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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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, $6\times$ 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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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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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 $2000 \times g$ 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 EcoRI and BamHI, 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.

2.5. SDS-PAGE and Western blot analysis

The rSARS-N proteins were analyzed by SDS-PAGE according to the Laemmli's method (Laemmli, 1970). Protein samples were resolved by electrophoresis through 10% SDS-PAGE gels. After electrophoresis, proteins were visualized by staining with Coomassie brilliant blue or transferred to a nitrocellulose membrane (Bio-rad). The blotted membrane was blocked with 2% skim milk in PBS containing 0.05% Tween 20 (PBST) overnight at 4 °C. After the membrane was washed with PBST, it was incubated for 1 h at room temperature with a blend of SARS-N mAbs (1:4000) or serum samples (1:100). After washing, the membrane was incubated with HRP-conjugated goat anti-mouse IgG antibody (1:4000) or HRP-conjugated goat anti-human IgG antibody (1:5000) for 1 h at room temperature. Finally, the reaction results were visualized using the ECL detection system (Amersham Biosciences) or DAB (3,3′-diaminobenzidine tetrahydrochloride) substrate solution (Pierce) according to the instructions of the manufacturer.

2.6. Indirect ELISA

Hi-bind microplates (Coring) were coated with rSARS-N proteins (200 ng/well) in carbonate buffer (pH 9.6) overnight at 4 °C, and were then blocked with PBST containing 3% BSA for 1 h at room temperature. After the plates were washed three times with PBST, 100 μl of a blend of SARS-N mAbs (1:1000), positive serum samples (1:1000), or negative serum samples (1:100) diluted in PBST containing 1% BSA was added to each well, and the plates were incubated for 1 h at 37 °C. Then, after the plates were washed another three times with PBST, 100 μl of HRP-conjugated goat anti-mouse IgG (1:1000) or anti-human IgG (1:1000) was added to each well, and the plates were incubated at 37 °C for 1 h. After three more washes with PBST, 100 μl of diluted tetramethylbenzidine (TMB) substrate (Sigma) was added to each well and incubated in the dark at room temperature for 10 min. The reaction was then stopped by the addition of 100 μl of 1N H_2SO_4 to each well. The optical density at 450 nm (OD_{450}) for each well was measured in an EL340 ELISA reader (Bio-Tek Instruments Inc.). Each serum was tested independently in duplicate, more than three times, and the results were expressed as mean values ± the standard deviation from absorbance values.

2.7. Detection of phosphorylated rSARS-N

The purified rSARS-N proteins were used after they had been boiled for 5 min in 2× SDS sample buffer. These protein samples were resolved by 10% SDS-PAGE, and total proteins were visualized by staining with Coomassie brilliant blue or phosphorylated proteins were visualized by staining with a GelCode Phosphoprotein Staining kit (Pierce) according to the instructions of the manufacturer. To confirm the phosphorylation of BrSARS-N, we also further analyzed by western blot analysis using anti-phosphoserine antibody (Chemicon).

2.8. In vitro dephosphorylation assay of rSARS-N

The BrSARS-N protein (0.1 mg/ml) was dialyzed with protein phosphatase buffer (50 mM Tris–HCl buffer, pH 7.5, 5 mM DTT, 0.1 mM Na_2EDTA, 1mM $MnCl_2$, 0.01% Brij 35) and then incubated with or without protein phosphatase 1 (PP1, 2 μg/ml) for 2 h at 30 °C. The reaction was terminated by the addition of 2× SDS sample buffer. After 10% SDS-PAGE, the gel mobility shifts of dephosphorylated proteins were detected by staining with Coomassie brilliant blue. The dephosphorylated rSARS-N samples were stored at −70 °C or directly used to examine the specificity and immunoreactivity of SARS-N protein by Western blot and indirect ELISA, respectively, as described above.

3. Results

3.1. Expression and purification of the BrSARS-N protein

Sf21 cells were infected with recombinant baculovirus and the expression of the BrSARS-N was optimized by varying the cell number at infection time, the MOI, and the incubation time. Maximal expression was achieved at 5×10^5 cell/ml of Sf21 cells, 10 MOI, and 48 h post-infection. Under optimum conditions, the BrSARS-N proteins were successfully expressed in insect cells (1 mg per 1×10^7 cells) and purified by using a Ni-NTA affinity column under physiological conditions. Analysis of crude extracts in insect cells by SDS-PAGE and Coomassie brilliant blue staining revealed that double protein bands of approximately 46 and 48 kDa were markedly expressed by insect cells infected with recombinant baculovirus (Fig. 1A). These isoforms were also observed on SDS-PAGE analysis of the purified recombinant proteins, and were confirmed to be the SARS-N protein by Western blot analysis using SARS-N mAbs (Fig. 1B). This result and Fig. 1C suggested that the BrSARS-N proteins are expressed as the major isoforms at 48 kDa and minor isoforms at 46 kDa in insect cells such as MrSARS-N protein expressed in mammalian cells, while ErSARS-N proteins expressed in *E. coli* have shown a protein bands of approximately 46 kDa such as previously reported (Yu et al., 2005).

3.2. Antigenic specificity of BrSARS-N protein compared with ErSARS-N

Some reports have shown that when a recombinant protein is produced, the influence of the expression system is a major variable, as it may affect the sensitivity and specificity of the serologic assay (Marcipar et al., 2004). Therefore, we examined the antigenic specificity of BrSARS-N protein compared with ErSARS-N using an indirect ELISA on five SARS positive sera and 180 SARS negative sera. ErSARS-N protein included for comparison in the present study was expressed in the same cDNA construct at the ORF of BrSARS-N protein. This protein was previously observed to be cross-reactive with SARS negative serum (Sun and Meng, 2004; Woo et al., 2004; Yu et al., 2005). In reactions in which equal amounts of each rSARS-N protein were coated onto ELISA plates, higher specificity was observed in the BrSARS-N protein than in the ErSARS-
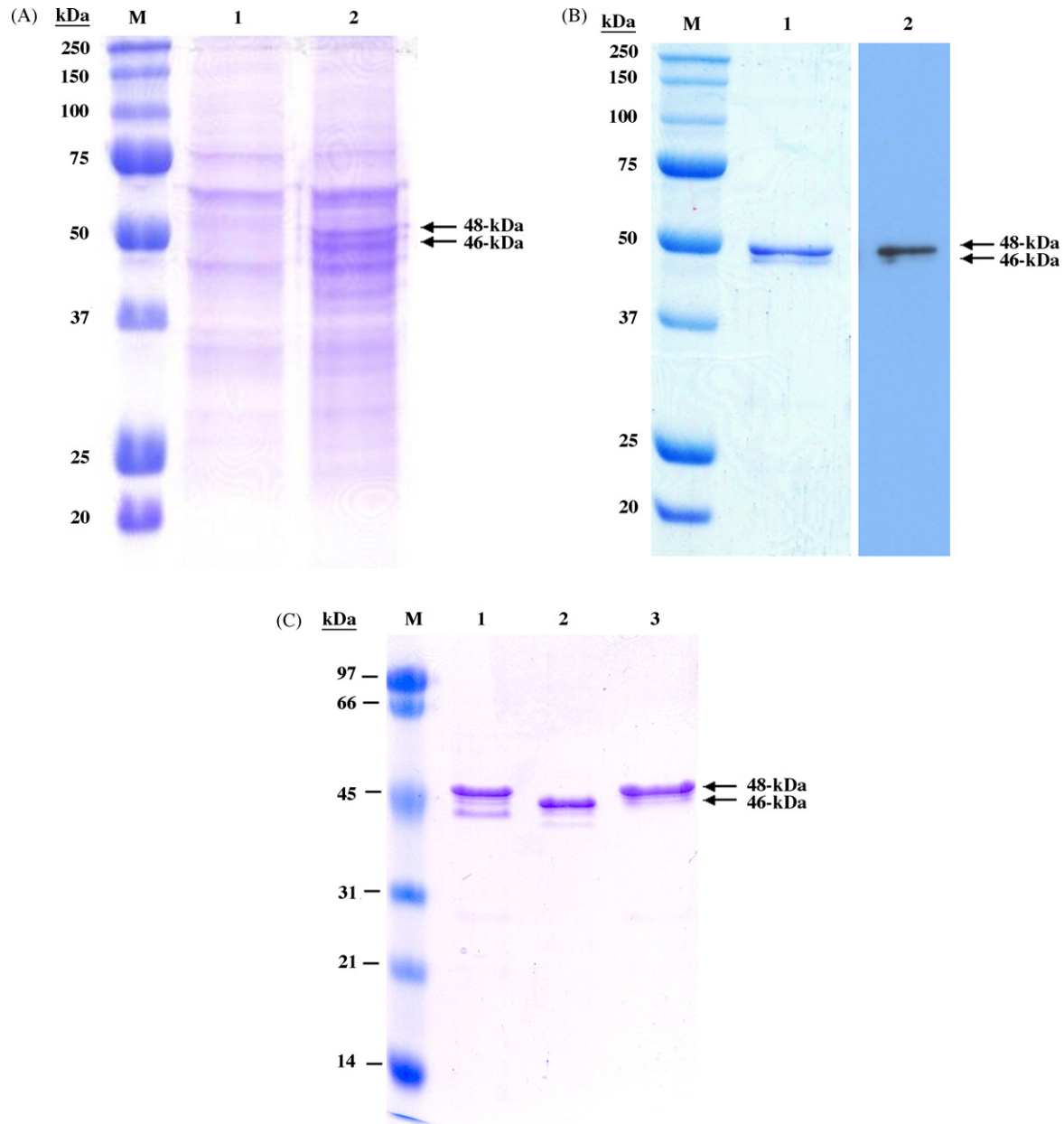
Fig. 1. Expression of SARS-N protein in insect cells using recombinant baculovirus. (A) Full-length SARS-N protein produced in Sf21 suspension cultures. Uninfected (lane 1) and infected cells with a recombinant baculovirus expressing the full-length SARS-N gene of the SARS-CoV Urbani strain (lane 2) were collected at 48 h post-infection. Cell lysates were analyzed in a 10% SDS-PAGE gel and revealed with Coomassie brilliant blue staining, revealing a major 46 and 48 kDa band expressed in insect cell-infected recombinant baculovirus. (B) Western blot analysis of purified SARS-N protein. The purified recombinant proteins were separated by SDS-PAGE (lane 1) and transferred to a nitrocellulose membrane (lane 2). The membrane was incubated with SARS-N mAbs, followed by HRP-conjugated anti-mouse IgG, and detected by chemiluminescence staining. A strong immunoreactive band and a minor band were detected at 48 and 46 kDa, respectively, suggesting that those proteins are SARS-N proteins. (C) The comparison of mass differences among BrSARS-N, ErSARS-N, and MrSARS-N protein by SDS-PAGE analysis. This result revealed that BrSARS-N was showed to a similar mass pattern with MrSARS-N protein, but molecular weight of ErSARS-N protein was slightly lower than BrSARS-N and MrSARS-N.

N protein. Thirty-four of the 180 SARS negative sera showed OD_{450} values higher than 0.500, ranging from 0.500 to 1.000 in ErSARS-N-based ELISA. In contrast, when the BrSARS-N protein was used, only four of 180 SARS negative sera showed OD_{450} values below 0.400 (data not shown). To further analyze the specificity of rSARS-N protein, we chose five SARS negative sera that had showed high OD_{450} values (>0.500) on ErSARS-N-based ELISA. The specificity of rSARS-N protein

was confirmed by indirect ELISA (Fig. 2A) and Western blotting using these negative sera and five SARS positive sera. For positive serum, ErSARS-N protein showed strong positive bands at approximately 46 kDa, while BrSARS-N protein showed two major antigenic bands of approximately 46 and 48 kDa (Fig. 2B, left panel). For negative serum, all of the five negative sera showed a strong false-positive reaction with ErSARS-N protein at the same size band of 46 kDa as result of SARS positive

serum, while none of the five negative sera showed false-positive reactions at the two major antigenic bands of BrSARS-N protein (Fig. 2B, right panel). This result indicated that the high value in indirect ELISA using ErSARS-N protein was not to the result of the potential interaction between residual *E. coli* antigens and antibodies against *E. coli* in human serum. Thus, the ErSARS-N protein has a higher cross-reactivity with SARS negative serum than BrSARS-N protein.

3.3. Immunoreactivity of BrSARS-N protein compared with ErSARS-N

Because human normal serum may contain antibodies that cross-react with SARS-N proteins (Schmidt et al., 1986; Woo et al., 2004; Yu et al., 2005), immunoreactivity analyses of ErSARS-N and BrSARS-N proteins were achieved by using a blend of SARS-N mAbs. The analysis of immunoreactivity was examined by Western blot under denaturing conditions. Both purified rSARS-N proteins were serially diluted from 125 to 1000 ng, and were examined by SDS-PAGE and Western blot using SARS-N mAbs. When both serially diluted rSARS-N proteins were loaded in the same amounts in each well of the SDS-PAGE gel, the BrSARS-N protein was significantly detected to 250 ng by SARS-N mAbs, but ErSARS-N protein was detected to 500 ng by SARS-N mAbs (Fig. 3A), which suggests that the BrSARS-N has higher antigenicity than the ErSARS-N.

To further examine the immunoreactivity of rSARS-N proteins under nondenaturing conditions, indirect ELISA was performed by using a serially diluted SARS-N mAbs. In order to determine the cut-off values, serum diluents (PBST with 1% BSA) were used to determine the baseline of each rSARS-N-based indirect ELISA, in which was found to be 0.118 at OD_{450}. Therefore, the cut-off value for detection of SARS-N antibody was set to 0.200, which is equal to the mean + two times the standard deviation of the OD_{450} for serum diluents. According to the cut-off threshold (0.200), the lower limits of the antibody detectable with BrSARS-N and ErSARS-N protein in the indirect ELISA were at the same concentration at 1.6 and 3.1 nM/well, respectively. At maximum, the immunoreactivity of BrSARS-N under nondenaturing conditions was 2.5-fold higher than that of ErSARS-N (Fig. 3B). This result indicated that BrSARS-N protein has a remarkably higher antigenicity than ErSARS-N, and smaller amounts of this protein could be
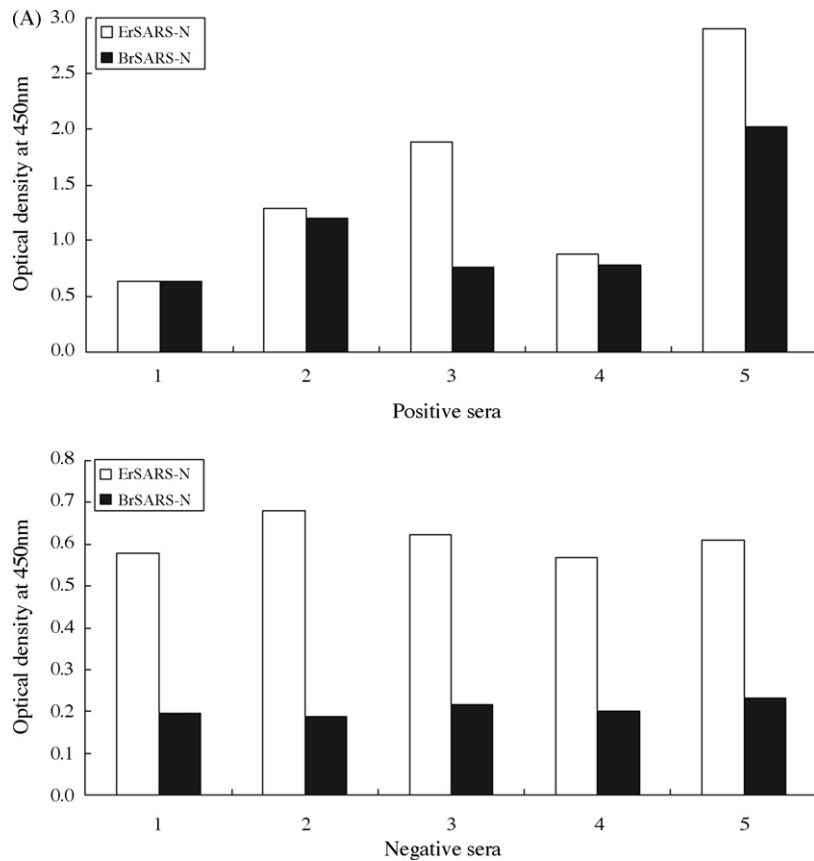

Fig. 2. Antigenic specificity analysis of recombinant SARS-N protein against human serum. (A) Purified BrSARS-N and ErSARS-N (200 ng/well) were coated onto the each microtiter plates, respectively. SARS positive (upper panel) and negative sera (lower panel) were incubated in each coated well, followed by HRP-conjugated anti-human IgG, and detection using TMB substrate solution, which indicated that all five SARS negative sera were highly cross-responsive with ErSARS-N protein, but not with BrSARS-N. (B) Purified BrSARS-N and ErSARS-N proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane separately. The membranes were cut into strips and incubated with five SARS positive sera and five SARS negative sera, followed by HRP-conjugated anti-human IgG, and proteins were detected using DAB staining. All five SARS negative sera showed strong positive bands with ErSARS-N protein, but not with BrSARS-N, suggesting that BrSARS-N protein is more specific than ErSARS-N protein.
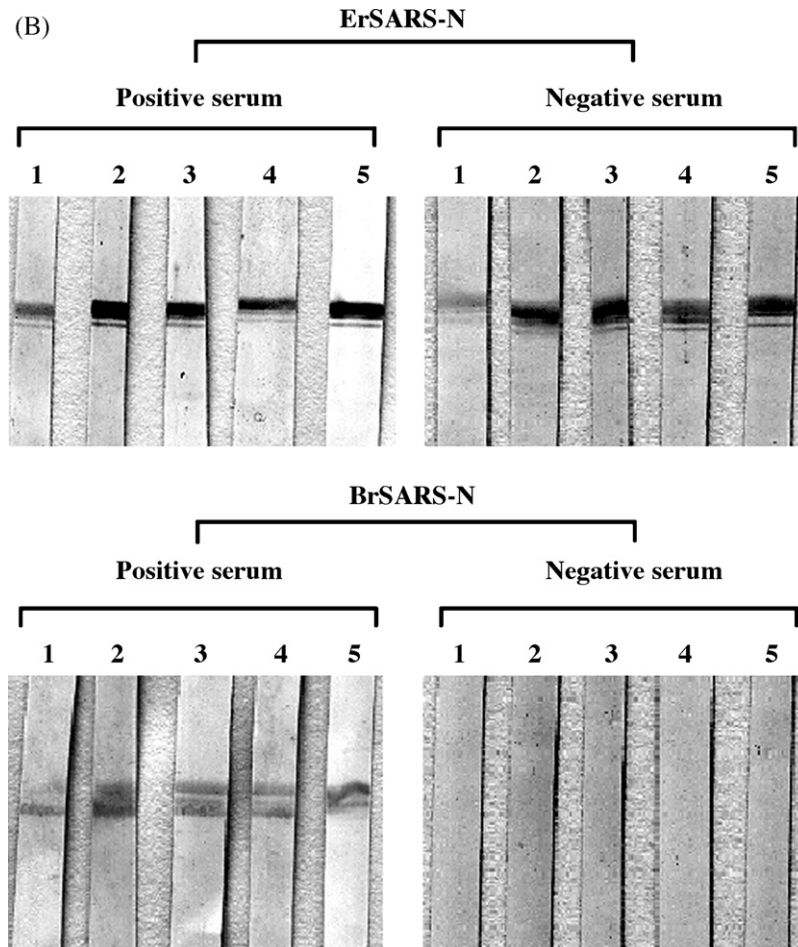
(B)

ErSARS-N

Positive serum

1 2 3 4 5

Negative serum

1 2 3 4 5

BrSARS-N

Positive serum

1 2 3 4 5

Negative serum

1 2 3 4 5

Fig. 2. (*Continued*).

used to detect antibody and thus may be more economical than ErSARS-N.

3.4. The effect of phosphorylation on specificity and immunoreactivity of rSARS-N protein

Since SARS-N protein expressed in mammalian cells has been reported to be a highly phosphorylated protein (Surjit et al., 2005; Zakhartchouk et al., 2005) and a baculovirus expression system can provide correct folding of recombinant protein and other important post-translational modifications similarly to that of mammalian cells, we examined the phosphorylation of BrSARS-N. Equal amounts of the BrSARS-N and ErSARS-N protein were subjected to SDS-PAGE. Total rSARS-N proteins were visualized by Coomassie brilliant blue staining (Fig. 4A, upper panel), and the phosphorylated rSARS-N proteins were investigated by GelCode Phosphoprotein Staining kit (Fig. 4A, lower panel) and western blot analysis using anti-phosphoserine antibody (Fig. 4B). This result was indicated that BrSARS-N protein was highly phosphorylated, while ErSARS-N protein was not.

We performed further analysis to confirm the phosphorylation of BrSARS-N protein. The BrSARS-N proteins that had been treated or not treated with PP1 were subjected to SDS-PAGE, and protein bands were visualized by Coomassie brilliant blue staining. There was no decrease in the intensity of the asterisk band indicating contaminated protein from insect cells during purification of recombinant protein (Fig. 5A, upper panel), which suggests that the enzyme specifically removed only the phosphate residues from the protein. In addition, a shift of the 48 kDa bands was observed, with a drop to a lower mass of 46 kDa after treatment with PP1 (Fig. 5A, upper panel); the two isoforms were confirmed as SARS-N protein by Western blot using SARS-N mAbs (Fig. 5A, middle panel). This result indicated that the isoforms of 48 kDa and 46 kDa in BrSARS-N protein represent the phosphorylated and nonphosphorylated form, respectively. These data also revealed that isoforms of 48 kDa were expressed more abundantly than isoforms of 46 kDa (Figs. 1B and 5A), and suggested that BrSARS-N protein is highly phosphorylated in insect cells. This feature of BrSARS-N protein may be the cause of greater immunoreactivity and specificity than that expressed in the prokaryotic system, as shown in Figs. 2 and 3.

To determine the correlation between the phosphorylation and the specificity of SARS-N protein, the BrSARS-N proteins treated or not treated with PP1 were examined by Western blot analysis using SARS negative serum that had shown a cross-reaction with ErSARS-N protein. As shown in the lower panel
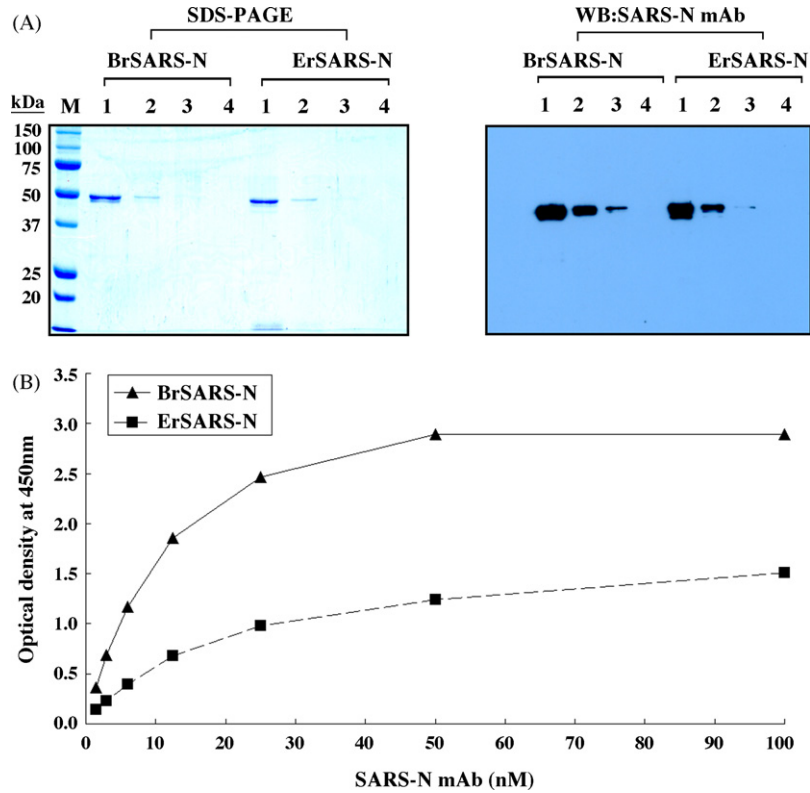
Fig. 3. Immunoreactivity analysis of recombinant SARS-N protein using mAbs. (A) Immunoreactivity analysis of BrSARS-N and ErSARS-N protein by Western blot under denaturing conditions. The serial diluents of both recombinant proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with SARS-N mAbs, followed by HRP-conjugated anti-mouse IgG, and proteins were detected using chemiluminescence staining. Findings suggested that BrSARS-N is more highly antigenic than ErSARS-N under denaturing conditions. (B) Immunoreactivity analysis of BrSARS-N and ErSARS-N protein by indirect ELISA under nondenaturing conditions. SARS-N mAb was incubated in each coated well with the rSARS-N proteins, followed by HRP-conjugated anti-mouse IgG, and proteins were detected using TMB substrate solution, which indicated that BrSARS-N protein has also higher antigenicity than ErSARS-N protein under nondenaturing conditions.
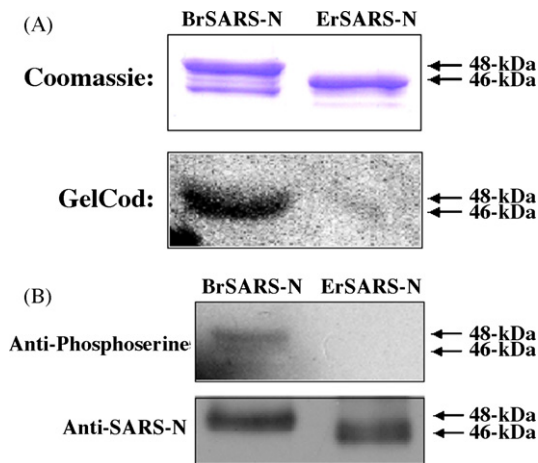

Fig. 4. Phosphorylation of SARS-N protein expressed in insect cells. (A) Purified BrSARS-N and ErSARS-N proteins were separated by SDS-PAGE. The proteins were visualized by Coomassie brilliant blue staining (upper panel) or the phosphorylated N proteins were detected using a GelCode phosphoprotein kit as described in Materials and Methods (lower panel). (B) The gel transferred to a nitrocellulose membrane and analyzed by Western blot using anti-phosphoserine antibody (upper panel) or SARS-N mAbs (lower panel), followed by HRP-conjugated anti-mouse IgG, and proteins were detected using chemiluminescence staining. This result indicated that BrSARS-N protein was highly phosphorylated, but ErSARS-N was not.

of Fig. 5A, phosphorylated 48 kDa protein did not cross-react with SARS negative serum, but dephosphorylated and nonphosphorylated 46 kDa protein showed a significant cross-reaction, suggesting that the phosphorylation is required to preserve the specificity of SARS-N protein.

Finally, we investigated whether the immunoreactivity of BrSARS-N protein was dependent on the phosphorylation of SARS-N protein. Protein samples treated or not treated with PP1 were prepared using the same methods used in the specificity analysis mentioned above, and the immunoreactivity of these protein samples was examined by indirect ELISA using serially diluted SARS-N mAbs. As shown in Fig. 5B, BrSARS-N protein treated with PP1 showed a significant decrease in the immunoreactivity against SARS-N mAbs compared to non-treated BrSARS-N protein, which suggests that the phosphorylation also affects the immunoreactivity of SARS-N protein.

4. Discussion

The SARS-N protein was previously demonstrated to be a major antigen in the human immune response to SARS, a sufficient antigen for serological diagnosis, and a candidate for the development of a SARS vaccine (Chan et al., 2005a,b; Kim et al.,
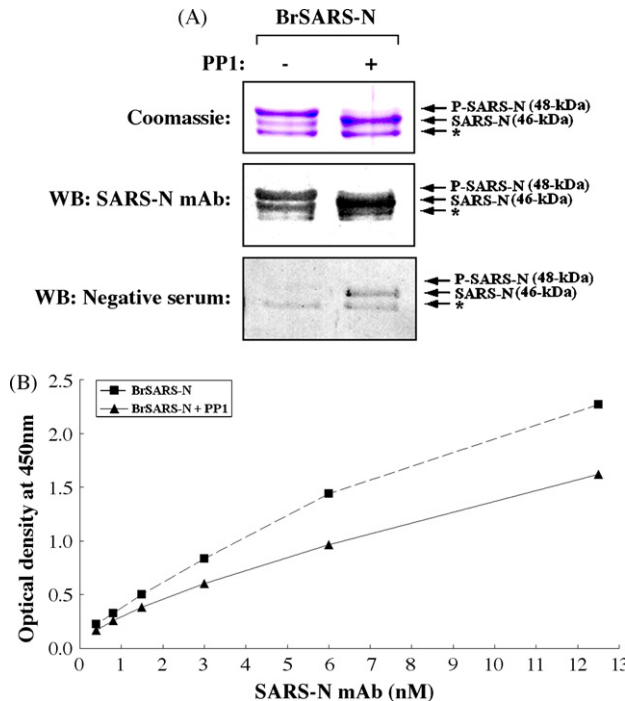
Fig. 5. Phosphorylation of SARS-N protein is important for the preservation of specificity and immunoreactivity. (A) Specificity analysis of BrSARS-N proteins treated with or without PP1. The purified BrSARS-N protein treated with or without PP1 were separated by SDS-PAGE (upper panel) and transferred to a nitrocellulose membrane. The membrane was incubated with SARS-N mAbs (middle panel) or SARS negative serum (lower panel), followed by HRP-conjugated anti-mouse or human IgG, and proteins were detected by DAB staining. Data shown are representative of five SARS negative sera with similar results, suggesting that both dephosphorylated and nonphosphorylated proteins are cross-reacted with SARS negative serum. (B) Immunoreactivity analysis of BrSARS-N protein treated with or without PP1. Aliquots of the protein samples treated with or without PP1 used in specificity analysis were coated onto the microtiter plates and incubated with SARS-N mAb, followed by HRP-conjugated anti-mouse IgG, and detection was performed using TMB substrate solution. Finding indicated that dephosphorylation considerably reduced the immunoreactivity of SARS-N protein.

2004; Shi et al., 2003; Wu et al., 2004). However, the antigenicity of the SARS-N protein is not yet wholly understood. Recent reports have suggested that SARS negative serum contains with cross-reacting antibodies, such as antibodies against other coronaviruses, and these antibodies are cause false-positive reactions with recombinant SARS-N protein (Sun and Meng, 2004; Woo et al., 2004; Yu et al., 2005). Some investigators have reported that the cross-response of full-length recombinant N protein with antibodies to other coronaviruses is caused by highly conserved motifs in the N-terminus of this protein (Yu et al., 2005). In the present study, BrSARS-N was also contained within these N-termini, and all of the human serum samples used in this study contained high titers (approximately 1:320 HAI unit) of antibodies against human coronavirus OC43 and 229E (data not shown). The BrSARS-N protein showed little cross-reactivity with SARS negative sera in Western blot analysis as well as indirect ELISA. Indeed, BrSARS-N protein reacted more strongly to SARS-N mAbs than to ErSARS-N, which has been widely used for the development of SARS diagnostic methods. These results

suggest that there are other factors affecting the antigenicity of SARS-N protein.

The BrSARS-N proteins were expressed from the same cDNA construct, with ErSARS-N covering the full-length SARS-N protein coding region and the same fusion partner, $6\times$ histidine, in the N-terminal of this protein. However, the BrSARS-N and ErSARS-N proteins were showed different levels of antigenicity in the present study. The differential antigenicity of both rSARS-N proteins was demonstrated by Western blot under denaturing conditions, and did not result from the conformation of SARS-N protein. The phosphorylation of N protein is a well-documented phenomenon in many coronaviruses (Calvo et al., 2005; Chen et al., 2005; Surjit et al., 2005; Wilbur et al., 1986; Zakhartchouk et al., 2005). Given that this phosphorylation event is restricted to the eukaryotic system, the ErSARS-N protein would have properties different from those of the native N protein. In contrast with the prokaryotic expression system, the baculovirus-insect cell expression system can provide this type of post-translational modification similarly to that of mammalian cells. In the present study, the BrSARS-N protein was abundantly phosphorylated. This suggests that this N protein may have similar antigenicity to that of the native N protein. Therefore, we investigated whether the phosphorylation of SARS-N protein could affect the cross-reactivity with SARS negative serum and immunoreactivity with SARS-N antibodies.

Previous investigators have reported that unphosphorylated recombinant proteins show distinct antigenic character compared to the phosphorylated form and *in vitro* phosphorylation of bacterially expressed proteins considerably reduced the reactivity of some specific mAbs against the protein (Kee et al., 1998; Kumar and Spandau, 1995). These previous reports suggest that the phosphorylation is closely related to the antigenicity of recombinant protein. In this study, we were able to observe that the phosphorylated BrSARS-N protein shows little cross-response with SARS negative serum under denaturing conditions, but BrSARS-N protein dephosphorylated by PP1 remarkably enhance a false-positive reaction against SARS negative serum. This probably occurs because the phosphorylation at conserved epitopes may inhibit non-specific binding of cross-reacting antibodies, which may result in higher specificity of the BrSARS-N protein than the ErSARS-N protein. Therefore, these data indicate that phosphorylation is required for preservation of the antigenic specificity of SARS-N protein under denaturing conditions.

Although phosphorylated BrSARS-N protein showed a higher specificity than nonphosphorylated ErSARS-N protein under denaturing conditions, the BrSARS-N proteins in Western blot analysis under native condition showed high antigenic cross-reactivity with SARS negative serum, similar to that shown by ErSARS-N protein (data not shown). This result indicates that some conformational epitopes of SARS-N protein might cause the cross-response with SARS negative serum; this occurs independently upon the phosphorylation of SARS-N protein. Thus, the use of phosphorylation BrSARS-N protein in Western blot analysis under denaturing conditions would be more suitable for use in SARS serology than compared to other

diagnostic methods. Furthermore, SARS negative and positive serum could be clearly distinguished by indirect ELISA using BrSARS-N protein under physiological conditions, similar to that observed for Western blot under denaturing conditions. SARS-N protein containing no cysteine residues and no disulphide bonds would be less stable than other structural proteins of SARS-CoV (Wang et al., 2004) and the surface of microtiter plate used in indirect ELISA is highly charged. Therefore, the conformational epitopes of SARS-N against cross-reacting antibodies may be destroyed by the surface of the plate, and/or cross-reacting epitopes may be hidden by the surface. As ELISA-based serology is less labor-intensive and easier to standardize than Western blot assay, Western blot confirmation of positive ELISA results is probably a better choice for routine use in clinical laboratories.

In the present study, nonphosphorylated SARS-N proteins with a molecular mass of 46 kDa were also expressed in insect cells, along with the major phosphorylated protein of 48 kDa. Thus, it is possible that this nonphosphorylated protein may be respond to cross-reacting antibodies in human serum. As shown in the result of indirect ELISA, four of 180 SARS negative sera showed reactivity against the BrSARS-N protein, although with low titer. Therefore, further studies should be performed to standardize BrSARS-N-based ELISA for more sensitive and specific diagnosis.

Some reports have demonstrated that *in vitro* phosphorylated peptides show higher avidity with human antibodies than untreated peptides (Machida et al., 1991; Vapalahti et al., 1996). The present study also revealed that the phosphorylated SARS-N protein showed higher reactivity with specific mAbs than did the nonphosphorylated protein. Indeed, the immunoreactivity of BrSARS-N protein was remarkably reduced when this protein was dephosphorylated with PP1, which suggests a correlation between the phosphorylation and the antigenic reactivity of SARS-N protein. Since coronavirus N proteins exist as phosphorylated forms in mature viral particles and most of the SARS-N proteins expressed in eukaryotic cells are highly phosphorylated (Surjit et al., 2005; Wilbur et al., 1986; Zakhartchouk et al., 2005), the major antibodies against N protein in SARS patients may be those that act against the phosphorylated form. Therefore, it appears that the phosphorylated SARS-N protein would be a more useful antigen for detecting SARS-N antibodies in the sera of SARS patient than the nonphosphorylated proteins expressed in a prokaryotic system.

In conclusion, we can successfully express phosphorylated rSARS-N protein in the baculovirus-insect cell system, and this protein could be obtained at a maximum of up to 1 mg per 1×10^7 cells, which is similar to the previously reported finding for rSARS-N protein (Ren et al., 2004). We can also demonstrate that phosphorylation of the N protein plays an important role in the antigenicity and specificity of SARS-N protein. Therefore, it is believed that the BrSARS-N protein may be a more suitable antigen for the development of sensitive and specific diagnostic methods than ErSARS-N protein, and will be useful in further investigations of SARS-N pathology.

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