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A Small Region of Porcine Hemagglutinating Encephalomyelitis Virus Spike Protein Interacts with the Neural Cell Adhesion Molecule

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Key Words
Porcine hemagglutinating encephalomyelitis virus · Spike protein · Neural cell adhesion molecule

Abstract
Objective: The spike (S) protein of porcine hemagglutinating encephalomyelitis virus (PHEV) may mediate infection by binding to a cellular neural cell adhesion molecule (NCAM). This study aimed to identify the crucial domain of the S1 subunit of the S protein that interacts with NCAM. ***Methods:*** Three truncated segments ($S_{1–291}$, $S_{277–794}$ and $S_{548–868}$) of the S gene of PHEV and the NCAM gene were cloned individually into the *Escherichia coli* expression vectors and yeast two-hybrid expression vectors. The interaction between $S_{1–291}$, $S_{277–794}$, $S_{548–868}$ and NCAM were detected by a GST pull-down experiment and yeast two-hybrid assay. ***Results:*** Three fusion proteins ($S_{1–291}$, $S_{277–794}$ and $S_{548–868}$) were screened for their interactions with NCAM by protein-protein interaction assays. The results of these assays clarified that $S_{277–794}$ interacted with NCAM, while $S_{1–291}$ and $S_{548–868}$ did not. ***Conclusions:*** A small fragment (258-amino-acid fragment, residues 291–548) on the PHEV S protein was posited to be the minimum number of amino acids necessary to interact with NCAM. This fragment may be the receptor-binding domain that mediates PHEV binding to NCAM.

Introduction

Porcine hemagglutinating encephalomyelitis virus (PHEV) is a positive, nonsegmented, single-stranded RNA coronavirus belonging to the *Betacoronavirus* genus within the Coronaviridae family. Other related members are mouse hepatitis virus, bovine coronavirus, human coronavirus OC43, human coronavirus HKU1, etc. [1]. PHEV causes vomiting and wasting disease, as well as encephalomyelitis in piglets younger than 3 weeks, particularly in those lacking PHEV antibodies such as SPF pigs [2]. Recently, more research has focused on PHEV because infection rates have risen in some countries [2–6]. PHEV is a highly neurovirulent virus that spreads to the central nervous system via peripheral nerves, where nerve cells are a target for viral replication [7], and this spread could result in high neuropathogenicity. However, the mechanism by which PHEV enters the nerve cells remains largely unknown.

The coronavirus spike (S) protein is a key protein for determining the host and the tissue specificity of the virus, and plays an important role in coronavirus infection and pathogenicity [8–10]. The receptor of the coronaviral S protein is responsible for viruses entering into cells via

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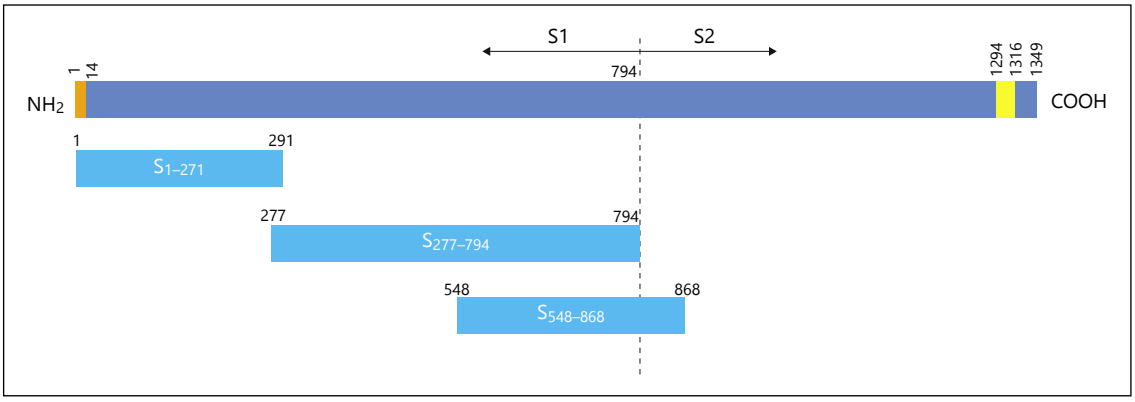

Fig. 1. Diagram of the expression constructs for the expression of soluble, truncated PHEV S glycoprotein.

fusion of viral envelope and the cellular membrane [11]. For example, the entry of SARS-CoV into cells is initiated by the binding of the S protein to the cellular receptor angiotensin-converting enzyme 2 [12], and the S protein of MERS-CoV targets the cellular receptor dipeptidyl peptidase 4 [13]. The neural cell adhesion molecule (NCAM, also known as CD56) is a homophilic glycoprotein expressed on the surface of nerve cells. NCAM has been implicated as having a role in cell-cell adhesion, neurite outgrowth and synaptic plasticity [14]. In 2010, Gao et al. [15] confirmed that NCAM participates in the process by which PHEV infects neurons. Therefore, NCAM may be a receptor for PHEV in N2a cells.

The coronaviral receptor-binding domain (RBD), which mediates the binding of viruses to receptors on susceptible cells, is the essential segment for triggering viral entry into target cells. A 193-amino-acid fragment of the SARS-CoV S protein has been identified as the RBD, which can efficiently bind angiotensin-converting enzyme 2 [16] and induce potent neutralizing antibodies and protection against SARS-CoV infection in an animal model [17]. Recently, a 286-amino-acid fragment (residues 377–662) of the MERS-CoV S protein that contains the viral RBD has also been successfully identified [18].

In a preliminary investigation, the coronaviral RBD of viruses such as SARS-CoV and MERS-CoV was found to be located on the S1 subunit of the S protein [12, 13]. To identify the crucial domain of the S1 subunit that interacts with NCAM, three truncated fusion proteins spanning the entire S1 subunit were prepared. These recombinant proteins were screened using a GST pull-down experiment, and the interactions were further confirmed by a yeast two-hybrid system assay. The results showed that the $S_{277–794}$ fragment could interact with NCAM, and a smaller fragment (258-amino-acid fragment, residues 291–548) located in $S_{277–794}$ may be the RBD of the PHEV S protein. These results will be the foundation for researching the mechanism of PHEV infection. In addition, recombinant proteins derived from the RBD could serve as targets for antiviral chemotherapy or a vaccine.

Table 1. Sequence of primers used in this work

Gene	Orientation	Sequence
$S_{1–291}$	F	5′-ggatccATGTTTTTTATACTTTTAA-3′
	R	5′-ctcgagTCATAAAATCACTAGCAC-3′
$S_{277–794}$	F	5′-ggatccGGTGTTTTATACCATGCTGT-3′
	R	5′-ctcgagAATGAACTCAAATTACC-3′
$S_{548–868}$	F	5′-ggatccGGTTTGGGTCTTGTGGAG-3′
	R	5′-ctcgagCTTGGTACTAAGGGTGAC-3′

Nucleotide sequences underlined are restriction sites.

Materials and Methods

Viruses and Cells

The HEV-67N strain of PHEV (ATCCVR741) [20] was conserved by the veterinary pathology laboratory of Jilin University and was propagated and assayed by the plaque method in N2a cell culture, as described previously [21].

PCR Amplification and Construction of the Expression Vector

According to the published standard strains of the HEV-67N gene sequence (Genbank accession No. AY078417), these primers were designed and synthesized to amplify the three truncated segments (fig. 1; table 1). The RT-PCR products were transferred into the expression vector pGEX-4T-1 (denoted pGEX $S_{1–291}$, pGEX $S_{277–794}$, pGEX $S_{548–868}$).

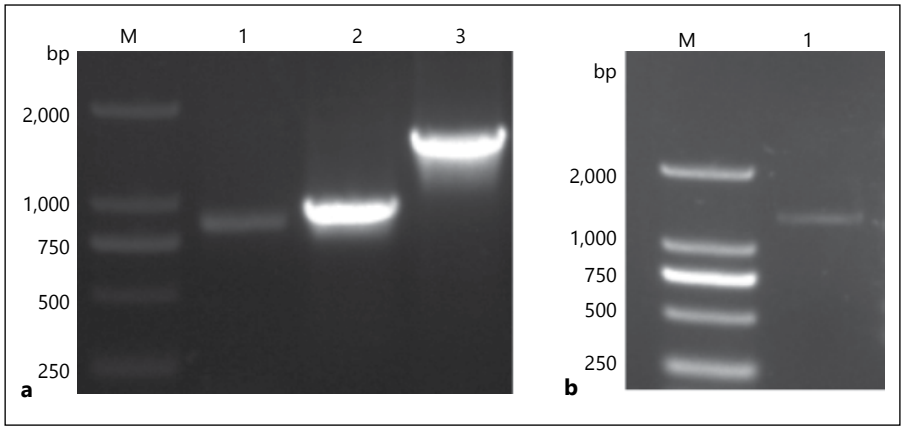

Fig. 2. Electrophoretic pattern of RT-PCR products. **a** Electrophoretic pattern of RT-PCR products of S_{1-291}, lane 1 (873 bp); $S_{548-868}$, lane 2 (960 bp), and $S_{277-794}$, lane 3 (1,548 bp). Lane M, DNA marker DL-2000. **b** Electrophoretic pattern of RT-PCR products of NCAM, lane 1 (1,281 bp). Lane M, DNA marker DL-2000.

The primers were designed according to Gao et al. [15] and the forward primer for the NCAM gene (5′-CGGAATTCGTG CCATCTATTAGCTCTGAAGT-3′) and the reverse primer for the S gene (5′-TTGCGGCCGCAAGTATGCCCTGGCCTGTA ATG-3′) introduced *Bam*HI and *Eco*RI sites, respectively, using total RNA from the N2a cells as a template for PCR amplification. The PCR product was ligated into the expression vector pET28a (denoted as pET-NC).

Fusion Protein Expression

The plasmids pGEX-4T-1, $pGEXS_{1-291}$, $pGEXS_{277-794}$, $pGEXS_{548-868}$ and pET-NC were individually transformed in *Escherichia coli* BL21 cells separately. The GST alone, S_{1-291}-GST, $S_{277-794}$-GST, $S_{548-868}$-GST and the NC-His fusion proteins were induced by 0.1 mmol/l isopropyl-β-D-thiogalactopyranoside. Then, GST alone, S_{1-291}-GST, $S_{277-794}$-GST and $S_{548-868}$-GST were purified with Glutathione Sepharose 4B beads (Amersham-Pharmacia Biotech). The NC-His fusion protein was purified using a His-Trap HP column. (Amersham Biosciences AB, Uppsala, Sweden). The identities of the proteins were analyzed by SDS-PAGE electrophoresis.

GST Pull-Down Assay

The Glutathione Sepharose 4B beads which adsorbed fusion protein S_{1-291}-GST, $S_{277-794}$-GST or $S_{548-868}$-GST were washed three times with PBS, and suspended the deposits with 200 μl of NETN buffer [50 mmol/l Tris-HCl (pH 7.5), 150 mmol/l NaCl, l mmol/l EDTA, 0.1 mmol/l DTT, 0.1% NP-40, 20% glycerol and 0.1% protease inhibitor]. The NC-His fusion protein was added to the solution and the purified GST protein, as a control, at 4° for more than 8 h. The tubes were spun at 900 *g* at 4° for 5 min. Then, the supernatant was removed. The pellets of the Sepharose 4B beads were washed four times with H buffer. Then, the pellets were boiled in 80 μl of loading buffer, subjected to 15% SDS-PAGE, transferred to PVDF and blotted with His-specific antibodies.

Yeast Two-Hybrid Assay

Three gene segments containing the S_{1-291}, $S_{277-794}$ and $S_{548-868}$ genes were excised from the plasmids $pGEXS_{1-291}$, $pGEXS_{277-794}$ and $pGEXS_{548-868}$. The excision was blunt and was repaired with T4 DNA polymerase. The segments were ligated with the yeast two-hybrid expression vectors to create the plasmids $pGADT7S_{1-291}$, $pGADT7S_{277-794}$ and $pGADT7S_{548-868}$. Similarly, the NCAM gene was cloned into pGBKT7 to create the plasmid pGBKT7-NC. The yeast strain Y187 was transformed with the $pGADT7S_{1-291}$, $pGADT7S_{277-794}$ and $pGADT7S_{548-868}$ plasmids, respectively, and the yeast strain Y2HGold was transformed with the pGBKT7-NC plasmid. The yeast Y187 containing $pGADT7S_{1-291}$, $pGADT7S_{277-794}$ or $pGADT7S_{548-868}$ was coated on SD/-Leu, SD/-Leu/Xa-Gal, SD/-Leu/-Trp and SD/-Leu/Xa-Gal/AbA solid plates, respectively, and the yeast Y2HGold containing pGBKT7-NC was coated on SD/-Trp, SD/-Trp/Xa-Gal, SD/-Leu/-Trp and SD/-Trp/Xa-Gal/AbA solid mediums at 30° for 3–4 days. The strains containing S_{1-291}, $S_{277-794}$, $S_{548-868}$ and the NCAM were monitored for autoactivation and toxicity to the yeast.

Each positive clone was chosen from Y187 containing $pGADT7S_{1-291}$, $pGADT7S_{277-794}$ and $pGADT7S_{548-868}$, or Y2HGold containing pGBKT7-NC, and was cotransferred into a 1.5-ml centrifuge tube with 500 μl of 2 × YPDA, mixed and stored at 30° for 20–24 h. The appropriate yeast were coated onto SD/-Trp, SD/-Leu and SD/-Leu/-Trp solid mediums and incubated at 30° for 3–5 days. Positive clones were chosen on the SD/-Leu/-Trp solid mediums and were coated onto SD/-Leu/-Trp/Xa-Gal/AbA and SD/-Ade/-His/-Leu/-Trp/Xa-Gal/AbA solid medium, incubated at 30° for 3–5 days and examined for the growth of yeast strains.

Results

PCR Amplification and Vector Construction

According to the published standard strains of the HEV-67N gene sequence (Genbank accession No. AY078417), we designed and synthesized three sections, and the target genes were amplified by PCR amplification. These gene products were purified by gel extraction and were the expected sizes (fig. 2). Three recombinant vectors, pGEX S_{1-291}, pGEX $S_{277-794}$ and pGEX $S_{548-868}$, were created by ligating the three products into the pEGX-4T-1 (fig. 3a).

Total RNA was extracted from the N2a cells and used as a template for PCR amplification. A resulting product

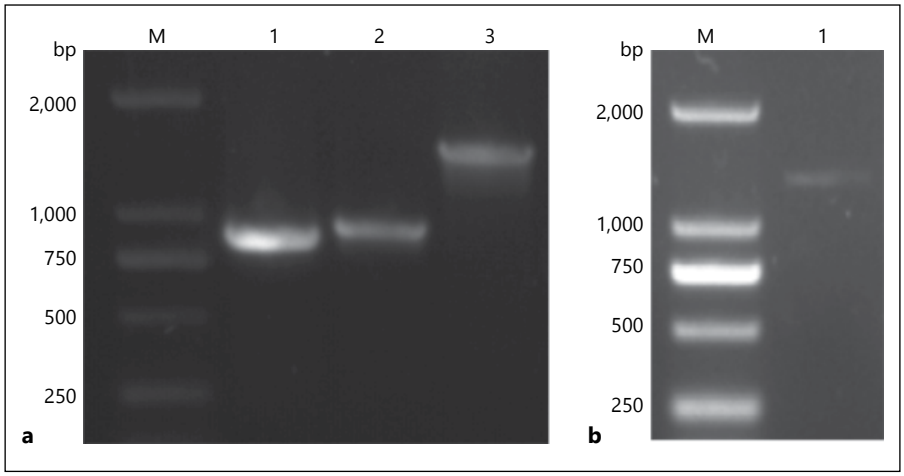

Fig. 3. Analysis of PCR of recombinant plasmids. **a** Analysis of PCR of pGADT7$_{S1-291}$, lane 1 (873 bp); pGADT7S$_{548-868}$, lane 2 (960 bp), and pGADT7S$_{277-794}$, lane 3 (1,548 bp). Lane M, DNA marker DL-2000. **b** Analysis of PCR of pGBKT7NC, lane 1 (1,281 bp). Lane M, DNA marker DL-2000.

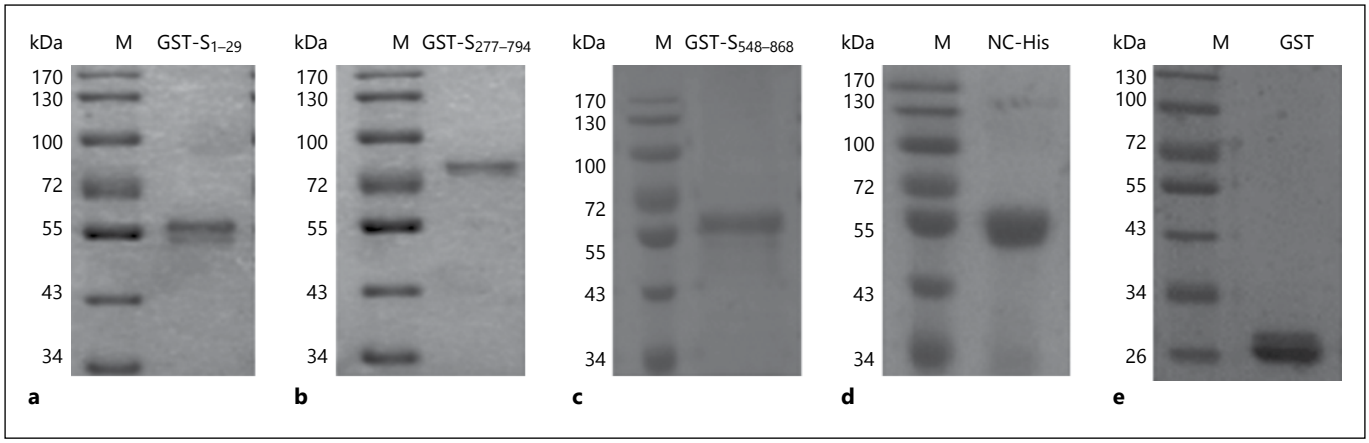

Fig. 4. Fusion proteins of GST-S$_{1-291}$, GST-S$_{277-794}$, GST-S$_{548-868}$, GST and NC-His were expressed and purified. **a** Fusion proteins of GST-S$_{1-291}$ (58 kDa) were expressed and purified. **b** Fusion proteins of GST-S$_{277-794}$ (84 kDa) were expressed and purified. **c** Fusion proteins of GST-S$_{548-868}$ (61 kDa) were expressed and purified. **d** Fusion proteins of NC-His (48 kDa) were expressed and purified. **e** Fusion proteins of GST were expressed and purified.

of approximately 1,281 bp was obtained (fig. 2). A recombinant vector, pET-NC, was constructed by ligating this product with pET28a (fig. 3b).

GST Pull-Down Experiment

The recombinant plasmids pGEX S$_{1-291}$, pGEX S$_{277-794}$, pGEX S$_{548-868}$, pGEX-4T-1 and pET-NC were transformed into *E. coli* BL21 cells. After isopropyl-β-D-thiogalactopyranoside induction, the fusion proteins (S$_{1-291}$-GST, S$_{277-794}$-GST, S$_{548-868}$-GST and NC-His) were obtained (fig. 4). The results of the GST pull-down experiment showed that S$_{277-794}$ could interact with NC-His, but there were no interactions between NCAM and S$_{1-291}$, or S$_{548-868}$ (fig. 5).

Identification of Interaction by Yeast Two-Hybrid System

The recombinant plasmids pGADT7 S$_{1-291}$, pGADT7 S$_{277-794}$ and pGADT7 S$_{548-868}$ were transformed individually into yeast Y187. Simultaneously, the plasmid pGBKT7NC was transformed into Y2HGold, and we observed no autoactivation (table 2). The pGADT7 S$_{277-794}$ plasmid was transferred into the yeast Y187, noted as b1, and the pGBKT7NC plasmid was transferred into the yeast Y2HGold, noted as NC (table 3). The transformed yeast b1 and NC only grew on SD/-Leu plates and SD/-Trp plates, respectively (tables 2, 3). The diploid yeast (pGADT7 S$_{7-522}$ × pGBKT7 NC) is denoted as Y2, and the diploid yeast (pGBKT7NC × pGADT7), (pGADT7

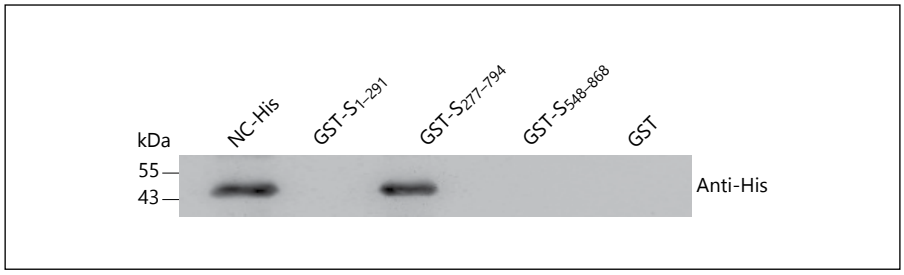

Fig. 5. Interactions of GST-$S_{1–291}$, GST-$S_{277–794}$ and GST-$S_{548–868}$ with NC-His were analyzed by GST pull-down assay. GST-$S_{277–794}$ binds to NC-His, but not to GST-$S_{1–291}$, GST-$S_{548–868}$ or GST alone. The expression product of NC-His was used as a control.

Table 2. Autoactivation results of the transformed yeast strains

Yeast strain (plasmid)	Selective agar plate	Colony	Color
Y187(pGADT7$S_{1–291}$)/Y187(pGADT7$S_{277–794}$)/ Y187(pGADT7$S_{548–868}$)	SD/-Leu	Yes	White
	SD/-Leu/X-a-Gal	Yes	White
	SD/-Leu/-Trp	No	No
	SD/-Leu/X-a-Gal/AbA	No	No
Y2Hgold(pGBKT7NC)	SD/-Trp	Yes	White
	SD/-Trp/X-a-Gal	Yes	White
	SD/-Leu/-Trp	No	No
	SD/-Trp/X-a-Gal/AbA	No	No
Y2HGold(pGBKT7-53)×Y187(pGADT7-T)	SD/-Leu/-Trp	Yes	White
	SD/-Leu/-Trp/X-a-Gal/AbA	Yes	Blue
Y2HGold(pGBKT7-Lam)×Y187(pGADT7-T)	SD/-Leu/-Trp	Yes	White
	SD/-Leu/-Trp/X-a-Gal/AbA	No	No

$S_{277–794}$ × pGBKT7), (pGBKT7-Lam × pGADT7-T) and (pGBKT7-53 × pGADT7-T) are denoted as n, b2, N and P (table 3). The results showed that Y2 grew blue colonies on SD/-Leu/-Trp/X-a-Gal/AbA plates and on SD/-Ade/-His/-Leu/-Trp/X-a-Gal/AbA plates (table 3; fig. 6). These results suggested that $S_{277–794}$ could interact with NCAM.

Similarly, we also transferred pGADT7 $S_{1–291}$ and pGADT7 $S_{548–868}$ separately into yeast Y187, denoted as a1 and c1 (table 3). Y1 and Y3 failed in growth on SD/-Ade/-His/-Leu/-Trp/X-a-Gal/AbA plates (table; 3, fig. 6), indicating that $S_{277–794}$ could interact with NCAM but $S_{1–291}$ and $S_{548–868}$ could not.

Discussion

PHEV is a member of the Coronaviridae family. Compared to other coronaviruses, the mechanism of its pathogenesis and the characterization of its RBD are relatively unknown. It is widely recognized that the main determinant of coronavirus tropism is the viral S protein which mediates binding to a cell surface receptor [22]. The S proteins of coronaviruses have an S1 and an S2 subunit. The S1 subunit initiates entry of viruses into cells by binding to cell surface receptors, and the S2 subunit helps viral fusion with cellular membranes [23–25]. The RBDs present in the S1 subunit is important for interaction with receptors. Wong et al. [16] reported that a 193-amino-acid fragment of the SARS-CoV S protein (residues 318–510) has been shown to interact with angiotensin-converting enzyme 2, and recently, Mou et al. [22] found that a 231-amino-acid fragment of the MERS-CoV S protein (residues 358–588) could interact with the cellular receptor dipeptidyl peptidase 4. Therefore, the S1 subunits of S proteins are crucial for the interactions of coronaviruses with specific receptors.

The PHEV S protein is composed of 1,349 amino acids. Based on bioinformatics analysis and the S proteins of other coronaviruses, there is a transmembrane helix between the 1,294 amino acid and 1,316 amino acid in the PHEV S protein. Residues 1–1,293 are located in the extracellular region, and residues 1,317–1,349 are intracellular. Residues 1–794 of the PHEV S protein compose the

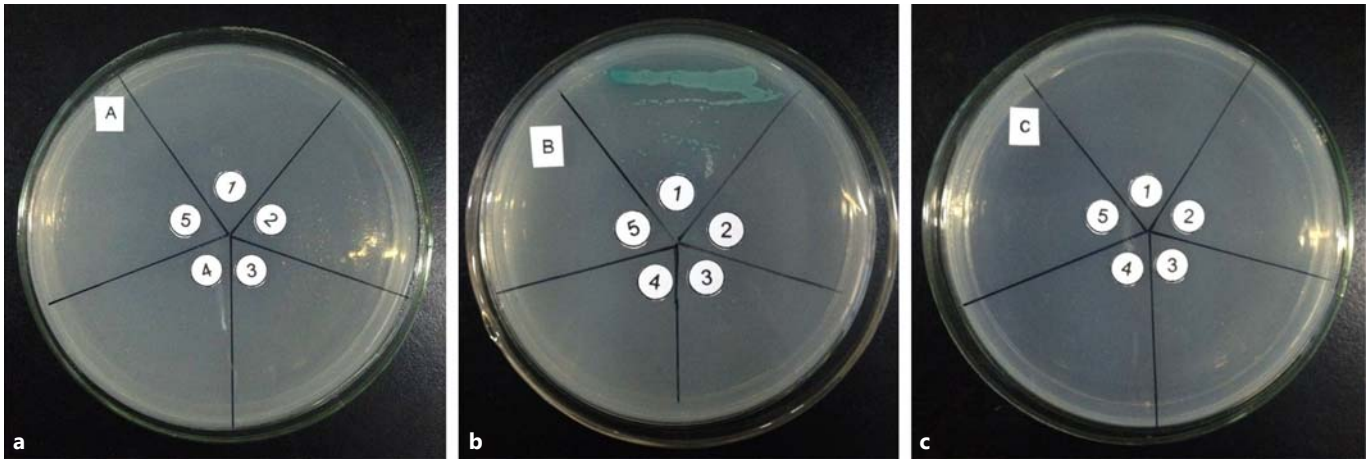

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Fig. 6. Analysis of the interactions between S protein and NCAM in yeast on the SD/–Ade/–His/–Leu/–Trp/X-a-Gal/AbA plate. **a** 1:Y2HGold (pGBKT7NC)×Y187(pGADT7S$_{1–291}$), 2:Y2HGold(pGBKT7)×Y187(pGADT7S$_{1–291}$), 3:Y2HGold(pGBKT7NC)×Y187(pGADT7),4:Y187(pGADT7S$_{1–291}$),and5:Y2HGold(pGBKT7NC). **b** 1:Y2HGold(pGBKT7NC)×Y187(pGADT7S$_{277–794}$), 2:Y2HGold(pGBKT7)×Y187(pGADT7S$_{277–794}$), 3:Y2HGold(pGBKT7NC)×Y187(pGADT7), 4:Y187(pGADT7S$_{277–794}$), and 5:Y2HGold(pGBKT7NC). **c** 1:Y2HGold(pGBKT7NC)×Y187(pGADT7S$_{548–868}$), 2:Y2HGold(pGBKT7)×Y187(pGADT7S$_{548–868}$), 3:Y2HGold(pGBKT7NC)×Y187(pGADT7), 4:Y187(pGADT7S$_{548–868}$), and 5:Y2HGold(pGBKT7NC).

Table 3. Analysis of the interactions between S protein and NCAM in yeast

No.	Yeast plasmid	Selective agar plate	Colony	Color
a1, b1, c1	Y187(pGADT7S$_{1–291}$), Y187(pGADT7S$_{277–794}$), Y187(pGADT7S$_{548–868}$)	SD/-Leu	Yes	White
		SD/-Leu/-Trp	No	No
NC	Y2Hgold(pGBKT7NC)	SD/-Trp	Yes	White
		SD/-Leu/-Trp	No	No
P	Y2HGold(pGBKT7-53)×Y187(pGADT7-T)	SD/-Leu/-Trp	Yes	White
		SD/-Ade/-His/-Leu/-Trp	Yes	White
		SD/-Ade/-His/-Leu/-Trp/X-a-Gal/AbA	Yes	Blue
N	Y2HGold(pGBKT7-Lam)×Y187(pGADT7-T)	SD/-Leu/-Trp	Yes	White
		SD/-Leu/-Trp/X-a-Gal/AbA	No	No
n	Y2HGold(pGBKT7NC)×Y187(pGADT7)	SD/-Leu/-Trp	Yes	White
		SD/-Leu/-Trp/X-a-Gal/AbA	No	No
a2, b2, c2	Y2HGold(pGBKT7)×Y187(pGADT7S$_{1–291}$), Y2HGold(pGBKT7)×Y187(pGADT7S$_{277–794}$), Y2HGold(pGBKT7)×Y187(pGADT7S$_{548–868}$)	SD/-Leu/-Trp	Yes	White
		SD/-Leu/-Trp/X-a-Gal/AbA	No	No
Y1	Y2HGold(pGBKT7NC)×Y187(pGADT7S$_{1–291}$)	SD/-Leu/-Trp	Yes	White
		SD/-Ade/-His/-Leu/-Trp	No	No
		SD/-Ade/-His/-Leu/-Trp/X-a-Gal/AbA	No	No
Y2	Y2HGold(pGBKT7NC)×Y187(pGADT7S$_{277–794}$)	SD/-Leu/-Trp	Yes	White
		SD/-Ade/-His/-Leu/-Trp	Yes	White
		SD/-Ade/-His/-Leu/-Trp/X-a-Gal/AbA	Yes	Blue
Y3	HGold(pGBKT7NC)×Y187(pGADT7S$_{548–868}$)	SD/-Leu/-Trp	Yes	White
		SD/-Ade/-His/-Leu/-Trp	No	No
		SD/-Ade/-His/-Leu/-Trp/X-a-Gal/AbA	No	No

S1 subunit, and residues 795–1,349 compose the S2 subunit. Although it has been reported that the PHEV S protein interacts with NCAM [15], the crucial binding domain of the PHEV S protein is unknown. Therefore, in this study, we prepared three truncated fusion proteins spanning the entire S1 subunit of the PHEV S protein to identify the crucial domain of interaction with NCAM.

In our work, we used the GST pull-down assay to elucidate the interaction between the PHEV S protein segments and NCAM. The NCAM that was generated using pET28a with a His-tag formed a stable complex with the GST-$S_{1–291}$, GST-$S_{277–794}$ and GST-$S_{548–868}$. The results showed that the fragment $S_{277–794}$ interacted with NCAM. Additionally, these results are supported by the yeast two-hybrid system assay. The yeast two-hybrid study showed that the fragments $S_{1–291}$, $S_{277–794}$ and $S_{548–868}$ are not autoactivating (table 2). The diploid yeast Y1, Y2, Y3, N and P, which were coated on SD/-Leu/-Trp plates, respectively, could grow white colonies (table 3), indicating that these plasmids were successfully transferred to the diploid yeast. The white colonies were inoculated separately on SD/-Leu/-Trp/Xa-Gal/AbA plates. However, only the diploid yeast Y2 and the positive control P grew colonies, which were blue (table 3). The diploid yeast Y2 could also grow colonies on an SD/-Ade/-His/-Leu/-Trp/Xa-Gal/AbA plate, and these colonies were also blue (table 3; fig. 6). These findings further demonstrate that the amino acid fragment $S_{277–794}$ may specifically interact with NCAM; however, there was no interaction between NCAM and the fragments $S_{1–291}$, or $S_{548–868}$.

Based on these results, we found an association between NCAM and $S_{277–794}$; however, $S_{1–291}$ and $S_{548–868}$ showed no such association. $S_{277–794}$ is located in the S1 subunit. In addition, because $S_{1–291}$ and $S_{548–868}$ could not interact with NCAM, and there are overlapping regions between $S_{277–794}$ and $S_{1–291}$ as well as $S_{277–794}$ and $S_{548–868}$, we conclude that the S1 subunit may contain a cellular receptor-binding region up to 258 amino acids long. These findings suggested that the 258-amino-acid fragment, residues 291–548, may be the NCAM RBD of the S protein. This region may mediate PHEV binding to NCAM, but further studies are needed to confirm these results. Meanwhile, $S_{1–291}$ and $S_{548–868}$ did not interact with NCAM and might be less relevant to bind the cellular receptor.

These amino acid residues should be further investigated to further identify the potentially crucial amino acids that interact between the S protein and NCAM. In addition, these findings may contribute in identifying the effective target epitope for an S protein-based subunit vaccine.

Acknowledgements

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