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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.


*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

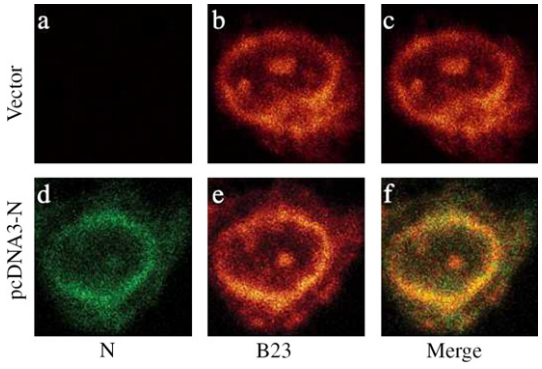


Fig. 1. Co-localization of SARS-CoV N protein and B23 protein. HeLa cells were transfected with vector or pcDNA3-N plasmid for 24 h. The cells were double-labeled with anti-N antibody (a and d) and anti-B23 antibody (b and e). The cells were examined by confocal microscopy. The two colors were then merged (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.)