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Virus Research 127 (2007) 71–80
Virus Research
www.elsevier.com/locate/virusres

Antigenic characterization of severe acute respiratory syndrome-coronavirus nucleocapsid protein expressed in insect cells: The effect of phosphorylation on immunoreactivity and specificity

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Received 5 September 2006; received in revised form 19 March 2007; accepted 21 March 2007
Available online 11 May 2007

Abstract

The nucleocapsid (N) protein of severe acute respiratory syndrome-coronavirus (SARS-CoV) is involved in the pathological reaction to SARS and is a key antigen for the development of a sensitive diagnostic assay. However, the antigenic properties of this N protein are largely unknown. To facilitate the studies on the function and antigenicity of the SARS-CoV N protein, 6× histidine-tagged recombinant SARS-CoV N (rSARS-N) with a molecular mass of 46 and 48 kDa was successfully produced using the recombinant baculovirus system in insect cells. The rSARS-N expressed in insect cells (BrSARS-N) showed remarkably higher specificity and immunoreactivity than rSARS-N expressed in *E. coli* (ErSARS-N). Most of all, BrSARS-N proteins were expressed as a highly phosphorylated form with a molecular mass of 48 kDa, but ErSARS-N was a nonphosphorylated protein. In further analysis to determine the correlation between the phosphorylation and the antigenicity of SARS-N protein, dephosphorylated SARS-N protein treated with protein phosphatase 1 (PP1) remarkably enhanced the cross-reactivity against SARS negative serum and considerably reduced immunoreactivity with SARS-N mAb. These results suggest that the phosphorylation plays an important role in the immunoreactivity and specificity of SARS-N protein. Therefore, the BrSARS-N protein may be useful for the development of highly sensitive and specific assays to determine SARS infection and for further research of SARS-N pathology.
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Keywords: Severe acute respiratory syndrome; Coronavirus; Nucleocapsid protein; Phosphorylation; Antigenicity

1. Introduction

Severe acute respiratory syndrome (SARS) is a newly emerging disease that is caused by the SARS-coronavirus (SARS-CoV). SARS-CoV appeared with high virulence and mortality, affecting 29 countries, with more than 8000 cases and over 916 deaths (Peiris et al., 2003; World Health Organization, 2003). Regarding the development of an efficient vaccine and a method for serological diagnosis to prepare for the reemergence of SARS, many investigators have spared no efforts towards the safe provision of large quantities of recombinant proteins that can be used as vaccine candidates and diagnostic reagents (Liu et al., 2003, 2004; Ren et al., 2004; Timani et al., 2004).

During SARS infection, the SARS-CoV nucleocapsid (SARS-N) protein is the most abundantly expressed of the structural proteins. Antibodies to the SARS-N protein are highly detectable in SARS patients, and the antigenicity of SARS-N protein is better than that of the SARS-CoV spike (SARS-S) protein (Chan et al., 2005a,b; Shi et al., 2003; Wu et al., 2004). Furthermore, the N proteins are capable of inducing protective immune responses against SARS-CoV infection (Kim et al., 2004). These features make it a suitable candidate for the development of diagnostic agents, and possibly subunit vaccines. Because the SARS-N gene contains no glycosylation sites (Marra et al., 2003), SARS-N proteins have been expressed mostly in *E. coli* (ErSARS-N proteins) for use as a diagnostic reagent. However, some cross-reactive responses of this recombinant protein with the antibodies against other coronaviruses have been detected by Western blot and ELISA (Sun and Meng, 2004; Woo et al., 2004; Yu et al., 2005). Since serum antibodies against the other coronaviruses are widespread within the human

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doi:10.1016/j.virusres.2007.03.019

population (Hruskova et al., 1990; Schmidt et al., 1986), it is important to clear the antigenic properties of this recombinant protein.

The SARS-N protein contains 423 amino acids and has been predicted to be a phosphoprotein with a molecular mass of approximately 46 kDa (Marra et al., 2003). The phosphorylation of N protein has been implicated in a variety of functions, including translocalization of N protein from nucleus to cytoplasm and membrane, encapsidation of the viral RNA genome, viral transcription and replication, and regulation of numerous signal transduction pathways in host cells (Huang et al., 2004; Surjit et al., 2005). This phosphorylation of SARS-N protein may also affect the antigenicity of the protein. However, the phosphorylation of proteins expressed in prokaryotic systems has not been reported, while N protein expressed in mammalian cells is mainly phosphorylated at serine/threonine residues by multiple kinases (Surjit et al., 2005). Thus, ErSARS-N protein could be antigenically different from native SARS-N protein in virus-infected cells and phosphorylated rSARS-N protein expressed in a eukaryotic system.

In the present report, we examined this possibility using the SARS-N protein expressed in insect cells (BrSARS-N); this protein was a highly phosphorylated protein. Our results suggest that the phosphorylation of this protein affects both immunoreactivity against SARS-N antibodies and specificity against cross-reacting antibodies in normal human serum.

2. Materials and methods

2.1. Reagents and sera

ErSARS-N was purchased from Biovendor Laboratory Medicine, Inc. (Heidelberg, Germany). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-human IgG were purchased from Abcam (Cambridgeshire, UK). The five SARS positive sera were collected at the Robert Koch Institute in Berlin, Germany acting within the European Network for Diagnostics of Imported Viral Diseases (ENVID). These sera were kindly obtained from Prof. M. Peiris (University of Hong Kong, China) and Dr. M. Zambon (Health Protection Agency, London). All five sera were from patients with SARS confirmed according to the WHO criteria, and had documented titers in immunofluorescence, ELISA, and neutralization assays. These specimens were used for quality control of different serological assays performed in different European laboratories acting as National Reference Centers for SARS diagnosis. Sera from 20 healthy donors and 160 non-SARS patients that were confirmed to be SARS negative by microneutralizing assay as the gold standard method for SARS serology were used for specificity analysis of the rSARS-N protein (Chan et al., 2005a,b). The SARS-N mAbs were used to examine the immunoreactivity of rSARS-N protein. This mAbs were mixed with 07-19-11 (N-terminus) and 21-10-06 mAbs (C-terminus) that were produced and characterized previously (Shin et al., 2006).

2.2. RNA extraction

SARS-CoV Urbani strain, provided from W. Bellini of the Center for Disease Control and Prevention, was propagated in a vero cell line maintained at 37 °C in Eagle's minimum essential medium supplemented with 2% fetal bovine serum for 4 days. Upon observation of a 90% cytopathic effect (CPE), the infected culture supernatant was clarified by centrifugation at 2000 × *g* for 10 min. Viral RNA was extracted from 140 μl of infected culture supernatant by using the QIAamp viral RNA mini kit (Qiagen) according to the instructions of the manufacturer. All experiments with live viruses were performed in a biosafety level 3 laboratory.

2.3. Preparation of recombinant baculovirus and mammalian expression construct

The complete coding sequence for the N protein (Urbani strain, GenBank accession No. AY278741, 28120–29388 bp) was amplified by RT-PCR as described previously (He et al., 2004), digested with *Eco*RI and *Bam*HI, and then inserted into a baculovirus expression vector, His-tagged pEntr_BHRNX vector (Neurogenex, Republic of Korea). The resulting plasmid construct, pEntr_NP7, was confirmed by restriction endonuclease digestion and DNA sequence analysis. It was then co-transfected into Sf21 insect cells with linearized baculovirus DNA using the BaculoGoldTM system (BD Biosciences) according to the instructions of the manufacturer. The mammalian expression construct of SARS-N gene was prepared as described previously (Kim et al., 2004).

2.4. Expression and purification of rSARS-N

Sf21 insect cells (5×10^5 cells/ml) infected with recombinant baculoviruses at a multiplicity of infection (MOI) of 10 were grown in suspension cultures with Sf900II-SFM medium (Invitrogen) supplemented with 5% fetal bovine serum (Invitrogen) at 27 °C. The cells were harvested at 48 h post-infection and broken by three freezing-thawing cycles in phosphate-buffered saline (PBS). After centrifugation, the supernatants were either immediately processed or were stored at −70 °C. The recombinant SARS-N protein from mammalian cells (MrSARS-N) was expressed as described previously (Kim et al., 2004). Purification of the recombinant proteins was achieved using a Ni-NTA resin and buffer kit (Merk Bioscience) according to the instructions of the manufacturer. The supernatants were applied to Ni-NTA bind resin equilibrated with a 1× Bind buffer containing 10 mM imidazole at room temperature. The column was then washed with an 8-column volume 1× Wash buffer containing 20 mM imidazole. The recombinant 6× histidine-tagged protein was finally eluted with 1× Elute buffer containing 250 mM imidazole and 1 μM DTT. The purified proteins were either dialyzed overnight or desalted with D-Salt Excellulose Desalting column (Pierce) in PBS containing 1 μM DTT.

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