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Japanese encephalitis virus NS2B-NS3 protease binding to phage-displayed human brain proteins with the domain of trypsin inhibitor and basic region leucine zipper

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Abstract

Flavivirus NS2B-NS3 proteases are associated with neurovirulence, becoming an important target for insight into the virus-induced pathogenesis. In this study, a phage-displayed human brain cDNA library was used to detect possible interaction between brain proteins and the Japanese encephalitis virus (JEV) NS2B-NS3 protease. After six rounds of biopanning, eight high-affinity NS2B-NS3 protease-interacting phages were identified. Identified NS2B-NS3 protease-interacting brain proteins contained several repeats of the consensus motifs E(R/K)(R/K)K and G(R/K)(R/K) with the dibasic residues, being similar to the conserved cleavage sites among flavivirus proteases. In addition, three identified brain proteins (phage-24, 34, and 44) were predicted as the domain of trypsin inhibitor and basic region leucine zipper (bZIP) using the SMART genome search. Immunoprecipitation and cleavage of two brain fusion proteins (phage-24 and phage-46) by the NS2B-NS3 protease confirmed the specific interaction between identified brain proteins and the JEV NS2B-NS3 protease. Fluorogenic peptide substrate assays revealed dose-manner inhibitory effects of these two brain fusion proteins on the *trans*-cleavage activity of NS2B-NS3 protease. Moreover, in vitro signaling pathway assay revealed that the JEV NS2B-NS3 protease significantly inhibited the signaling pathway of activator protein 1(AP1), a member of the bZIP family. Our results provide an insight into the protein interaction network of the JEV NS2B-NS3 protease in human brain.

Keywords: Japanese encephalitis virus; NS2B-NS3 protease; Phage-display; Human brain cDNA library; Trypsin inhibitor; Basic region leucine zipper

1. Introduction

Japanese encephalitis virus (JEV), a member of the *Flaviviridae* family, is a mosquito-borne neurotropic flavivirus (Burke and Monath, 2001; Lindenbach and Rice, 2001). JEV causes severe central nerve system diseases such as poliomyelitis-like acute flaccid paralysis, aseptic meningitis and encephalitis (Solomon et al., 2000). Japanese encephalitis (JE) has a high fatality rate of 30% and around half of the JE survivors have severe neurological sequela (Solomon et al., 2000). Approximately 50,000 JE cases with 10,000 deaths are reported annually in Asian countries (Burke and Monath, 2001; Solomon et al., 2000).

JEV contains a single-stranded, positive-sense RNA with about 11,000 nucleotides in length (Lindenbach and Rice, 2001). The JEV genome encodes for three structural proteins (capsid (C), membrane (prM/M), and envelope (E)) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). The N-terminal one third of NS3 contains the protease active sites at His51, Asp75, and Serl35 (Ryan et al., 1998), requiring NS2B as the cofactor of the viral serine protease (Chambers et al., 1993; Falgout et al., 1993). Sequence analysis of the JEV variants indicated that mutations at NS2B-NS3 correlated with the neurovirulence (McMinn, 1997; Chiou and Chen, 2001). Recent, the NS2B-NS3 protease of dengue viruses and Langat virus has been demonstrated to induce apoptosis

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(Prikhod'ko et al., 2002; Shafee and AbuBakar, 2003), being in line with the relevance of viral proteases to the virus-induced pathogenesis (Li et al., 2002; Barco et al., 2000). Investigating the protein interaction networks of the JEV NS2B-NS3 protease in human brain will be helpful for elucidating the JEV pathology.

Phage display provides a powerful tool for the study of the ligand-protein interaction from in vitro selection of cDNA libraries with the target protein (Smith, 1985; Danner and Belasco, 2001; Benhar, 2001). Each unique protein in a cDNA library could be expressed and fused with the coat protein gene VI in M13 phage (Hufton et al., 1999), the head protein D in λ phage (Santini et al., 1998) and the capsid protein gene 10B in T7 phage (Yamamoto et al., 1999). Phage display cDNA libraries have been demonstrated to identify the binding proteins of the interest ligands, such as the 2-methylnorharman-interacting brain proteins (Gearhart et al., 2002) and the adaptor protein 2-assaociated kinase (Conner and Schmid, 2002). Screening cDNA libraries displayed on the phage surface appears to be convenient, rapid and effective for identification of the interest ligand-interacting proteins.

In this study, a phage-displayed library encoding sequences from human brain cDNA was used to detect possible interaction between brain proteins and the JEVNS2B-NS3 protease. Sequence analysis of identified JEV NS2B-NS3 protease-interacting brain proteins were carried out using the BLASTP program and the SMART genome search. The interaction of the JEV NS2B-NS3 protease with identified JEV NS2B-NS3 protease-interacting brain proteins were confirmed by the co-immunoprecipitation assay and the in vitro cleavage assay. Moreover, the cellular effect of the JEV NS2B-NS3 protease was examined using in vitro signaling pathway assay. This study demonstrated the specific interaction of brain proteins with the JEV NS2B-NS3 protease that could be involved in the JEV-induced pathogenesis.

2. Materials and methods

2.1. Purification of recombinant NS2B-NS3 protease synthesized in E. coli

The JEV strain T1P1 was used in this study as previously described (Chiou and Chen, 2001). Viral genome was extracted from the culture supernatants of infected Vero cells using a TRIzol kit (Invitrogen). The NS2B-NS3 protease gene encoding full-length NS2B and residues 1–180 of NS3 was amplified by the reverse-transcriptase polymerase chain reaction (RT-PCR). Oligonucleotide primers used were 5′-ATCCGAATTCGGGTGGCCAGCTACTGAGTTTTTG-3′ and 5′-GGTGCTCGAGGGTGTAAGCTTCTGGGACTGGTTC-3′ The PCR product was cloned into the *Eco*RI-*Xho*I site of the bacterial expression vector pET24a (Novagen). The transformed *E. coli* BL21(DE3) cells with pET24a-NS2B-NS3 were grown in LB medium containing 100 μg of ampicillin and grown at 37 °C. Once the cells reached an absorbance at 600 nm of 0.6, they were induced by the addition of 0.5 mM isopropyl-*β*-D-thiogalactopyranoside (IPTG) at 20 °C for 4 h. Finally, the bacteria were harvested by centrifugation at 9000 rpm for 15 min at 4 °C, and resuspended in the denature buffer (10 mM imidazole, 8 M urea and 1 mM β-mercaptoethanol) for sonication. The supernatant after centrifugation (10,000 × *g* for 20 min) was applied into the His-Bind Resin column (Novagen). For decreasing concentrations of urea, recombinant NS2B-NS3 protease slowly renatures in-gel with a gradient washing by the refolding buffer (10 mM imidazole and 1 mM β-mercaptoethanol) at 4 °C overnight. After washing with 100 mM imidazole, recombinant NS2B-NS3 protease was eluted using 400 mM imidazole, and then further dialyzed against phosphate-buffered saline.

2.2. SDS-PAGE and Western blotting

The samples from each purification step were dissolved in 2× SDS-PAGE sample buffer without 2-mercaptoethanol, and boiled for 10 min. Proteins were resolved on 12% SDS-PAGE gels, and then electrophoretically transferred to nitrocellulose paper. The resultant blots were blocked with 5% skim milk, and then reacted with the 1:1000 dilution of Anti-His Tag monoclonal antibody (mAb) (Serotec) for 3-h incubation. After three-time washing with TBST (Tris-buffered saline, pH 7.5, 0.1% Tween-20), the blots were overlaid with a 1:1000 dilution of rabbit anti-mouse IgG antibodies conjugated with alkaline phosphatase (KPL). Following another 1-h incubation at room temperature, the blots were developed with NBT/BCIP (Invitrogen).

2.3. Biopanning of a phage display brain cDNA library with the NS2B-NS3 protease

A human brain cDNA library that displayed on the C terminus of phage capsid protein 10B was purchased from Novagen (Madison, WI). Six rounds of biopanning were carried out for screening the JEV NS2B-NS3 protease-interacting phages, as described in our previous report (Lin and Wu, 2004). Recombinant NS2B-NS3 protease was coated onto microplates (5 μg/well). After blocking with 5 mg/ml BSA in TBST, the phage clones reacted with NS2B-NS3 protease coated in microplates at room temperature for 1-h. The JEV NS2B-NS3 protease-interacting phages were eluted with the soluble NS2B-NS3 protease, and then amplified in *E. coli* for next round of biopanning.

2.4. Affinity binding ELISA assay

10^{10} p.f.u. of phages that individually amplified from the sixth-round selection were coated onto each well of microplates. After blocking, 20 μg/ml of the NS2B-NS3 protease was added into each well and reacted with phages at room temperature for 1-h. After washing with TBST, bound NS2B-NS3 protease was detected using the ELISA with the anti-His tag monoclonal antibody and anti-mouse IgG antibodies conjugated to peroxidase (Pharmacia). ELISA product was developed using a chromogen solution containing ABTS (2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonate), and hydrogen peroxide and then measured atA405 nm.