Acta Pharmaceutica Sinica B 2015;5(5):487–492

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Acta Pharmaceutica Sinica B

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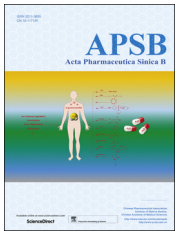

SHORT COMMUNICATION

Interactions among SARS-CoV accessory proteins revealed by bimolecular fluorescence complementation assay

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Received 15 December 2014; revised 13 April 2015; accepted 28 April 2015

KEY WORDS

SARS-CoV;
Accessory proteins;
Y2H;
Bimolecular fluorescence complementation assay

Abstract The accessory proteins (3a, 3b, 6, 7a, 7b, 8a, 8b, 9b and ORF14), predicted unknown proteins (PUPs) encoded by the genes, are considered to be unique to the severe acute respiratory syndrome coronavirus (SARS-CoV) genome. These proteins play important roles in various biological processes mediated by interactions with their partners. However, very little is known about the interactions among these accessory proteins. Here, a EYFP (enhanced yellow fluorescent protein) bimolecular fluorescence complementation (BiFC) assay was used to detect the interactions among accessory proteins. 33 out of 81 interactions were identified by BiFC, much more than that identified by the yeast two-hybrid (Y2H) system. This is the first report describing direct visualization of interactions among accessory proteins of SARS-CoV. These findings attest to the general applicability of the BiFC system for the verification of protein-protein interactions.

Abbreviations: aa, amino acids; AD, activation domain; BD, binding domain; BiFC, bimolecular fluorescence complementation; Co-IP, co-immunoprecipitation; E, envelope; EYFP, enhanced yellow fluorescent protein; M, membrane; N, nucleocapsid; NLS, nuclear localization signal; ORFs, open reading frames; PCR, polymerase chain reaction; PPIs, protein-protein interactions; PUPs, predicted unknown proteins; S, spike; SARS-CoV, severe acute respiratory syndrome coronavirus; Y2H, yeast two-hybrid

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Peer review under responsibility of Institute of Materia Medica, Chinese Academy of Medical Sciences and Chinese Pharmaceutical Association.

http://dx.doi.org/10.1016/j.apsb.2015.05.002

1. Introduction

The severe acute respiratory syndrome coronavirus (SARS-CoV) is an enveloped, positive-stranded RNA virus with a very large genomic size (about 30 kb) and constitutes 14 open reading frames (ORFs)[1,2]. The two large overlapping (ORF1a and ORF1b) are translated to produce polyproteins that are involved in viral RNA replication and transcription. The downstream ORFs encode the structural proteins in the following order: spike (S), envelope (E), membrane (M) and nucleocapsid (N). In addition to these two groups of proteins, there is another group including accessory proteins (3a, 3b, 6, 7a, 7b, 8a, 8b, 9b and ORF14) varying in length from 39 to 274 amino acids (aa) and with no significant sequence homology to proteins in other coronaviruses[1,2]. The third group of proteins, named as predicted unknown proteins (PUPs), is unique to SARS-CoV[1,2].

Although the precise functions of these accessory proteins are still obscure, it seems clear that many of them play important roles in various biological processes mediated by interactions with their partners. For example, the 3a protein can interact with structural proteins like S, M and N proteins, and another accessory protein 7a in SARS-CoV infected cells; the resulting multiple complexes may be inferred to be important for viral assembly[3]. Additionally, the interaction of SARS-CoA protein 6 and another accessory protein 9b was also confirmed to be present in SARS-CoV infected cells[4]. Thus, the identification of the occurrence and components of protein-protein interactions (PPIs) among accessory proteins provided invaluable insights into the cellular functions of these proteins. However, very little is known about other interactions among these accessory proteins[5]. Given that several accessory proteins have been shown to exhibit functions in virus-host interactions during SARS-CoV infection *in vivo*, it is necessary to understand the putative interactions among accessory proteins[6,7].

A wide range of methods, including the yeast two-hybrid (Y2H) analysis[8], co-immunoprecipitation (Co-IP) assay[9] and bimolecular fluorescence complementation (BiFC) system[10,11], have been developed for the identification and analysis of PPIs *in vitro* and *in vivo*. Each method has its own advantages and limitations. Co-IP is one of the most commonly used methods for examining PPIs. This technique often requires the preparation of specific antibodies against each of the analyzed proteins, making it an expensive and time-consuming process[12]. Also, Co-IP can be performed using lysates of the cells, which generally prevents the determination of the exact subcellular localization of the interacting proteins[13]. Y2H is another means of assessing whether two single proteins interact. Although used extensively, Y2H suffers from several drawbacks, such as high occurrence of false-positives and the requirement that the interacting proteins accumulate in the cell nucleus[8]. Among these methods, the fluorescent protein–based BiFC is an effective and straightforward tool to study PPIs, enabling direct visualization of the occurrence and subcellular localization of PPIs with simple equipment. Moreover, this approach can avoid the possibility of non-physiological protein interactions caused by cell lysis and mixing the contents of different cellular compartments. Due to its stronger signal and direct readout, this assay has become widely accepted over the past decade[10,11,14,15].

In the present investigation, characterization of the interactions among accessory proteins of SARS-CoV was performed using BiFC and Y2H. Biochemical evidence revealed the interactions existed in accessory proteins.

2. Materials and methods

2.1. Strains and plasmids

Escherichia coli (*E. coli*) TG1 was used as a host strain for subcloning. The strain was grown in Luria–Bertani medium (10 g/L Bacto–Tryptone, 5 g/L Bacto-yeast extract, 10 g/L NaCl) supplemented with ampicillin (100 μg/mL) when required for selection.

BiFC vectors pFA6a-VN-KanMX6 and pFA6a-VC-KanMX6 were kindly supplied by Prof. Won-Ki Huh (Seoul National University, Republic of Korea)[16]. The haploid *Saccharomyces cerevisiae* (*S. cerevisiae*) strains AH109 (*MATa*, *his3-200*, *leu2-3*, *112*, *trp1-901*, *ura3-52*, *gal4Δ*, *gal80Δ*, *LYS2:: GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3*, *GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2*, *URA3:: MEL1$_{UAS}$-MEL1$_{TATA}$-lacZ*, *MEL1*)[17] and W303-1B (*MATa*, *ura3-1*, *trp1-1*, *ade2-1*, *leu2-3*, *112*, *his3-11*, *15*)[18] were used to determine PPIs. The GAL4 DNA binding domain (BD) vector pGBKT7 and pGBT9, and the activation domain (AD) vector pGADT7 were from Clontech Laboratories, Inc. (Mountain View, USA) and were used throughout. The yeast expression vector pYeDP60 was kindly provided by Prof. Werck-Reichhart (France). pYeDP60 is a 2 μm plasmid with *GAL10-CYC1* promoter, *URA3* and *ADE2* marker[19,20]. All yeast strains were grown either in the non-selective YPD medium (10 g/L yeast extract, 20 g/L bactopeptone and 20 g/L glucose) or in the selective SD medium (0.7% yeast nitrogen base without amino acids, 0.1% casamino acids and 2% glucose) with appropriate amino acids dropped out at 30 °C. The detailed plasmids and strains used in the work are listed in Table S1 in Supporting information.

2.2. Enzymes and chemicals

In-Fusion® HD Cloning Kit, Restriction enzymes and X-α-gal were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan). KOD Plus Taq DNA polymerase was purchased from Toyobo Co., Ltd. (Osaka, Japan). All other fine chemicals were of analytical grade and commercial available.

2.3. Cloning of SARS-CoV accessory genes

ORF3a, *ORF3b*, *ORF6*, *ORF7a*, *ORF7b*, *ORF8a*, *ORF8b*, *ORF9b* and *ORF14* genes were chemically synthesized respectively, according to the genome sequence of SARS-CoV Tor2 isolate (GenBank accession number AY274119.3)[2]. After the sequence confirmation, the genes were subcloned into the vector pMD™18-T to acquire nine vectors named pMD3a, pMD3b, pMD6, pMD7a, pMD7b, pMD8a, pMD8b, pMD9b and pMDORF14, which were used as template to construct Y2H expression vectors and BiFC plasmids by the In-Fusion method.

2.4. Plasmids construction for Y2H assay

Specific PCR primers (Table S2 in Supporting information) were designed according to the sequences of SARS-CoV accessory genes and used to amplify each accessory gene by using of KOD Plus Taq DNA polymerase. The PCR products were ligated into the *Nde*I and *Eco*RI double digested vectors pGADT7 and pGBKT7, respectively, using the In-Fusion method. The resulting constructs pGAD-APs and pGBK-APs (APs refer to *ORF3a*, *ORF3b*, *ORF6*, *ORF7a*, *ORF7b*, *ORF8a*, *ORF8b*, *ORF9b* and *ORF14* genes) (Figs. S1 and S2 in Supporting information) containing SARS-CoV accessory genes were verified by custom sequencing.