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The nucleocapsid protein of SARS-associated coronavirus inhibits B23 phosphorylation

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Abstract

Severe acute respiratory syndrome-associated coronavirus (SARS-CoV) is responsible for SARS infection. Nucleocapsid (N) protein of SARS-CoV encapsidates the viral RNA and plays an important role in virus particle assembly and release. In this study, the N protein of SARS-CoV was found to associate with B23, a phosphoprotein in nucleolus, *in vitro* and *in vivo*. Mapping studies localized the critical N sequences for this interaction to amino acid residues 175–210, which included a serine/arginine (SR)-rich domain. *In vitro* phosphorylation assay showed that the N protein inhibited the B23 phosphorylation at Thr199.
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Keywords: SARS coronavirus; Nucleocapsid protein; B23; Protein–protein interaction; Serine/arginine-rich domain; B23 phosphorylation

Introduction

Severe acute respiratory syndrome (SARS) is a human infectious disease that first emerged in Southern China in mid-November 2002 [1]. A novel coronavirus named SARS-associated coronavirus (SARS-CoV) was identified as the causative agent of SARS [2]. The nucleocapsid (N) protein of SARS-CoV consists of 422 amino acids and shares 20–30% homology with N proteins of other coronaviruses [3]. The N protein is the major antigen recognized by convalescent antisera and can be used as a diagnostic marker for the detection of SARS [4]. The N protein is reported to be phosphorylated [5] and the phosphorylation plays a role in the immunoreactivity and specificity of SARS-CoV N protein [6].

The N protein plays a role in viral packaging, viral core formation and regulation of signal transduction. The N protein of SARS-CoV encapsidates the viral RNA and binds N protein itself and the membrane protein of SARS-CoV [7–9]. SARS-CoV N protein has been reported to localize in the cytoplasm of SARS-CoV infected cells with a weak presence in the nucleus [10]. N protein also has the capability to regulate cellular signal pathways such as arresting cell cycle progression [11], inducing apoptosis and actin reorganization [12] and activating the AP-1 signal transduction pathway [13]. It has previously been shown that the N protein of SARS-CoV interacts with cellular proteins, including cyclophylin A [14], human cellular heterogeneous nuclear ribonucleoprotein A1 [15], human ubiquitin-conjugating enzyme [16] and CDK–cyclin complex proteins [11].

The nucleolar phosphoprotein B23 is originally identified as a high-level phosphoprotein in granular regions of the nucleolus [17]. B23 protein can bind nucleic acids [18] and physically interact with maturing preribosomal ribonucleoprotein particles [19]. This protein also functions as a shuttle protein in protein nuclear import [20,21]. It is reported that B23 protein forms a specific complex with several viral proteins such as Rex protein of human T-cell leukemia virus [22], Rev protein [23], and Tat protein [24] of human immunodeficiency virus and Hepatitis delta virus

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antigens [25]. B23 protein is also identified as one of the substrates of CDK2/cyclin E and plays a critical role in centrosome duplication during cell cycle progression, which is regulated by the phosphorylation of B23 at Thr199 [26,27]. Furthermore, B23 protein possesses molecular chaperoning activities, including preventing protein aggregation, protecting enzymes during thermal denaturation, and facilitating renaturation of chemically denatured proteins [28].

In this study, we discovered that the N protein of SARS-CoV interacted with human phosphoprotein B23 both *in vitro* and *in vivo*. Furthermore, mutational analysis indicated that the SARS-CoV-N/B23 interaction required the serine/arginine (SR)-rich domain (aa 175–210) in the N protein. We found that the N protein inhibited the phosphorylation of B23 at Thr199.

Materials and methods

Plasmids construction. The full-length SARS-CoV N gene and the truncated mutants N1 (1–225), N2 (1–293), N3 (210–422), N4 (175–422) and N5 (353–422) were obtained by polymerase chain reaction (PCR) using the plasmid pGEMT-N containing the full-length N gene as a template (GenBank Accession No. AY365036). The PRC products were cloned into the vector pET-GST. This vector was used for expression of GST fusion proteins.

The human B23.1 gene in pBluescriptII SK plasmid was kindly provided by Dr. Stephan W. Morris (St. Jude Children's Research Hospital, USA). The B23 coding sequence was amplified by PCR and then cloned into the vectors of pET-GST and pET-His. pET-His vector was used for expression of proteins with a 6His-tag at the C-terminus.

All the recombinant plasmids were confirmed by PCR, restriction digestion and DNA sequencing.

Indirect immunofluorescence assay and confocal microscopy. HeLa cells grown on coverslips were transfected with 2 μg of pcDNA3 vector or pcDNA3-N plasmid [10] for 6 h. At 24 h post-transfection, cells were fixed with ice cold 50% methanol–50% acetone at −20 °C. Coverslips were incubated for 1 h at 37 °C with anti-N antibody [4] and anti-B23 antibody (Sigma–Aldrich, St. Louis, MO), followed by incubation with FITC-conjugated goat anti-rabbit IgG (Sigma) and TRITC-conjugated goat anti-mouse IgG (Sigma) for 1 h at 37 °C, respectively. The final fluorescence images were visualized under laser confocal scanning microscope (Leica Laser Technik, Germany) with appropriate filters.

Expression and purification of recombinant proteins. Recombinant 6His-tagged B23 protein, GST-fused SARS-CoV N protein and deletion mutants were expressed in *Escherichia coli* (*E. coli*) BL21 (DE3) cells after induction with 1 mM IPTG for 6 h in LB-medium at 37 °C. The bacterial cells were spun down and resuspended in lysis buffer (20 mM Tris–HCl [pH 7.9], 500 mM NaCl, 5 mM Imidazole, 1 mM NaF and 1 mM PMSF) and then sonicated and centrifuged at 12,000*g* at 4 °C for 30 min. The recombinant proteins were purified by a Ni–NTA affinity column (Qiagen, Chatsworth, USA) according to the manufacturer's instructions.

Preparation of cell lysates and GST pull-down assay. To prepare HeLa cell lysates, approximate 10^7 HeLa cells were harvested, washed with PBS buffer and sonicated in cellular lysis buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM PMSF, and 1 mM DTT) with 1× protease inhibitor cocktail (Sigma, St. Louis, MO). The lysates were centrifuged at 12,000*g* for 20 min at 4 °C.

Equal amount of either GST or GST-fused proteins bound to glutathione–sepharose beads was mixed with 6His-tagged B23 protein or HeLa cell lysates. After incubation for 3 h at room temperature, the beads were washed five times with PBS. Proteins bound to the beads were removed by boiling in 4× SDS sample buffer for 10 min. Proteins were analyzed by SDS–PAGE and detected by Western blot analysis.

Co-immunoprecipitation. At 24 h post-transfection with plasmid pcDNA3-N [10], HeLa cells were washed twice with PBS buffer and then lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl [pH 8.0]) with 1× protease inhibitor cocktail (Sigma, St. Louis, MO). The lysates were centrifuged at 12,000*g* for 20 min at 4 °C. The anti-N antibody [4] was added to the supernatants precleared by protein A-agarose beads and incubated for 1 h at room temperature. The antibody–antigen complexes were then precipitated with protein A-agarose beads at 4 °C for 4 h. The beads were collected by centrifugation and washed with RIPA buffer three times. Proteins bound to the beads were eluted by boiling in 4× SDS sample buffer for 10 min, analyzed by SDS–PAGE and then detected by Western blot analysis.

In vitro phosphorylation assay. For examination of the phosphorylation of B23 by CDK2 kinase, HeLa cell lysates were subjected to immunoprecipitation using anti-CDK2 antibody (Neomarker). The antibody–antigen complexes were collected with protein A-agarose. The immunoprecipitated cell lysates were washed twice with kinase buffer (20 mM Tris–HCl [pH 7.5], 1 mM dithiothreitol, 5 mM glycerolphosphate, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$) and then incubated with the indicated substrate along with 1 mM ATP for 30 min at 37 °C. Samples were then subsequently boiled for 10 min in 4× SDS sample buffer, analyzed by SDS–PAGE and then detected by Western blot assay using anti-phospho-B23 (Thr199) antibody (Cell signaling).

Results

Co-localization of N protein and cellular protein B23

Previous study has demonstrated that SARS-CoV N protein bears three nuclear localization signals (NLS) and a nuclear export signal, acting as a shuttle protein between nucleus and cytoplasm [10]. Since B23 protein can bind NLS and facilitate protein nuclear import [20,21], we investigated the possibility of co-localization of these two proteins in pcDNA3-N transfected HeLa cells by indirect immunofluorescence staining using anti-N antibody and anti-B23 antibody, respectively, visualized under laser confocal scanning microscope. Consisting with previous findings [10], N protein was localized in the cytoplasm, mostly in perinuclear region (Fig. 1d). B23 protein was

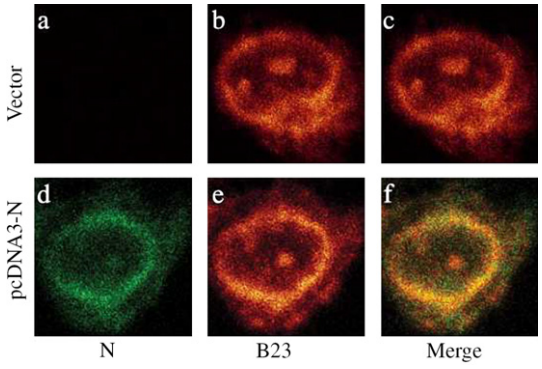

Fig. 1. Co-localization of SARS-CoV N protein and B23 protein. HeLa cells were transfected with vector or pcDNA3-N plasmid for 24 h. The cells were double-labeled with anti-N antibody (a and d) and anti-B23 antibody (b and e). The cells were examined by confocal microscopy. The two colors were then merged (c and f) and the yellow region is the area where the two proteins co-localized. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

localized both in cytoplasm and in nucleus and the N protein did not affect the B23 localization (Fig. 1b and e). The overlayed result indicated that the SARS-CoV N protein co-localized with B23 protein in the perinuclear region of HeLa cells (Fig. 1f). The same experiment was repeated in VeroE6 cells and a similar result was observed (data not shown).

The interaction between N protein and B23 protein in vitro and In vivo

Co-localization of SARS-CoV N protein and cellular B23 protein suggested that N protein might interact with B23 protein. To examine this possibility, a protein binding assay of GST-N fusion protein to cellular B23 protein was performed. As shown in Fig. 2A, the endogenous B23 was pulled-down by GST-N fusion protein but not by GST protein.

Because both N protein and B23 protein bind RNA [7,18], it is possible that N and B23 protein interact through RNA. To test whether RNA was required for the interaction between B23 and N protein, the lysates of HeLa cells were treated with RNaseA prior to the GST-pull down assay. This treatment did not affect the amount of B23 bound to GST-N protein compared to that of the untreated extracts (Fig. 2B), indicating that N protein interacted with B23 independent of cellular RNA.

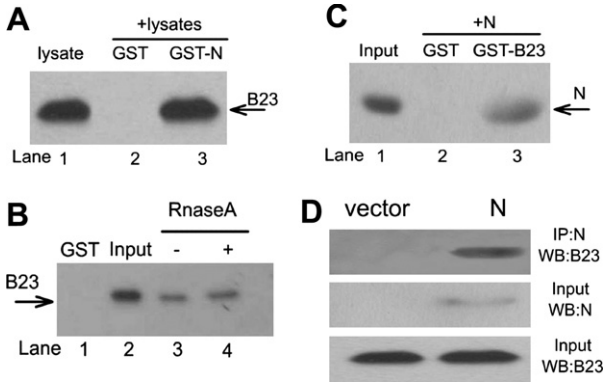

Fig. 2. The interaction of the SARS-CoV N protein and B23 *in vitro and in vivo*. (A) HeLa cell lysates were incubated with glutathione–sepharose beads bound to either GST (lane 2) or GST-N fusion protein (lane 3). After washing, the bound protein was detected using anti-B23 antibody. The B23 protein in cell lysates served as a molecular mass marker (lane 1). (B) The untreated HeLa cell lysates (lane 3) or the HeLa cell lysates treated with RNaseA (lane 4) were used in GST pull-down assay, respectively. After washing, the bound protein was detected using anti-B23 antibody. Lane 2 was the GST control and The B23 protein in cell lysates served as a molecular mass marker (lane 1). (C) N protein with 6His-tag was incubated with glutathione–sepharose beads bound to either GST (lane 2) or GST-B23 fusion protein (lane 3). After washing, the bound protein was detected using anti-N antibody. The purified N protein served as a molecular mass marker (lane 1). (D) HeLa cells were transfected with pcDNA3 (vector) and pcDNA3-N (N) plasmid. At 24 h post-transfection, the cells were lysed and co-immunoprecipitation was performed using anti-N antibody, the immunoprecipitated protein was detected using anti-B23 antibody (first panel). Cell lysates was detected using anti-N antibody (second panel) and anti-B23 antibody (third panel) to confirm the expression of the interested protein.

GST pull-down assay was repeated using *E. coli*-expressed 6His-B23 protein instead of HeLa cell lysates and a similar result was obtained (data not shown). In addition, the GST pull-down assay using GST-B23 protein and 6His-N protein was performed. As what expected, N protein could bind GST-B23 fusion protein, but no N protein could bind GST protein alone (Fig. 2C). All the results above demonstrated that N protein of SARS-CoV interacted directly with B23 *in vitro*.

To investigate whether the interaction between N protein and B23 protein occurred in N-expressing cells, we performed co-immunoprecipitation assay using anti-N antibody to co-immunoprecipitate the N protein and any other possible interacting proteins in cellular extracts of pcDNA3-N transfected HeLa cells. The bound proteins were detected by anti-B23 antibody. Consisting with the above results, B23 protein together with N protein could be co-inmmunoprecipitated by the anti-N antibody while no B23 was precipitated in the control sample (Fig. 2D), confirming that SARS-CoV N protein interacted with B23 protein *in vivo*.

Mapping the B23-binding domain of SARS-CoV N protein

To identify the B23-interactive region within the N protein, a series of deletion mutants were generated (Fig. 3A). As shown in Fig. 3B, the mutant N1 (1–225), N2 (1–293) and N4 (175–422) bound B23 (Fig. 3C, lanes 3, 4, and 6),

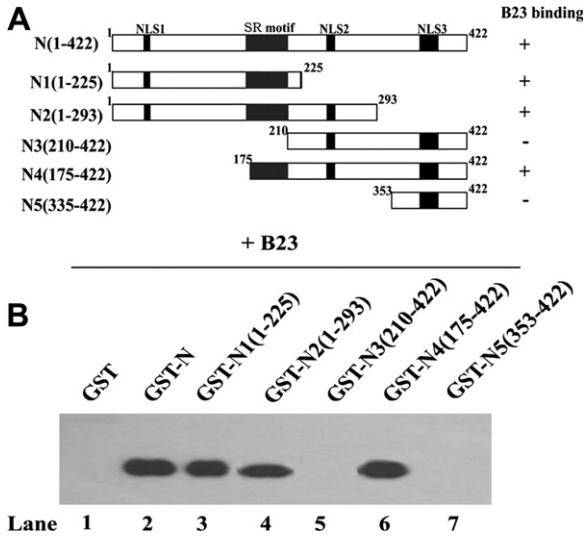

Fig. 3. Identification of B23-binding domain on SARS-CoV N protein. (A) Schematic representation of deletion mutants of SARS-CoV N and their respective capacity of binding B23. Boxes represented the structure of different constructs of SARS-N protein with the amino acid residues indicated above. The capacity of each SARS-CoV N mutant to interact with B23 was indicated by a plus or minus sign. NLS, nuclear localization signal; SR, serine/arginine-rich domain. (B) Dissection of the B23-interacting domain of SARS-CoV N protein. The interaction between GST-N fusion mutant and B23 protein was determined in a GST pull-down assay. Proteins were analyzed by SDS–PAGE followed by immunoblotting with anti-B23 antibody. Names of individual GST fusion mutants were indicated on the top.

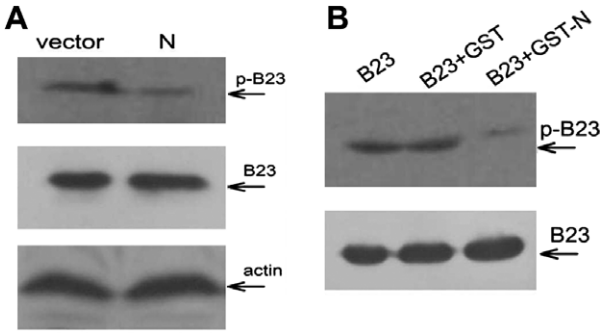

Fig. 4. The N protein of SARS-CoV inhibited the phosphorylation of B23 at Thr199 (A) HeLa cells were transfected with vector and pcDNA3-N plasmid. At 24 h post-transfection, total cell lysate was harvested and detected using anti-phospho-B23 (Thr199) antibody (first panel), anti-B23 antibody (second panel) and anti-β-action antibody (third panel). (B) HeLa cell lysate was immunoprecipitated with CDK2 antibody and used for *in vitro* phosphorylation assay along with B23 (lane1), B23 and GST (lane 2) or B23 and GST-N (lane 3), respectively. Proteins were immunoblotted with anti-phospho-B23 (Thr199) antibody (upper panel) or anti-B23 antibody (lower panel).

but the mutant N3 (210–422) and N5 (353–422) did not (Fig. 3B, lanes 5 and 7), indicating that amino acid residues 175–210 in the N protein, which included a serine/arginine (SR)-rich domain, was responsible for the interaction between the N protein and B23 protein.

N protein inhibited phosphorylation of B23 at Thr199

B23 plays a critical role in centrosome duplication during cell cycle progression, which is regulated by the phosphorylation of B23 at Thr199 [26,27]. Interestingly, the N protein can arrest the cell cycle progression [10,11]. To study whether the direct binding of B23 by N protein had a functional effect in cell cycle progression, first we examined the effect of the N protein on the phosphorylation of B23. As shown in Fig. 4A, the amount of B23 protein in control cells and N-expressing cells were the same but the amount of phosphorylated B23 was significantly decreased in N-expressing cells compared to that in the control cells. However, there are many factors which can influence the phosphorylation of B23 *in vivo*. To further test whether the inhibition of the phosphorylation of B23 was caused by the N protein of SARS-CoV, an *in vitro* phosphorylation assay using immunoprecipitated CDK2 kinase was performed. As shown in Fig. 4B, GST protein did not affect the phosphorylation of B23 at Thr199 but GST-N protein exhibited an inhibitory effect on the phosphorylation of B23 at Thr199. The above results indicated that the N protein inhibited the phosphorylation of B23 at Thr199.

Discussion

In our previous research, we found that SARS-CoV N protein contained three NLS motifs and a nuclear export signal, acting as a shuttle protein between nucleus and cytoplasm, and it could arrest the cell cycle progression [10]. B23 protein can bind NLS, facilitate protein nuclear import and play a role in centrosome duplication during cell cycle progression [20,21,26]. Therefore, we studied the interaction of these two proteins and the functional effect of this interaction. We discovered that B23 interacted with SARS-CoV N protein *in vitro* and *in vivo*. Somewhat surprisingly, we mapped the B23-binding domain within the N protein to a SR-rich domain (aa 175–210) rather than any putative NLS motif. B23 usually plays a role in nuclear import of the viral proteins to which it binds [24,25]. However, according to our previous study [10], the deletion mutant N4 (175–422), which could interact with B23 protein, was shown to localize in cytoplasm and the deletion mutant N1 (1–225), which also could interact with B23 protein, localized in the cytoplasm and nucleus. Moreover, overexpression of B23 protein in HeLa cells did not change the cytoplasmic localization of N protein of SARS-CoV (data not shown). Therefore, we speculated that unlike other B23-binding viral proteins, the binding of N protein with B23 protein might not involve in sub-cellular localization of N protein.

B23 protein is also identified as one of the substrates of CDK2/cyclin E and plays a role in centrosome duplication during cell cycle progression. B23 associates specifically with unduplicated centrosomes and this association is controlled by CDK2–cyclin E-mediated phosphorylation. B23 loses its affinity to centrosomes in its phosphorylated form. Dissociation of the centrosomal B23 is essential for the centrosome to initiate duplication [26]. The Thr199 amino acid in B23 is the phosphorylation site essential in this regulation of centrosome duplication [27]. In our previous research, we have shown that the N protein of SARS-CoV can arrest cell cycle progression [10], which is in accordance with the result of Surjit et al. [11]. Here we demonstrated that N protein inhibited the phosphorylation of B23 at Thr199 in HeLa cells and in *in vitro* phosphorylation assay (Fig. 4). Therefore, we hypothesize that when N protein interacts with B23, N protein may competitively inhibit the interaction of B23 and CDK2 kinase or the phosphorylation site of B23 may be masked by the bound N protein, resulting in lack of phosphorylation of B23 protein. This can lead to cell cycle arresting since the centrosome can not initiate duplication. In this model, the N protein of SARS-CoV manipulates the host cell cycle machinery by acting as a competitive inhibitor to B23 phosphorylation through its interaction with B23 protein.

B23 protein has been shown to participate in various other cellular events such as ribosome assembly [19] and acting as a molecular chaperone [28]. The interaction of B23 and N protein of SARS-CoV might influence the ribosome biogenesis in cells and resulted in suppression of host gene expression to create a more favorable milieu for virus survival. This interaction also might affect the molecular chaperoning activities of B23 and leaded to incorrect protein folding.

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