 cells infected with SARS-CoV, there were some virus-likes particles in the nucleus [23]. Previous study showed that association with the nucleolus is a common feature of the N protein from the order *Nidovirales* [13]. The virus-like particle may be the N protein. The function of N protein in the nucleolus is unknown. The intracellular localization may play a role in the regulation of the cell cycle, and could be part of a general virus strategy to sequester ribosomal subunits for preferential translation of virus proteins.

Acknowledgements

We are grateful to Wang XS for helpful comments and Qin J for providing reagents and fluorescent analysis.

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Edited by
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