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Full Length Research Paper

Interaction of severe acute respiratory syndrome (SARS) nucleocapsid protein with macrophage migration inhibitory factor protein (MIF)

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The nucleocapsid (N) protein of SARS coronavirus (SARS-CoV) is a major structural component of virions, which appears to be a multifunctional protein involved in viral RNA replication and translation. However, how N protein interacts with host protein remains largely elusive. To identify cellular proteins that interact with the N protein and to elucidate the possible involvement of N protein in SARS-Cov replication and translation, a human lung cDNA library was screened using a yeast two-hybrid system assay. In this study, we have identified Macrophage migration inhibitory factor protein (MIF) as a novel interaction partner of N protein by yeast two-hybrid system. The direct interaction and co-localization of N protein with MIF were confirmed by immunoprecipitation and confocal microscopy analysis, respectively. The mapping studies localized the critical N sequences for this interaction to amino acid 140-210 including SR motif by yeast two -hybrid system. To the best of our knowledge, this is the first report that SARS-N protein interacts with the MIF within host cells, which enhance our understanding of the molecular mechanisms of SARS replication.

Key words: Severe acute respiratory syndrome (SARS) coronavirus, nucleocapsid protein, Macro|phage migration inhibitory factor protein, yeast two-hybrid.

INTRODUCTION

Severe acute respiratory syndrome (SARS) is a novel worldwide infectious disease, which is caused by a newly identified member of the *coronaviridae* family, SARS coronavirus (SARS-CoV) (Ksiazek et al., 2003; Marra et al., 2003; Peiris et al., 2003; Rota et al., 2003). Like other known coronaviruses, SARS-CoV is an enveloped virus containing three outer structural proteins, namely the membrane (M), nucleocapsid (N), and spike (S) proteins (Lai et al., 1997; Versteeg et al., 2007). The N protein is the most abundant viral protein in coronaviruses which 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 (He et al., 2004; Hiscox et al., 2001).

Mapping virus–host protein interactions can provide important clues on the initial stages of infection. For coronaviruses, the N protein plays an important role during host cell entry and virus particle assembly and release (Hatakeyama et al., 2008; Narayanan et al., 2001). It has been reported that the N protein of SARS interacts with human cellular heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) (Wang et al., 1999), which shown that N protein plays a crucial role in viral RNP assembly and replication by interaction with host proteins. However, how N protein interacts with host protein is still unclear. To obtain a more detailed insight into the N protein and host relationship, a yeast two-hybrid system was employed in the present study to identify host proteins which bind to N protein of SARS.

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MATERIALS AND METHODS

Strains and general techniques

The strain of *Saccharomyces cerevisiae* used in this study was AH109 from Clontech (USA). Yeast cells were cultured at 30°C either in a complete YPD medium (1% yeast extract, 1% peptone, 2% glucose) or in a synthetic defined (SD) medium supplemented with required essential nutrients. Plates contained 2% agar. Transformation of yeast cells was performed by the lithium acetate. *Escherichia* coli KC8 were used for general cloning. DNA manipulation was performed according to established protocol. Beta-galactosidase assays were carried out according to the CLONTECH Matchmaker manual (PT3024-1, Clontech, USA).

Plasmids and construction of recombinant vectors

For bait construction, the full-length N gene of the SARS was PCR-amplified from a genomic construct of clone, and cloned into the pMD18-T vector (Takara, China) . The full- length N gene was subjected to DNA sequencing, and the inserts were verified against the corresponding region of the SARS coronavirus. The full-length N gene was excised from the pMD18-T –N construct using the restriction enzymes EcoRI and BamHI, and ligated into the pGBKT7 vector to generate an N-terminal in frame fusion with the GAL4 activation domain (BD), and the resultant plasmid was named as pGBKT7-N. To identify the putative domain of amino acid sequence required for MIF/N interaction. The truncated mutants N1–139, N140–280, N281–422, N140–210 and N211–280 were subcloned into the yeast two-hybrid vector pGBKT7. The MIF gene was obtained by PCR from the rat liver cDNA library, and subcloned into the yeast vector pGADT7. For mammalian cell expression, the full-length N gene and MIF were subcloned into the pCMV-Myc vector (Clontech, USA) and pCMV–HA, and fluorescence vector pEGFP-N1 and pDsRed-N1, respectively. All DNA manipulations were performed as described by Sambrook et al. (2001). All constructs were verified by restriction digestion and sequencing.

Yeast two-hybrid screening

Yeast two-hybrid experiments were performed as described in the Clontech manual for the MATCHMAKER GAL4 two-hybrid system and in the Clontech yeast protocols handbook (Clontech, USA). The rat liver Matchmaker cDNA library (Clontech, USA) cloned in frame with the GAL4 activation domain in the PGAD vector was used as a prey. The bait pGBKT7-N was transformed into yeast strain AH109. The bait construct did not show any toxic effect and autonomous transcriptional activation on the host strain. The prey PGAD- library was then transformed into the bait-transformed AH109 cells, and the cells were incubated on minimal synthetic dropout medium for yeast (SD)/-His/-Leu/-Trp at 30°C. The fresh growing clones were assayed for -galactosidase activity by replica plating the yeast transformants onto Whatman filter papers; the filters were snap-frozen in liquid nitrogen for 10 sec twice and incubated in a buffer containing 5-bromo-4- chloro 3-indolyl- -D-galactopyranoside solution at 30°C for 1–8 h. Positive interactions were detected by the appearance of blue clones. The liquid - galactosidase activity was determined using the substrate ONPG as described standard Protocols Handbook (PT3024-1, Clontech, USA). Data for quantitative assays were collected for yeast cell number and are the mean ± S.E.M. of triplicate assays. Appropriate positive/negative controls and buffer blanks were used. The positive pGAD-cDNA plasmids were isolated from positive yeast transfor-mants by culture in leucine-deficient medium, which resulted in spontaneous loss of the plasmid pGBK-N and transformed into *E. coli* KC8 for sequence analysis. Auto sequencing assay was performed in Takara Company (China) and the resulting sequence was analyzed in the database of EMBL\Gene Bank by the BLAST program.

In vivo co-immunoprecipitation and western blotting

To reaffirm the results observed from yeast two-hybrid assays, another independent assay, co-immunoprecipitation was carried out. Vero cells were co-transfected with the plasmids expressing pCMV-Myc-MIF and pCMA-HA-N using the lipofectamine^{TM} transfaction reagent (Invitrogen, USA). At 48 h post- transfection, cells were washed with PBS and then lysed in lysis buffer. Cell lysate was then mixed with anti-HA magnetic microbeads for 30 min on ice. 100 l of 10% suspension of protein A–Sepharose was then added to the samples. The mixture was allowed to shake for 1 h at 4°C, after following which the beads were washed four times in lysis buffer, and protein was eluted in 2×SDS dye by boiling the sample for 5 - 10 min. Samples were resolved by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked using 0.5% BSA in PBST for 1 h, and incubated overnight with anti-Myc antibodies (1:1000; Clontech, USA). The blot was then washed three times in PBST, incubated with anti-mouse IgG HRPO for 1 h and washed three times in PBST and the proteins were visualized with 5- bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. The Vero cells transected with the pCMV-Myc and pCMV-HA vectors were used as negative controls.

Confocal microscopy

Hela cells were grown on coverslips in a 6-well chamber and simultaneously transfected with the recombinant fluorescence plasmids pEGFP-N and pDsRed-MIF. After 24 h transfection, the cells were washed with PBS three times and fixed in 4% paraformaldehyde for 20 min at room temperature. The coverslips were then washed with PBS and mounted. Intracellular localization of the N protein and MIF was observed under a Leica confocal microscope (Germany).

RESULTS

Yeast two-hybrid identified MIF as an N interacting protein

A yeast two-hybrid approach was used to identify host proteins that interact with N protein. For this purpose, the complete N gene was cloned into a two-hybrid bait expression vector. The resulting Gal4-N fusion protein was used to screen a human lung cDNA library. Approximate 2.2×10^6 transformants were screened for His-Ade-Trp-Leu independent growth and blue colony formation in the -galactosidase assay. Thirty-six positive clones were obtained. As some AD-cDNA fusion products can activate reporter gene transcription without interacting with the BD-N fusion protein, this false-positive clone can be identified using the technique of segregation analysis. Only 4 of 36 clones survived all genetic tests and were considered to be genuine positive clone, DNA sequence analysis of the fragment revealed that the four cDNA fragments inserted have a high identity with four genes in the GeneBank database. One of these clones was identified as MIF, which encodes a 158-amino acid protein.

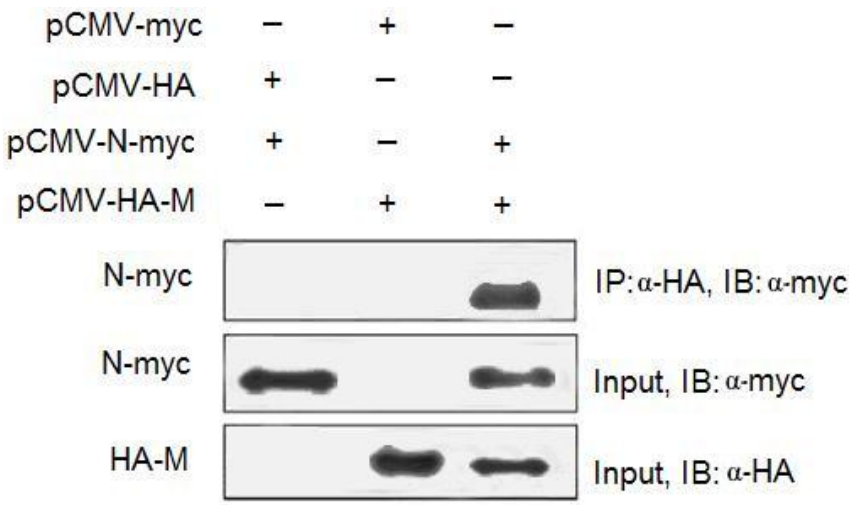

Figure 1. MIF protein was immunoprecipitated with the MHV-N protein. Indicated plasmids were simultaneously transfected into Vero cells. Twenty-four hours after transfection, coimmunoprecipitation was per-formed using anti-HA magnetic microbeads, the proteins immuno-precipitated (IP) were assayed with an anti-myc monoclonal antibody. Cell lysates were immunoblotted (IB) with anti-Myc to confirm the expression of the object proteins. M: MIF protein; N : N protein.

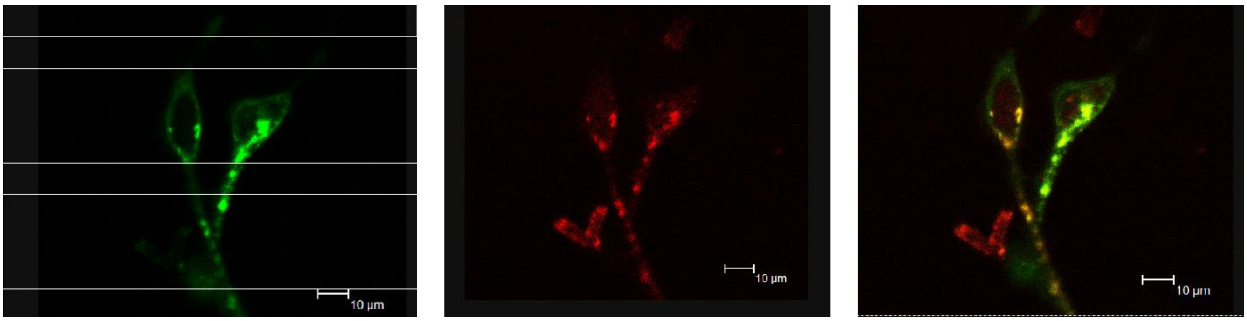

Figure 2. Co-localization of the N protein and MIF. pEGFP-N (green) and pDsRed-RACK1 (red) were co-transfected into HeLo cells. After 24 h, cells were fixed, mounted, and the localization of the proteins was observed with a Leica confocal microscope. As shown, the N protein and MIF were colocalized in the cytoplasm.

As shown in Figure 3B, the protein encoded by the pGAD-MIF clones interacted specifically with the N protein and did not interact with the unfused GAL4-BD protein expressed from the parental pGBKT7 vector.

Co-immunoprecipitation determined the interaction of the N protein and MIF

To confirm the specific interaction between N protein and MIF, co- immunoprecipitation was performed. The N protein was fused at the amino terminus with a Myc-tag, and MIF was fused at the carboxyl terminus with a HA tag. The two plasmids were cotransfected into Vero cells and immunoprecipitated. The immunoprecipitated complexes were separated on SDS–PAGE, and analyzed by Western blot with anti-Myc monoclonal antibodies. As shown in Figure1, the Myc-fused N protein immunoprecipitates with HA-MIF. However, it does not immunoprecipitate with HA alone. These findings indicate that N protein interacts with MIF in mammalian cells

Confocal microscopy assay the N protein and MIF

To further confirm the interaction between N and MIF proteins, the localization patterns of the N protein and MIF were investigated in Vero cells using confocal microscopy technology. pEGFP-N and pDsRed-MIF were transfected simultaneously into Vero cells. As shown in Figure 2, MIF and N protein mainly were localized in the cytoplasm. The merged image revealed that the N protein and MIF co-localized in the cytoplasm of Vero cells indicating that N protein interacts with MIF in cells.

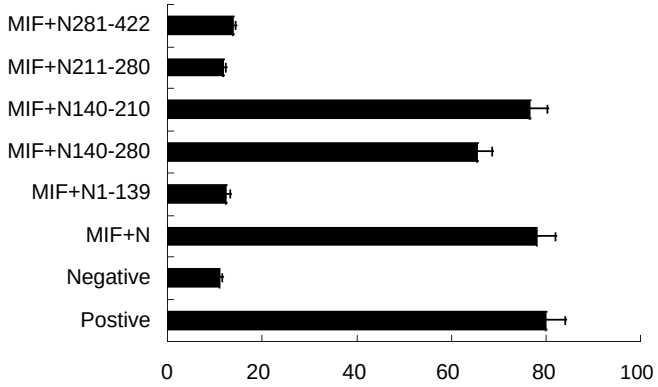

Figure 3. Mapping the interaction domain of N protein. The empty vectors pGBKT7 and pGADT7 co-transformed were used as the negative control and the pGBKT7-53 and pGADT7- T co-transformed were used as the postive con-trol. Every experiment was repeated for at least three times and the data were obtained by average. The error bars represent standard error of the mean. -galactosidase activity assay.

Mapping the MIF binding region of the N Protein by yeast two-hybrid assays

To map the involved regions of N protein in the N/MIF interaction, the full-length N protein was divided into three domains to study the binding domain of the N protein: the N-terminal domain N1–139, the middle domain N140–280 containing the SR-rich motif and the C-terminal domain N281–422 (Figure 3). These three truncated proteins were tested for MIF binding using yeast two-hybrid assay. The results indicated that N140–280, the middle domain of the N protein, is responsible for the interaction (Figure 3). To determine further the region of the N protein involved in MIF binding, the middle domain was divided into two parts, N140–280 containing the SR-rich motif and N140–210, In the yeast two-hybrid assay, the -galactosidase activity of N140–210 fragment is much higher than the activity of N211–280 fragment (Figure 3). These results implied that the SR-rich motif of the N protein is responsible for the majority of the binding to MIF.

DISCUSSION

In the present study, by employing a series of biochemical and biophysical methods, we have firstly reported that SARS-N protein has a specific binding to human MIF protein, and the further yeast two-hybrid assay demonstrated that the fragment amino acid 140–210 of SARS-N probably contribute to the N/MIF interaction. MIF was first discovered as a lymphokine involved in delayed hypersensitivity and various macrophage functions, including phagocytosis, spreading, and tumoricidal activity. It has been reported that MIF is associated with the pathogenesis of a variety of diseases (Crichlow et al. , 2009; Oddo et al., 2005). Multiple clinical studies have indicated the utility of MIF as a biomarker for different diseases that have an inflammatory component; these include systemic infections and sepsis, autoimmune diseases, cancer, and metabolic disorders such as type 2 diabetes and obesity (Shen et al., 2003; Wistow et al., 2005). Over the past few years, the significance of the role of MIF in a variety of both solid and hematologic tumors has been established. Both circulating and intracellular MIF protein levels are elevated in cancer patients and MIF expression reportedly correlates with stage, metastatic spread and disease-free survival.

To our knowledge, the fragment amino acid 140–210 contains SR- rich motif (containing rich serine and arginine) that is multifunctional and conserved in N protein of coronaviruses (Toney et al., 2004). SR-related proteins are often involved in protein-RNA and protein–protein interactions (Blencowe et al., 1999), and the SR-rich motif is conserved in the N protein of *coronavirus*. It has been reported that SR-rich motif is indispensable for SARS N oligomerization and for N protein interaction with SARS-CoV membrane protein. The SR-rich motif is also responsible for the interaction with hnRNPA1 in SARS (Haibin et al., 2005). In addition, this motif is also involved in interaction with Hubc9 in SARS (Fan et al., 2006). All of these facts indicate that the SR-rich motif of the N protein might play a crucial role in SARS infection.

In conclusion, our data have shown for the first time both *in vitro* and *in vivo* that the nucleocapsid protein of SARS has a high binding affinity to human MIF and such a protein–protein interaction involves the region amino acid 140–210 of MHV-N. However, the pathophysiological significance of the interaction between N and MIF is largely unknown; elucidation of these questions will depend on further studies. Moreover, the disruption of interaction between N and MIF proteins using RNA interference technology may provide further clues to the specific function of N and MIF protein. This current research contributes useful data that will shed light on the molecular mechanism of N phosphorylation and provide valuable clues for mutagenic studies in disrupting virion assembly and replication and developing antiviral agents.

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