SARS-CoV Nucleocapsid Protein Binds to hUbc9, a Ubiquitin Conjugating Enzyme of the Sumoylation System

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SARS-CoV is a newly identified coronavirus (CoV) that causes severe acute respiratory syndrome (SARS). The SARS-CoV nucleocapsid (N) protein is an important structural and functional protein. To identify cellular proteins that interact with the SARS-CoV N protein and to elucidate the possible involvement of N protein in SARS-CoV pathogenesis, a human lymphocyte cDNA library was screened using a yeast two-hybrid system assay. hUbc9, a ubiquitin conjugating enzyme of sumoylation system, was found to interact specifically with the N protein, implying the post-translational sumoylation of the N protein. Mapping studies localized the critical N sequences for this interaction to amino acids 170–210, which includes the SR-rich motif. However, the consensus motif of sumoylation $GK^{62}EE$ in the N protein is not responsible for binding to hUbc9. Mutations of hUbc9 at the enzyme active site C93A or C93S severely impair the interaction with the N protein. The two proteins were also shown to colocalize in the cytoplasm of the transfected 293T cells. This is the first report demonstrating the interaction of hUbc9 with a structural protein of plus-strand RNA viruses, indicating a new drug target for SARS-CoV. *J. Med. Virol. 78:1365–1373, 2006.* © 2006 Wiley-Liss, Inc.

KEY WORDS: severe acute respiratory syndrome; coronavirus; SARS-CoV; nucleocapsid (N) protein; hUbc9; interaction

INTRODUCTION

Severe acute respiratory syndrome (SARS) is a newly identified infectious disease, which led to over 8,000 infected cases and as many as 774 death cases worldwide before the 31st July 2003 (http://www.who.int/csr/sars/country/table2003-9-23/en/). The pathogen was soon identified as a new member of family *Coronaviridae* and named as SARS-associated coronavirus (SARS-CoV) [Ksiazek et al., 2003; Peiris et al., 2003]. The SARS-CoV is a single positive-stranded RNA virus with a genome of about 30 kb in length that has a 5′ cap structure and a 3′ polyadenylation tail. The 5′ two-thirds of the SARS-CoV genome encodes the replicase complex, ORF1a and ORF1b, the latter resulting from a −1 frameshift of 1a. The 3′ one-third of the SARS-CoV encodes the structural proteins of the virus, such as spike (S), envelope (E), membrane (M), nucleocapsid (N) protein, and other proteins of unknown functions [Marra et al., 2003; Rota et al., 2003].

The N protein is the most abundant viral protein in coronaviruses that is produced throughout infection and is an important multifunctional protein. Several functions have been postulated for the coronavirus N protein throughout the virus life cycle, including viral packaging, viral core formation, and signal transduction

[Hiscox et al., 2001; He et al., 2003]. Primarily, N protein complexes with the coronavirus genomic RNA to form a ribonucleocapsid structure, and it has been observed, together with the M protein, to be a component of the viral core [Risco et al., 1996].

The SARS-CoV N is an extensively phosphorylated highly basic structural protein with 422 amino acids, which shares 20–30% homology with N protein of other coronaviruses. A lysine-rich region in the C-terminal domain was predicted as the nuclear localization signal (NLS), and an SR-rich motif was identified in the middle domain that may be related to RNA binding and protein–protein interaction (Fig. 1). The N protein has been shown to form a multimer both in vivo and in vitro, and its C-terminal domain is responsible for this oligomerization [Surjit et al., 2004b; Yu et al., 2005]. The formation of N protein dimers is likely to be the first step in the formation of the nucleocapsid core and further oligomerization is likely to continue to drive assembly of the core. The N protein can also interact with the membrane protein of SARS-CoV, and this appears to be an essential process for viral assembly [He et al., 2004b]. The three-dimensional structure of the N-terminal RNA-binding domain of the SARS-CoV N protein has been described by NMR spectroscopy [Huang et al., 2004].

SARS-CoV is known to cause apoptosis in cell culture and during host infection [Yan et al., 2004; Ren et al., 2005]. Surjit et al. [2004a] have indicated that the SARS-CoV N protein is capable of inducing apoptosis. The SARS-CoV N protein has a variety of roles, including activation of the AP-1 signal transduction pathway [He et al., 2003] and actin reorganization in mammalian cells under stressed conditions [Surjit et al., 2004a]. The regulation of cell signaling pathways undoubtedly requires a complex interaction of both viral and host cell proteins. However, the proteins involved in this process have not been identified convincingly.

In many viruses, the N protein plays a crucial role in viral RNP assembly and replication. It is also possible that some cellular proteins are involved in these events. Such cellular proteins could function through an interaction with the N protein. It has been reported that the SARS-CoV N protein interacts with several cellular proteins, including cyclophylin A and human cellular heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) [Luo et al., 2004, 2005]. A yeast two-hybrid screen system was used to search for cellular proteins that may interact with SARS-CoV N protein. In this study, it is demonstrated that the SARS-CoV N protein specifically interacts with human ubiquitin-conjugating enzyme 2 (hUbc9), an important enzyme of sumoylation system. This result is consistent with a recent study [Li et al., 2005] that showed SARS-CoV N was sumoylated as hUbc9 is involved in the process of protein sumoylation. It is also shown that the binding site of the N protein is largely localized in the SR-rich motif, and that the enzymatic active site C93 of hUbc9 is responsible for the interaction with the N protein.

MATERIALS AND METHODS

Plasmids and Construction of Recombinant Vectors

The full-length SARS-CoV N gene and the truncated mutants N1–169, N170–295, N296–422, N170–220, N210–295 (constructs and putative functional domains were shown in Figure 1) were subcloned into the yeast two-hybrid vector pGBKT7. The *hUbc9* gene was obtained by PCR from the human lymphocyte cDNA library, and cloned into the yeast AD vector pACT2 and the bacterial expression vector pGEX-6p-1. For mammalian cell expression, the SARS-CoV N and hUbc9 were subcloned into pcDNA4-myc/HisA and pcDNA3-FLAG, and fluorescence vector pEGFP-N1 and pDsRed2-N1, respectively. The hUbc9 mutants C93A and C93S and the N mutant K62A were created using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

The Yeast Two-Hybrid System

The Matchmaker Two-hybrid System3 (Clontech, Mountain View, CA) was used to test the interaction
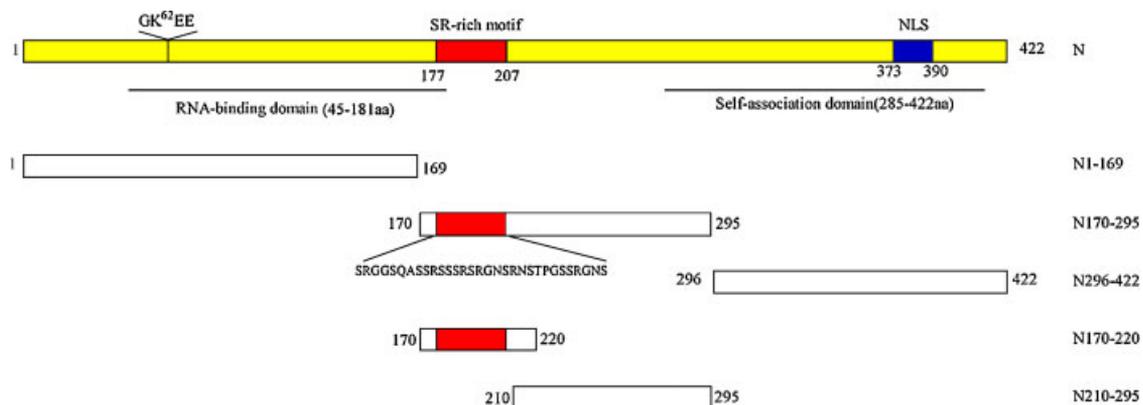
Fig. 1. Schematic representation of the SARS-CoV N protein and the truncated mutants used in the yeast two-hybrid system. The SUMO-1 conjugation site (K62), the SR-rich motif, the putative nuclear localization signal (NLS), the RNA-binding domain and the self-association domain are highlighted.

between the SARS-CoV N protein and hUbc9 in vitro. The human lymphocyte cDNA library in the yeast plasmid pACT2 was screened in the yeast strain AH109. All procedures for growing yeast, transformation, and selection followed the protocol provided by the manufacturer (Clontech). To perform quantitative two-hybrid tests between known proteins, two yeast expression plasmids were cotransformed simultaneously into the yeast strain SFY526. β-galactosidase assays were carried out according to the CLONTECH Matchmaker manual and repeated at least three times.

Cell Culture and Transient Transfection

293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, and cultured in 5% CO_2 at 37°C. Transient transfection assays were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Subcellular Localization

293T cells were grown on coverslips in a 12-well chamber and simultaneously transfected with the recombinant fluorescence plasmids pEGFP-N and pDsRed2-Ubc9. Twenty-four hours after transfection, the cells were washed with PBS three times and fixed in 4% paraformaldehyde for 15 min at room temperature. The coverslips were then washed with PBS and mounted. Intracellular localization of the N protein and hUbc9 was observed under a Leica confocal microscope.

Protein Expression, Purification, and GST-Pull Down Assays

For purification of GST-Ubc9, *E. coli* BL21(DE3)/pGEX-Ubc9 was cultured to mid-log phase in 500 ml of LB medium, isopropyl-1-thio-β-D-galactopyranoside (IPTG) was then added to the medium to a final concentration of 1 mM. Cells were harvested 4 hr later, suspended in ice-cold PBS buffer (pH 8.0), and homogenized by sonication. The cell lysate was then centrifuged at 14,000g for 10 min at 4°C. The supernatant was applied to a column containing 0.5 ml of sepharose 4B-glutathione (Amersham Pharmacia Biotech, Piscataway, NJ). The column was washed with ten column volumes of PBS buffer. GST-Ubc9 bound to the beads was used for pull down assays. GST-C93A, GST-C93S, and GST alone were purified by the same method.

The translation in vitro of the ^{35}S-labeled N protein was operated according to the protocol of the TNT Quick Coupled Transcription/Translation systems (Promega, Madison, WI). An equal amount of either GST or GST-fusion protein bound to sepharose 4B-glutathione was mixed with 10 μl of ^{35}S-labeled N protein translated in vitro or 1 ml of Vero E6 cell lysate infected by SARS-CoV, and incubated with shaking for 8 hr at 4°C. The beads were washed three times with PBS. Proteins bound to the beads were recovered by adding SDS sample buffer boiling for 5 min and were then analyzed by SDS–PAGE. Proteins were then visualized by autoradiography, or detected by Western blot with an anti-N protein monoclonal antibody.

Coimmunoprecipitation

Twenty-four hours after transfection, 293T cells were washed two times with PBS buffer and then lysed in 1 ml of RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH 8.0) with 0.5% protease inhibitor cocktail (Sigma, St. Louis, MO). The lysate was centrifuged at 12,000g for 20 min at 4°C. The supernatants were added to anti-FLAG M2 affinity gel (Sigma) and incubated with shaking for 4 hr at 4°C. The beads were collected by centrifugation and washed three times with PBS buffer. Proteins bound to the beads were eluted by adding 2 × SDS sample buffer and boiling for 5 min, the supernatant was analyzed by Western blot with anti-myc monoclonal antibody (Santa Cruz, Santa Cruz, CA).

RESULTS

Identification of hUbc9 as a SARS-CoV N Interacting Protein by Yeast Two-Hybrid System

A yeast two-hybrid screen of a human lymphocyte cDNA library was undertaken to identify proteins that interact with the SARS-CoV N protein. A bait expression vector was constructed by fusing the pGBKT7 encoded GAL4-DNA-binding domain (GAL4-DBD) to the full-length N protein. The pGBKT7-N bait was transformed into the yeast strain AH109, which uses three reporters-*ADE2*, *HIS3*, and *lacZ*. Expression of the N protein in the yeast cells transfected with pGBKT7-N was confirmed by Western blot analysis using an anti-GAL4-DBD antibody (data not shown). AH109 yeast cells transformed with pGBKT7-N alone or cotransformed with pGBKT7-N and the activation domain vector, pACT2, did not activate transcription of *LacZ* reporter gene. Thus, further experiments were carried out to screen a human lymphocyte cDNA library for proteins that interact with SARS-CoV N protein. Approximately 5×10^6 yeast transformants were screened, and ten colonies were obtained in the high stringency medium (SD/-Ade/-His/-Leu/-Trp/X-α-Gal).

The ten clones expressing putative N interacting proteins were characterized further by sequence analysis, and four of these clones were identified as human ubiquitin-conjugating enzyme 2 (hUbc9), which encodes a 158-amino acid protein with a calculated molecular mass of 19 kDa. As shown in Figure 2, the protein encoded by the pACT2-Ubc9 clones interacted specifically with the N protein and did not interact with the unfused GAL4-DBD protein expressed from the parental pGBKT7 vector.

GST-Pull Down Assays Detected the Interaction of the N Protein and hUbc9 In Vitro

To confirm that the N protein and hUbc9 interaction detected in the yeast two-hybrid system was due to a
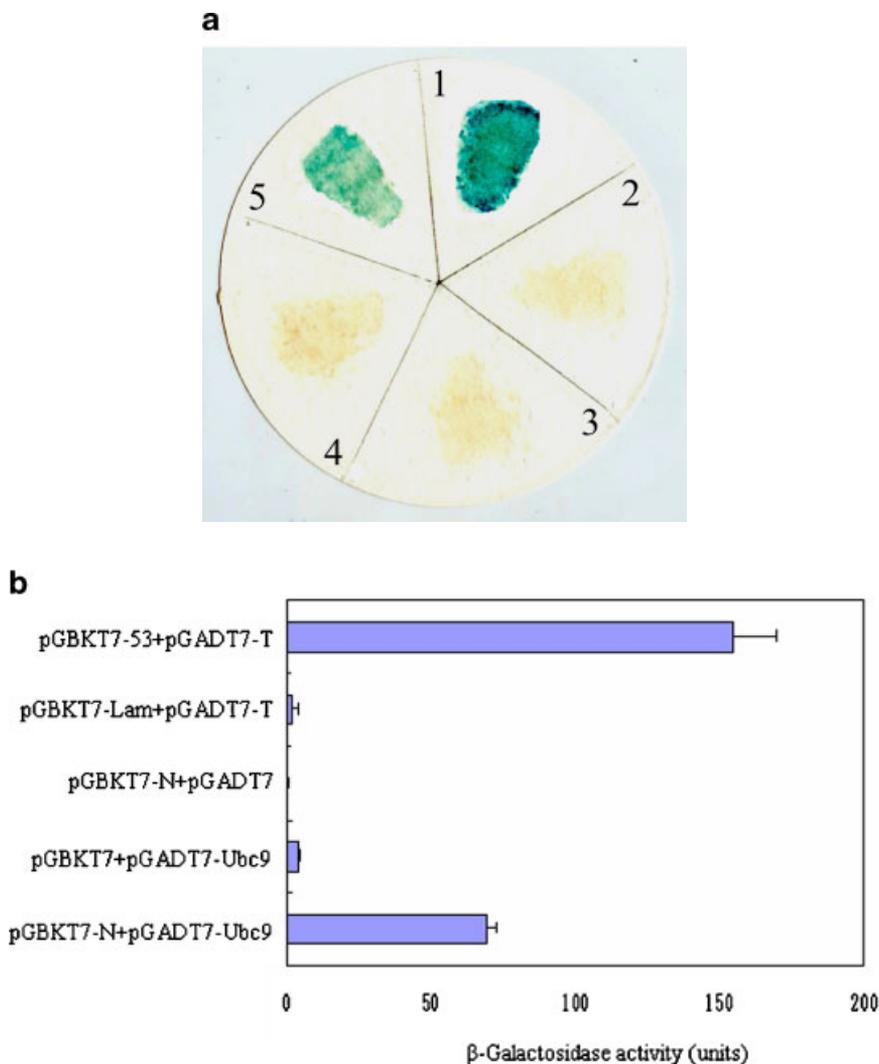
Fig. 2. Yeast two-hybrid analysis of the SARS-CoV N protein and hUbc9 interaction. **A**: β-galactosidase filter assay. 1, Positive control pGBKT7-53 + pGADT7-T; 2, negative control pGBKT7-Lam + pGADT7-T; 3, pGBKT7-N + pGADT7; 4, pGBKT7 + pGADT7-Ubc9; 5, pGBKT7-N + pGADT7-Ubc9. **B**: Yeast two-hybrid quantitation of β-galactosidase activity. The plasmid pairs above were cotransfected into the SFY526 yeast strain, and assessed for β-galactosidase activity. Results shown are the average units for triplicate assays. Error bars represent standard deviation.

direct binding of the N protein to hUbc9, GST-pull down assays were performed using GST-Ubc9 fusion protein and the SARS-CoV N protein derived from two different sources: (1) in vitro translation and (2) lysates of cells infected by SARS-CoV.

^{35}S-labeled N protein was incubated with an equal amount of GST alone or GST-Ubc9 fusion protein bound to glutathione-sepharose beads. After extensive washing, the ^{35}S-labeled protein bound to the beads was extracted and analyzed by autoradiography. As shown in Figure 3A, the N protein is associated with GST-Ubc9, but not with GST alone.

Further validation of the specific interaction between N and hUbc9 was obtained with the cell lysates infected by SARS-CoV. The lysates were incubated with GST alone or GST-Ubc9 fusion protein bound to glutathione-sepharose beads. After washing extensively with PBS,

the proteins bound to the beads were detected by Western blot analysis using anti-N monoclonal antibodies. Again, GST-Ubc9 strongly bound N protein, whereas GST alone was found not to bind the N protein (Fig. 3B). The results confirmed further that hUbc9 is capable of interacting with the SARS-CoV N protein.

The SARS-CoV N protein detected in the cell lysates by Western blot often appeared as several bands with two major bands of around 50 kDa (Fig. 3), this may be as a result of a post-translational modification, such as phosphorylation [Surjit et al., 2005] and the breakdown of the N protein which is common for the known coronaviruses [Eleouet et al., 2000]. The analysis of phosphorylation sites of the N protein with NetPhos 2.0 Server shows that there are 33 potential phosphorylation sites, including 22 serines, 8 threonines, and 3 tyrosines, of which 11 possible serine phosphorylation
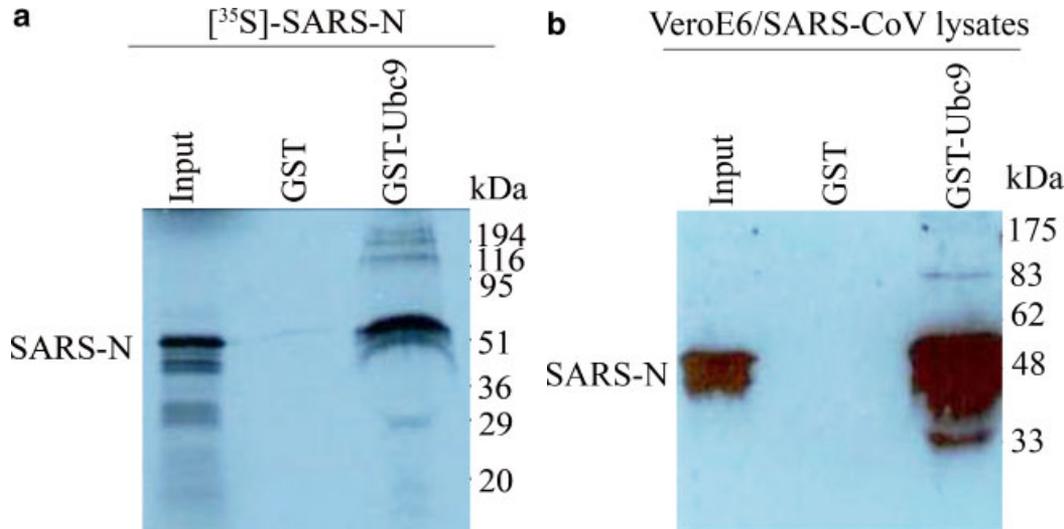
Fig. 3. GST-pull down assays for the interaction of the SARS-CoV N protein and hUbc9 in vitro. **A**: ^{35}S-labeled N protein translated in vitro was incubated with glutathione-sepharose 4B beads bound to either GST-Ubc9 or GST alone. After washing extensively, the bound proteins were resolved on 12% SDS–PAGE, and analyzed by autoradiography. **B**: The cell lysates infected with SARS-CoV were incubated with GST alone or GST-Ubc9 fusion protein bound to glutathione-sepharose 4B beads, after washing extensively with PBS, proteins bound to the beads were detected by Western blot analysis using anti-N protein monoclonal antibodies.

sites are found in the SR-rich motif. However, the residues responsible for the phosphorylation site are yet to be identified.

Immunoprecipitation Determined the Interaction of the N Protein and hUbc9

To examine further the interaction of the N protein and hUbc9, coimmunoprecipitation was performed. The SARS-CoV N protein was fused at the amino terminus with a myc tag, and hUbc9 was fused at the carboxyl terminus with a FLAG tag. The two plasmids were cotransfected into 293T cells and immunoprecipitated with anti-FLAG M2 affinity gel, and washed exten-

sively. The immunoprecipitated complexes were separated on SDS–PAGE, and analyzed by Western blot with anti-myc monoclonal antibodies. As shown in Figure 4, the myc-fused N protein immunoprecipitates with FLAG-Ubc9, however, it does not immunoprecipitate with FLAG alone and neither can myc alone immunoprecipitate with FLAG-Ubc9. These data confirm the results shown with the yeast two-hybrid system that the SARS-CoV N protein can interact with hUbc9.

Colocalization of the N Protein and hUbc9

The localization patterns of the SARS-CoV N protein and hUbc9 were investigated in 293T cells. pEGFP-N
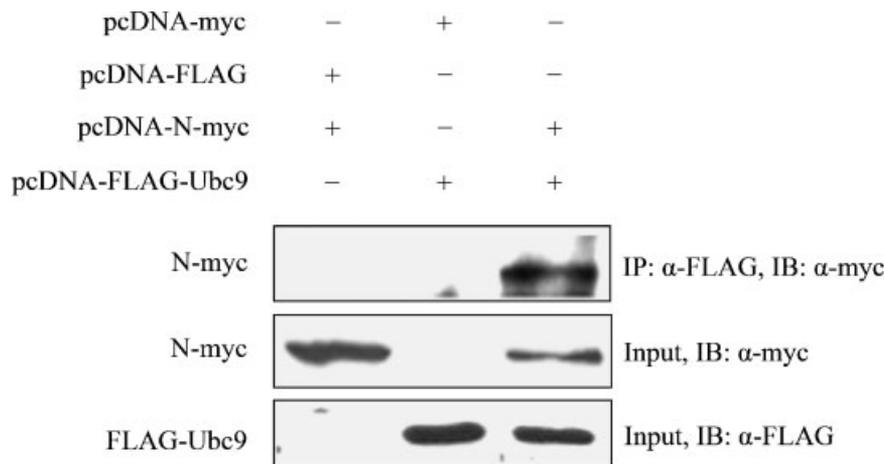
Fig. 4. hUbc9 immunoprecipitated with the SARS-CoV N protein. Indicated plasmids were simultaneously transfected into 293T cells. Twenty-four hours after transfection, the cells were lysed in RIPA buffer and coimmunoprecipitation was performed using anti-FLAG M2 affinity gel, the proteins immunoprecipitated (IP) were assayed with an anti-myc monoclonal antibody. Cell lysates were immunoblotted (IB) with anti-myc or anti-FLAG antibodies to confirm the expression of the interested proteins.
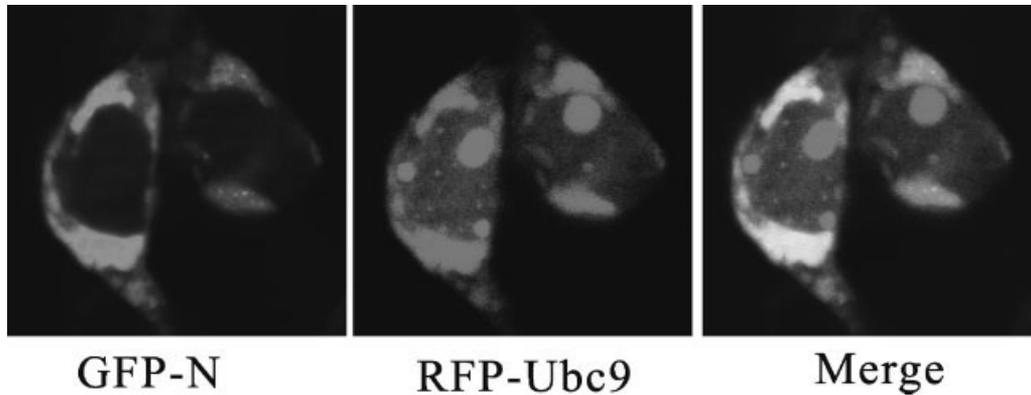
Fig. 5. Colocalization of the SARS-CoV N protein and hUbc9. pEGFP-N (green) and pDsRed-Ubc9 (red) were cotransfected into 293T cells. After 24 hr, cells were fixed, mounted, and the localization of the proteins was observed with a Leica confocal microscope. As shown, the N protein and hUbc9 were colocalized in the cytoplasm.

and pDsRed-Ubc9 were transfected simultaneously into 293T cells. As shown in Figure 5, hUbc9 lies in both the cytoplasm and the nucleus, the SARS-CoV N protein localized mainly in the cytoplasm. The combined results indicated that the SARS-CoV N protein and hUbc9 are colocalized in the cytoplasm of 293T cells.

Mapping the hUbc9 Binding Region of the N Protein

The consensus motif for sumoylation of target proteins has been defined as a tetrapeptide ψKXE (where ψ is usually a hydrophobic residue, and X is any amino acid), containing an acceptor lysine residue [Seeler and Dejean, 2003]. Nucleocapsid protein of some viruses can be sumoylated at this consensus acceptor lysine [Lee et al., 2003; Maeda et al., 2003]. Analysis of the SARS-CoV N protein sequence showed that it contains 27 lysine residues. Only one lysine residue at amino acid position 62, K62, lies roughly within the consensus SUMO-1 modification sequence (GK^{62}EE) [Li et al., 2005]. To determine whether this lysine was responsible for the interaction with hUbc9, it was mutated to an Ala by site-directed mutagenesis. As shown in Figure 6A, this mutation does not affect the binding of the N protein and hUbc9. The full-length N protein was divided into three domains to study the binding domain of the N protein: the N-terminal domain N1–169, the middle domain N170–295 containing the SR-rich motif and the C-terminal domain N296–422 (Fig. 1). These three truncated proteins were tested for hUbc9 binding using the two-hybrid assay in yeast (Fig. 6B). The results indicated that N170–295, the middle domain of the N protein, is responsible for the interaction. To determine further the region of the N protein involved in hUbc9 binding, the middle domain was divided into two parts, N170–220 and N210–295, the former containing the SR-rich motif. In the yeast two-hybrid assay, the β-galactosidase activity of N170–220 fragment is much higher than the activity of N210–295 fragment. These
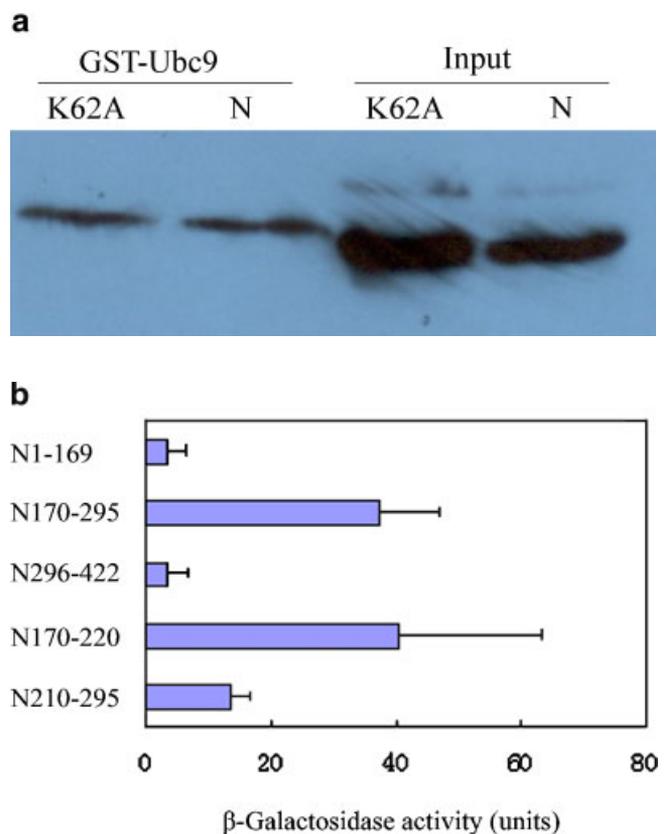
Fig. 6. Mapping the interaction domain of the SARS-CoV N protein. **A**: GST-pull down assay was used to detect whether the N protein mutation K62A affected binding to hUbc9. The results showed that K62A mutant of the N protein retained its ability to bind hUbc9. **B**: Yeast two-hybrid quantitation of β-galactosidase activity was used to assess the binding ability of the truncated N protein mutants. The fragments N170–295 and N170–220 showed intensive binding to hUbc9, whereas other fragments showed little or no binding. Results shown are the average units for triplicate assays. Error bars represent standard deviation.

results implied that the SR-rich motif of the N protein is responsible for the majority of the binding to hUbc9.

Determination of the Site in hUbc9 That Interact With the SARS-CoV N Protein

The enzyme active site of hUbc9 is centered on C93. To detect whether C93 is responsible for or has any effects on the interaction of hUbc9 with the SARS-CoV N protein, two mutants, C93S and C93A, were constructed and GST-fusion proteins were produced and purified. Lysates of 293T cells transfected with N-myc were incubated with the GST-fusion proteins GST-hUbc9, GST-C93A, GST-C93S, or GST alone, and pull down assays were performed. As shown in Figure 7, the mutant C93A has no interaction with the N protein, while the mutant C93S shows very weak binding to the N protein.

DISCUSSION

Using the yeast two-hybrid system, hUbc9, a SUMO-1-conjugating enzyme, was identified as a protein that interacts specifically with the SARS-CoV N protein. hUbc9 is an extraordinarily conserved protein in evolution from yeast to human. It is an important enzyme in the sumoylation pathway. hUbc9 catalyzes the modification of target proteins by covalent addition of a small ubiquitin-like modifier known as SUMO-1. Sumoylation is a post-translational modification process of proteins involving the formation of an isopeptide bond between the carboxyl terminus of SUMO-1 and a lysine side chain of the target protein. The SUMO-1 pathway involves ATP-dependent SUMO-1 activation catalyzed by the heterodimeric SUMO-1 activating enzyme (SAE1/SAE2). This is followed by transfer of SUMO-1

Fig. 7. Identification of the binding site of hUbc9 to the N protein determined by GST-pull down assays. Purified GST and GST-fusion proteins, such as GST-hUbc9, GST-C93A, and GST-C93S bound to the glutathione-sepharose 4B were incubated with the 293T cell lysates transfected with N-myc for 8 hr at 4°C. After washing, the bound proteins were eluted and analyzed by SDS–PAGE and Western blotted with anti-myc monoclonal antibodies. This clearly indicated that the mutation C93A abolished the binding, with only weak binding for the C93S mutant.

to the SUMO-1 conjugating enzyme hUbc9 in a transesterification reaction and the final transfer of SUMO-1 from the SUMO-1-Ubc9 complex to the target protein. Unlike ubiquitination, sumoylation does not cause protein degradation, but has been implicated in other cellular processes such as regulating the activity of transcription factors, mediating protein–protein interaction, enhancing protein stability and nuclear and cytoplasm trafficking [Muller et al., 2001; Seeler and Dejean, 2003]. Many viral proteins can interact with hUbc9 and sumoylated, which is important for virus replication [Rangasamy and Wilson, 2000; Lee et al., 2003; Maeda et al., 2003; Weldon et al., 2003; Chang et al., 2004; Gurer et al., 2005]. So far it seems that most of the viral proteins binding to hUbc9 are capsid proteins involved in virus genome binding and replication.

This study has shown that hUbc9 interacts specifically with the SARS-CoV N protein. The specificity of this interaction was initially observed in the yeast two-hybrid system and was substantiated by further experiments. Firstly, it was shown that purified GST-hUbc9 fusion proteins specifically bound the N protein obtained from either in vitro translation reactions or cell lysates infected by SARS-CoV. Secondly, FLAG-hUbc9 could be immunoprecipitated with an N-myc fusion protein using anti-FLAG M2 affinity gel. Finally and importantly, coexpression of pEGFP-N and pDsRed-Ubc9 in 293T cells revealed a reproducible colocalization both in the cytoplasm. These results strongly suggest that hUbc9 and the SARS-CoV N protein interact in the cytoplasm of mammalian cells. This is another example of viral capsid protein bound to hUbc9 but the exact role of this kind of interaction in virus replication needs to be further explored.

The interaction of N protein and hUbc9 implies the N protein is sumoylated, which has been reported recently and the sumoylation site was mapped to the consensus motif GK^{62}EE on the N protein [Li et al., 2005]. In order to determine whether the sumoylation site K62 is responsible for the binding to hUbc9, some site-mutagenesis work was undertaken and the result suggests that the consensus motif of sumoylation does not correspond with the binding site of the N protein to hUbc9. The yeast two-hybrid assays of the truncated N mutants with hUbc9 indicated that the binding site of the N protein to hUbc9 is focused instead on the SR-rich motif. SR-related proteins are often involved in protein-RNA and protein–protein interactions [Blencowe et al., 1999; Hertel and Graveley, 2005], and the SR-rich motif is conserved in the N protein of coronavirus. It has been reported recently that SR-rich motif is indispensable for SARS-CoV N oligomerization [He et al., 2004a] and for N protein interaction with SARS-CoV membrane protein [He et al., 2004b]. The SR-rich motif is also responsible for the interaction with hnRNPA1 [Luo et al., 2004]. All of these facts indicate that the SR-rich motif of the N protein has a crucial role in SARS-CoV infection.

As an essential E2-conjugating enzyme for sumoylation, hUbc9 is believed to play a central role in sumoylation-mediated cellular pathways. For example,

expression of dominant negative hUbc9 can suppress the expression of Bcl-2 and enhance apoptosis [Mo et al., 2005]. Some reports show that SARS-CoV N protein is associated with signaling pathways such as the AP-1 or Akt pathway to influence the cell cycle [He et al., 2003; Surjit et al., 2004a]. In COS-1 cells, SARS-CoV N protein expression down-regulated Bcl-2 levels and induced apoptosis in the absence of growth factors [Surjit et al., 2004a]. Therefore, it is possible that the interaction of the SARS-CoV N protein and hUbc9 may directly regulate the activity of hUbc9, affecting downstream signaling factors involved in the cell cycle, in addition to its function in the process of sumoylation, which might be important for protein trafficking, and nucleocapsid core assembly. However, the function of the interaction between the SARS-CoV N protein and hUbc9 in the virus life cycle and the host cell cycle remains to be clarified fully.

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