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Evidence of VP1 of duck hepatitis A type 1 virus as a target of neutralizing antibodies and involving receptor-binding activity

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ABSTRACT

The VP1 protein of the foot-and-mouth disease virus (FMDV) is a major target of neutralizing antibodies and is responsible for viral attachment to permissive cells via an RGD motif. VP1 of duck hepatitis A type 1 virus (DHAV-1) does not contain any RGD motif. To investigate the antibody and receptor-binding properties of DHAV-1, VP1 has been expressed as a His fusion protein (His-VP1) in baculovirus system. Sera against His-VP1 raised in rabbits effectively neutralized DHAV-1 infection *in vitro* and *in vivo*. A flow cytometry binding assay indicated that His-VP1 bound to duck embryo fibroblast cell (DEF) surface receptors. This binding was reduced in a dose-dependent manner by the addition of purified DHAV-1 virions, demonstrating the specificity of this interaction. A separate cell-binding assay also implicated His-VP1 in receptor binding. Importantly, anti-His-VP1 antibodies inhibited the binding of DHAV-1 virions to DEF cells, suggesting that these antibodies exert their neutralizing activity by blocking viral attachment. Similar to the counterpart of FMDV, DHAV-1 VP1 appears to be involved in receptor binding activity and a target of protective antibodies. This study confirms the potential of recombinant VP1 protein to serve as vaccine and diagnostic reagents for the control of DHAV-1 infections.

1. Introduction

Duck hepatitis A virus (DHAV), a member of the genus *Parechovirus* in the family *Picornaviridae,* consists of a single-stranded, positive-sense RNA. DHAV is genetically divided into three serotypes: the original worldwide type 1 virus (DHAV-1), a type 2 virus isolated in Taiwan (DHAV-2), and a type 3 virus isolated in South Korea and China (DHAV-3) (Tseng et al., 2007; Kim et al., 2008, 2007; Liu et al., 2011; Tseng and Tsai, 2007). Duck hepatitis A type 1 virus causes rapid spread of disease in ducklings primarily characterized by hepatitis (Woolcock, 2003). The disease was first reported on Long Island, New York, in 1950 (Levine and Fabricant, 1950). DHAV-1 has spread worldwide and continues to be a great threat to the duck-growing farms because of high potential mortalities when infection occurs. Mortality in the field often exceeds 50% and may reach 95% (Woolcock, 2003).

The DHAV-1 RNA comprises approximately 7623 to 7691 nt and contains a single open reading frame (ORF) encoding a polyprotein of 2249 amino acids (Tseng et al., 2007; Kim et al., 2006), which is processed into the capsid proteins VP0, VP3, and VP1. VP1 of the foot-and-mouth disease virus (FMDV) is the most external and immunodominant of the picornavirus capsid proteins, which interacts with cellular receptors and elicits neutralizing antibodies (Hewat et al., 1997; Verdaguer et al., 1995; Duque and Baxt, 2003). The majority of FMDV strains, coxsackievirus A9 (CAV-9), echovirus (EV), human parechovirus 1 (HPeV-1), and human parechovirus 2 (HPeV-2), contain highly conserved RGD motifs (Fowler et al., 2014; Williams et al., 2004; Oberste et al., 1999; Ito et al., 2004; Boonyakiat et al., 2001). Structural studies have shown that the RGD motif participates directly in the interaction with neutralizing antibodies and is involved in receptor recognition (Berinstein et al., 1995; Jackson et al., 2000; Neff, 1998; Verdaguer et al., 1995); however, DHAV-1 does not have an RGD motif in any region of the capsid proteins. It has been reported that the C terminus of FMDV VP1 includes a stretch of basic amino acids, 200-RHKQKI-205, which is similar to the heparan binding site of vitronectin

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(KKQRF) (Jackson et al., 2000), and that DHAV-1 possesses a similar stretch of amino acids (211-KKRWKPR-217) at a similar location within the VP1 protein.

In this study, we describe the expression of DHAV-1 VP1 as a His-fusion protein (His-VP1) in a baculovirus system. The expressed His-VP1 protein elicited strong neutralizing antibodies in immunized rabbits, and rabbit sera against His-VP1 could protect infected duck embryos from death. We also show that the baculovirus-expressed DHAV-1 His-VP1 binds to DEF in a manner that appears to mimic viral attachment.

2. Materials and methods

2.1. Cells and virus used in analysis

The DHAV-1 HP-1 isolate has been described previously (Liu et al., 2011). Duck embryo or duck embryo fibroblast cells (DEF) were used for virus replication as described previously (Liu et al., 2011). The virus was produced in DEF as follows: briefly, confluent DEF was infected by adding the virus to cells and allowing adsorption before the addition of Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO Life Technology) containing 1% FCS (GIBCO Life Technology). The supernatant was collected at 72 h post-infection and stored at −70 °C. Virus purification was prepared at 4 °C as follows: the DEF culture supernatant was clarified at 10,000 *g* for 20 min and then concentrated from at 100,000 *g* for 3 h. The pellet was suspended in TNE (0.01 M Tris-HCl [pH 8.0], 0.1 M NaCl, and 1 mM EDTA) containing 1% SDS and was pelleted through a 10% sucrose cushion at 100,000 *g* for 4 h. The re-suspended virus was then purified with 15–45% (w/v) sucrose gradient at 100,000 *g* for 4 h, and the gradient was collected in 1-ml fractions.

2.2. Cloning and expression of DHAV-1 VP1

The VP1 cDNA was amplified from the purified RNA of DHAV-1 HP-1 strain by reverse transcriptase PCR using synthetic oligonucleotide primers pVP1F: 5′TTTGGATCCGGTGATTCCAACCAGTTGGGGGATGAT3′ (*Bam*HI site is underlined) and pVP1R: 5′CCCCTCGAGTCATTCAATTTCCAGATTGAGTTC3′ (*Xho*I site is underlined). PCR products corresponding to the expected size of the VP1 were obtained and ligated into *Bam*HI/*Xho*I-digested baculovirus transfer vector a pFastBac1 vector (Novagen, Madison, WI). The derived plasmid, pFastBac-VP1 was proof sequenced and selected for recombinant baculovirus production. Isolated recombinant bacmid DNA and pFastBac DNA (as a control) were used to transfect *spodoptera frugiperda* (Sf9) cells according to the manufacturer's instructions. Briefly, 10 μg of pFastBac-VP1 plasmid was co-transformed into competent DH10Bac cells with 500 ng of bacmid DNA using CELLFECTIN Reagent (GIBCO) according to the supplier's instructions. The recombinant progeny virus (rBacmid-VP1) was selected, agar-plaque purified, and amplified in Sf9 cells. Working stocks of the selected rBacmid-VP1 recombinant virus was prepared from the first passage virus stock propagated in Sf9 cells. The VP1 fusion proteins in cell debris and supernatant were purified by using an affinity chromatography Ni-NTA kit (Qiagen, Valencia, CA) and then analyzed by SDS-PAGE and Western blotting.

2.3. Sera and IgG purification anti-DHAV-1

Polyclonal duck sera were prepared as described previously (Liu et al., 2010). Hyper immune rabbit sera were prepared from New Zealand White rabbits (Animal Center of Harbin Veterinary Research Institute, China) with completely UV-inactivated DHAV-1 HP-1 strain or purified His-VP1 protein. Rabbits were subcutaneously immunized with 50 μg of purified His-VP1 or 50 μg of UV-inactivated DHAV-1 virions emulsified in Freund's complete adjuvant (Sigma-Aldrich) and boosted twice at 4-week intervals with 30 μg of His-VP1 or 30 μg of DHAV-1 virions in Freund's incomplete adjuvant (Sigma), respectively. Prebleed rabbit sera (R_N) were collected before hyperimmune sera preparation. IgG from sera were purified by using Protein G Agarose (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.4. Characterization of recombinant protein by SDS-PAGE and Western blot analysis

Sf9 cells were infected with rBacmid-VP1 at a multiplicity of infection of 10–20 p.f.u. per cell. Monolayers were harvested 72 h post-infection, washed twice with phosphate-buffered saline (PBS), and finally suspended in TE 0.1 × (Tris–HCl 1 mM, EDTA 0.1 mM). Ten to the fifth power cells were diluted (v/v) in 2 × loading buffer (100 mM Tris–HCl pH 6.8, 4% SDS, 5% β-mercaptoethanol, 0.2% bromophenol blue, 20% glycerol). To obtain the fusion protein, whole cell pellets were disrupted by sonication at 4 °C; the expressed proteins were subsequently purified by Ni-NTA (Qiagen, Valencia, CA, USA). The pellet materials or purified His-VP1 protein were boiled for 5 min with an equal volume of reduced Laemmli sample buffer and subjected to electrophoresis in 10% acrylamide gels. The gels were stained by standard methods using Coomassie brilliant blue or electro transferred to nitrocellulose membranes (Millipore, Bedford, Mass.), and blocked overnight at 4 °C with 5% skim milk in PBS, pH 7.4. The membranes were probed with duck anti-DHAV-1 diluted 1:150 in PBS containing 0.05% Tween 20 (PBST) followed by reaction with horseradish peroxides conjugated goat anti-duck (1:500) (KPL) for 90 min at room temperature. The binding reaction was detected by the addition of the diaminobenzidine bromochloroindolyl phosphatenitro blue tetrazolium (BCI/NBT) color development reagent. For detection of His-tag in purified His-VP1 fusion proteins, the membrane was probed with anti-His mAb (Abbkine, Inc., Redlands, CA, USA) diluted 1:1000 in PBST, followed by reaction with horseradish peroxides conjugated goat anti-mouse (1:500) (KPL).

2.5. Serum neutralization (SN) assays in vitro and in vivo

The SN assay *in vitro* followed the method described previously (Woolcock, 2003; Liu et al., 2010). Serial twofold dilutions (initial dilution 1/10) of serum sample were made in 50 μl of DMEM in 96-well microtiter plates (Biofil, Canada JET Biochemicals). An approximately 100 tissue culture infective dose 50% ($TCID_{50}$) of the virus in 50 μl was added to each well, and the mixtures were incubated at 37 °C for 1 h. One hundred microliters of the mixture were added to confluent monolayer DEF (about 10^4 cells per well), and the plate was incubated for at 37 °C for 2 h. After incubation, the mixtures in the plates were removed. The plate was washed two times with PBS and incubated in DMEM containing 2% FCS at 37 °C in a humidified 5% CO_2 atmosphere. The cells were watched for CPE for up to 96 h. The end-point for serum neutralizing activity was expressed as the reciprocal of the highest dilution of serum that neutralized 100 $TCID_{50}$. Twelve-day old specific pathogen free (SPF) duck embryos (Harbin Vet Res Inst Experimental Animal Center, China) were used as the indicator system in the SN assay *in vivo*. Serial 2-fold dilutions (initial dilution 1/20) of rabbit anti-His-VP1 or rabbit anti-DHAV-1 sera sample were made in PBS. A 100 embryo lethal dose 50 (ELD_{50}) of the virus was added, and the mixture was incubated at 37 °C for 1 h. About 0.2 ml of the mixture was inoculated into the allantoic cavity of embryonated duck eggs. The eggs were observed for death over a 6-day period. The end-point for SN *in vivo* was calculated by the method of Reed and Muench as the