

Identification of amino acid residues involved in the interaction between *peste-des-petits-ruminants* virus haemagglutinin protein and cellular receptors

Xuelian meng, Xueliang Zhu, Niyokwishimira Alfred and Zhidong Zhang*

Abstract

Peste-des-petits-ruminants virus (PPRV) haemagglutinin (H) protein mediates binding to cellular receptors and then initiates virus entry. To identify the key residues of PPRV H (Hv) protein of the Nigeria 75/1 strain involved in binding to receptors, interaction of the Hv and mutated Hv (mHv) proteins with receptors (SLAM and Nectin 4) and their mutants (mSLAM1, mSLAM2, mSLAM3 and mNectin 4) was investigated using surface plasmon resonance imaging (SPRi) and coimmunoprecipitation (co-IP) assays. The results showed that the Hv protein failed to interact with mSLAM3, but interacted at a strong or medium intensity with SLAM, mSLAM2, Nectin 4 and mNectin 4, and at a low level with mSLAM1. The mHv protein was unable to interact with SLAM and its mutants, but bound to Nectin 4 and mNectin 4 with medium and weak intensity, respectively. Further analysis showed that the Hv protein could precipitate mSLAM1, mSLAM2 and mNectin 4, but not mSLAM3. The mHv protein failed to coprecipitate with SLAM and its mutants. The binding activities of mNectin 4 and Nectin 4 to mHv were less than 30.36 and 51.94% of the wild-type levels, respectively. Based on the results obtained, amino acids at positions R389, L464, I498, R503, R533, Y541, Y543, F552 and Y553 of H protein and I61, H62, L64, K76, K78, E123, H130, I210, A211, S226 and R227 in SLAM were identified to be essential for the specificity of H–SLAM interaction, while the critical residues of H–Nectin 4 interaction require further study. These findings would improve our understanding of the invasive mechanisms of PPRV.

INTRODUCTION

Peste-des-petits-ruminants virus (PPRV) belongs to the genus *Morbillivirus* of the family *Paramyxoviridae*, along with measles virus (MV), Rinderpest virus (RPV) and canine distemper virus (CDV). PPRV causes an acute and severe contagious disease, *peste-des-petits-ruminants* (PPR) in small ruminants, particularly goats and sheep [1]. PPR was first reported in Africa in the 1940s, and has since shown a trend to spread. In addition to Africa, Asia and the Middle East, PPR has now reached Europe [2–6]. Following the successful eradication of Rinderpest, PPR has been targeted as the next candidate for global elimination by the World Organization for Animal Health (OIE) and the Food and Agriculture Organization (FAO) [6–9]. Viruses enter susceptible cells and initiate the subsequent infection events by specifically

recognizing and binding receptors. Thus, the interaction between the viral proteins and receptors is the focus of virological study.

PPRV haemagglutinin (H) binding to receptors initiates the fusion of the viral membrane with the host cell membrane in the initial step of PPRV infection. The C-terminal ectodomain of PPRV H protein contains a membrane-proximal 96-residue stalk and a cuboidal 6-bladed β -propeller head domain that provide contact sites with the receptors [10–13]. Amino acids of MV H head domains are highly conserved and it is likely that H proteins of the other congeneric viruses may also utilize this region to infect cells. The virological data showed that amino acids 429–438 of MV H protein may be the functional domains interacting with signaling lymphocyte-activation molecule (CD150/SLAM) [14, 15].

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Author affiliations: ¹State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Animal Virology of Ministry of Agriculture, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Xujiaping 1, Yanchangpu, Chengguan District, Lanzhou 730046, PR China.

***Correspondence:** Zhidong Zhang, zhangzhidong@caas.cn

Keywords: surface plasmon resonance imaging; co-immunoprecipitation assay; haemagglutinin (H); receptor; peste-des-petits-ruminants virus.

Abbreviations: CDV, canine distemper virus; co-IP, coimmunoprecipitation; Hv, haemagglutinin protein of PPRV strain Nigeria 75/1; mHv, site-directed mutant of PPRV Hv; mNectin 4, site-directed mutant of Nectin 4; mSLAMs, site-directed mutants of SLAM; MV, measles virus; Nectin 4, poliovirus receptor related 4; PPRV, *Peste-des-petits-ruminants* virus; RPV, rinderpest virus; SLAM, signaling lymphocyte-activation molecule; SPRi, surface plasmon resonance imaging; WT, wild-type.

Three supplementary figures are available with the online version of this article.

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Recently, computational analysis results indicated that 21 amino acids at positions 191–196, 483 and 503–556 on MV and PPRV H proteins play a critical role in the H–SLAM interaction [12, 16]. Among these amino acids, amino acids at positions 503, 505, 507, 533, 543 and 552 were shared by human and ovine species for binding SLAM. Structurally, 22 amino acids in the 388–392, 458–506 and 524–550 domains of MV H showing strong attraction to human SLAM [11, 16] (Fig. S1, available in the online version of this article).

Cellular receptors determine the virus tropism and the host specificities. To date, two molecules, SLAM and poliovirus receptor-related 4 (Nectin 4/PVRL4) have been identified as receptors for PPRV [17, 18]. SLAM was also identified as a receptor for MV, CDV and RPV, while Nectin 4 for MV and CDV [19–22]. SLAM is expressed selectively in immune cells such as monocytes, dendritic cells and activated T and B cells [23–26]. SLAM contains an extracellular region with two Ig-like domains (V–C2 set). The V domain of SLAM is a critical domain for interacting with virus proteins and contains key sites specially binding to virus protein within spatial structures [27]. Hu *et al.* confirmed that amino acid 27–135 in the V domain was the functional domain that interacts with MV H [14] (Fig. S2). Ohishi *et al.* showed that eight (64, 67, 69, 73, 85, 119, 121 and 130) amino acid residues at positions 58–130 determined host–virus specificity and were shared by animal groups susceptible to the corresponding viruses in the genus *Morbillivirus* [28]. Molecular docking revealed the significance of 16 residues at positions 62–82, 123 and 127–131 of SLAM in determining caprine and human SLAM binding potentiality [12]. Intriguingly, amino acid residues at positions 58–63, 210–211 and 226–227 in human and ovine SLAM proteins have a key role in the receptor function of SLAM for PPRV and MV [14, 29, 30]. The 210, 211, 226 and 227 amino acids of ovine SLAM are I, A, S and R, respectively, different from the bovine, canine and human counterparts [31]. Nectin 4 is normally localized at adherent junctions and is expressed abundantly in epithelial cells [32, 33]. Nectin 4 contains three Ig-like ectodomains (V–C2–C2 sets), and has an important role in mediating cell–cell adhesion [34, 35]. The V domain of Nectin 4 is also involved in binding to viral proteins, serving as an entry factor [19, 36]. This receptor mediates virus infection in epithelial cells of the lungs and airways.

It is essential to accurately confirm the key amino acid residues mediating the interactional specificity of PPRV H and receptors in order to clarify viral invasion. The interactional amino acids between PPRV H and SLAM have been analysed based on a homology model of the complex. However, the key amino acid residues mediating the specificity of PPRV H interacting with the respective receptors remain largely unknown. Our previous study using surface plasmon resonance (SPR) determined the important heptad repeat region of PPRV F protein involved in intermolecular interaction in the fusion process [37]. Given these facts, in this study the recombinant plasmids expressing the wild-type (WT) and mutant receptors and PPRV H were constructed, and then SPR imaging (SPRi) and co-immunoprecipitation (co-IP) were applied to determine the key amino acids for the

interactions between PPRV Hv and the receptors, SLAM and Nectin 4. The results showed that amino acids at positions R389, L464, I498, R503, R533, Y541, Y543, F552 and Y553 of PPRV Hv protein and I61, H62, L64, K76, K78, E123, H130, I210, A211, S226 and R227 in caprine SLAM determine the specificity of the H–SLAM interaction. The results should provide important information for developing peptide-based vaccines and antiviral drugs against PPR.

RESULTS

Preparation of the recombinant proteins

The recombinant expression vectors Hv-HA, mHv-HA, SLAM-Myc, Nectin 4-Myc, mSLAM1-Myc, mSLAM2-Myc, mSLAM3-Myc and mNectin 4-Myc were constructed successfully and transiently expressed in HEK293 cells. The results of SDS-PAGE (Fig. S3) and Western blot (Fig. 1) showed that target proteins with a high purity (>90%) and specificity could be used for SPRi.

Characterization of binding affinity by SPRi

Biotin (positive) and DMSO (negative) were used as system controls interacting with streptavidin to determine the quality of the chip. The results showed that the chip quality was good and it could be used for follow-up SPRi (Fig. 2).

The target proteins Hv-HA and mHv-HA were used as ligands and printed on the chip surface in quadruplicate. SLAM-Myc, Nectin 4-Myc, mSLAM1-Myc, mSLAM2-Myc, mSLAM3-Myc and mNectin 4-Myc were used as analytes and injected at different concentrations (200, 400, 800, 1600 and 3200 nM) on the same SPRi chip. The raw sensorgrams and measurements of the binding process of ligands and analytes were recorded in real time. The kinetic parameters, the association rate constant (k_a/k_{on}), the dissociation rate constant (k_d/k_{off}), and the equilibrium dissociation constant ($K_D, k_d/k_a$) were calculated to describe the protein–protein interactions from the sensorgrams (Table 1). The SPRi signal of protein–protein interactions increased with increasing protein concentration (Figs 3 and 4). The sensorgrams and affinity parameters showed that the interaction intensity of Hv with SLAM, Nectin4 and mNectin 4 was strong, while that with mSLAM2 was of medium intensity, and that with mSLAM1 and mSLAM3 was very weak or nonexistent (Fig. 3). The K_D value of Hv interacting with mSLAM2 was comparable to that for SLAM, while the K_D values for Hv interacting with mSLAM1 and mSLAM3 were much greater than that for SLAM, showing an approximately 10^3 to 10^4 -fold difference. Moreover, the mHv protein did not interact with SLAM and its three mutants, but the interaction intensities of mHv with Nectin 4 and mNectin 4 were mild and weak, respectively (Fig. 4). The K_D value of mHv binding to SLAM and its three mutants was more than 10^{-2} M. The K_D value of Hv interacting with mNectin 4 was comparable to that for Nectin 4, while the K_D value of mHv interacting with Nectin 4 was much greater than the WT level, with an approximately 100-fold difference. The affinity of Hv–Hv or mHv–mHv interaction was caused by the formation of homodimers or tetramers. No

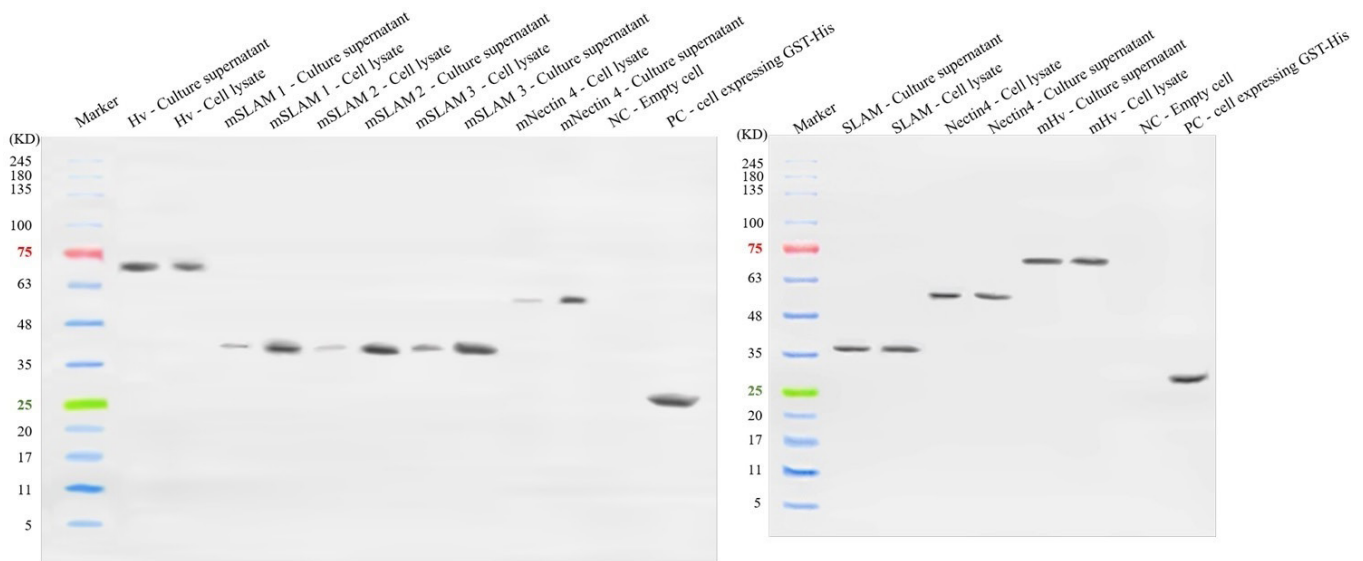


Fig. 1. Western blot of the recombinant protein.

binding was observed between Hv–PBS or mHv–PBS (Figs 3h and 4h).

These results indicated that the mutated amino acid residues of Hv, mSLAM1 and mSLAM3 are vital to the recognition and binding of the virus to SLAM. The mutated residues of Hv protein have an important effect on binding to Nectin 4, while the mutated amino acids sites of Nectin 4 may not play a decisive role in binding to Hv protein.

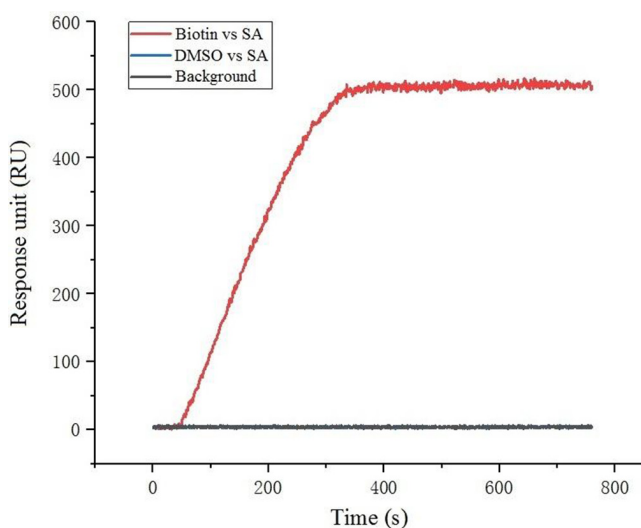


Fig. 2. Detection of microarray chip based on SPRI. SPRI graph showing interaction of biotin (positive control) and DMSO (negative control) with SA on the microarray chip.

Confirmation of binding affinity by co-immunoprecipitation assay

To further determine the key amino acid residues of Hv interacting with receptors, CHO cells co-transfected with different combinations of plasmids were subjected to immunoprecipitation, Western blot and grey value analysis by Alphaview SA. As shown in Fig. 5a, the Hv protein could not coprecipitate mSLAM3 and the mHv protein failed to coprecipitate SLAM and its three mutants. Although the Hv protein could coprecipitate mSLAM1 and mSLAM2, the interaction abilities were remarkably decreased, especially with mSLAM1, ranging from 36.99 to 55.07% of the WT level (Table 2). The results relayed in Fig. 5b and Table 3 showed that the binding activity of mNectin 4 to Hv remained above 70.5% of the WT level, while that of mNectin4 and Nectin 4 to mHv was less than 30.36 and 51.94% of the WT level, respectively. These results indicate that the mutated sites in mSLAM3 and mHv are the key amino acid residues for the interaction between SLAM and PPRV H protein, and the partially mutated residues in mSLAM1, mSLAM2 and mNectin 4 play key roles in receptors interacting with H, and the partially mutated residues of mHv are the critical sites for the interaction with Nectin 4.

Based on the results obtained, we identified that amino acids at positions R389, L464, I498, R503, R533, Y541, Y543, F552 and Y553 of PPRV H protein and I61, H62, L64, K76, K78, E123, H130, I210, A211, S226 and R227 in SLAM determine the specificity of the H–SLAM interaction. The key amino acids residues of PPRV H interacting with Nectin 4 could not be determined.

DISCUSSION

An accurate mechanism ensures timely and effective entry of viruses into host cells. PPRV H protein binding to viral

Table 1. Kinetic parameters of Hv and mHv proteins binding with receptors and mutants from SPRi

No.	analytes	ligands	Avg k_a	Avg k_d	Avg K_D	Interactions	ABS (tr_ K_D)
			(1/Ms)	(1/s)	(M)	intensity	
1	SLAM	Hv	8.88E+02	9.59E-04	1.08E-06	Strong	19.8205
2	Nectin 4	Hv	1.42E+03	4.35E-04	3.06E-07	Strong	21.6395
3	mSLAM1	Hv	1.78E+01	5.06E-02	2.85E-03	Weak	8.4558
4	mSLAM2	Hv	8.45E+01	4.41E-03	5.22E-05	Middle	14.2250
5	mSLAM3	Hv	7.21E+00	2.34E-01	3.25E-02	VW/None	4.9432
6	mNectin 4	Hv	6.21E+02	1.11E-03	1.79E-06	Strong	19.0881
7	Hv	Hv	1.81E+01	5.14E-02	2.83E-03	Weak	8.4626
8	PBS	Hv	1.30E+00	2.80E-01	2.16E-01	VW/None	2.2111
9	SLAM	mHv	4.29E+00	1.33E-01	3.09E-02	VW/None	5.0177
10	Nectin 4	mHv	9.85E+01	2.08E-03	2.11E-05	Middle	15.5312
11	mSLAM1	mHv	5.64E+00	1.58E-01	2.80E-02	VW/None	5.1585
12	mSLAM2	mHv	1.37E+01	3.52E-01	2.56E-02	VW/None	5.2851
13	mSLAM3	mHv	2.99E+00	3.29E-01	1.10E-01	VW/None	3.1839
14	mNectin 4	mHv	1.81E+01	7.21E-02	3.99E-03	Weak	7.9697
15	mHv	mHv	1.87E+01	4.61E-02	2.47E-03	Weak	8.6604
16	PBS	mHv	1.95E+00	1.82E-01	9.33E-02	VW/None	3.4220

Interaction intensity level: 10^{-13} – 10^{-8} , very strong; 10^{-8} – 10^{-5} , strong; 10^{-5} – 10^{-3} , medium; 10^{-3} – 2×10^{-2} , weak; 2×10^{-2} – 10^2 , very week (VW)/ none.

Avg k_a , average association rate constant; Avg k_d , average dissociation rate constant; Avg K_D , average equilibrium dissociation constant (k_d/k_a);

ABS (tr_ K_D), absolute affinity coefficient [$\log_2(K_D)$].

receptors is the ‘core’ mechanism and an initial step for virus entry. Identification of the key amino acids of PPRV H involved in binding to cellular receptors is essential to understand the mechanism of virus entry. The H proteins

of members of the family *Paramyxoviridae* have a globular head with a six-blade β -propeller structure that is responsible for receptor binding [11, 38–40]. A hydrophobic pocket of H protein is located at the boundary between blades β_4 and

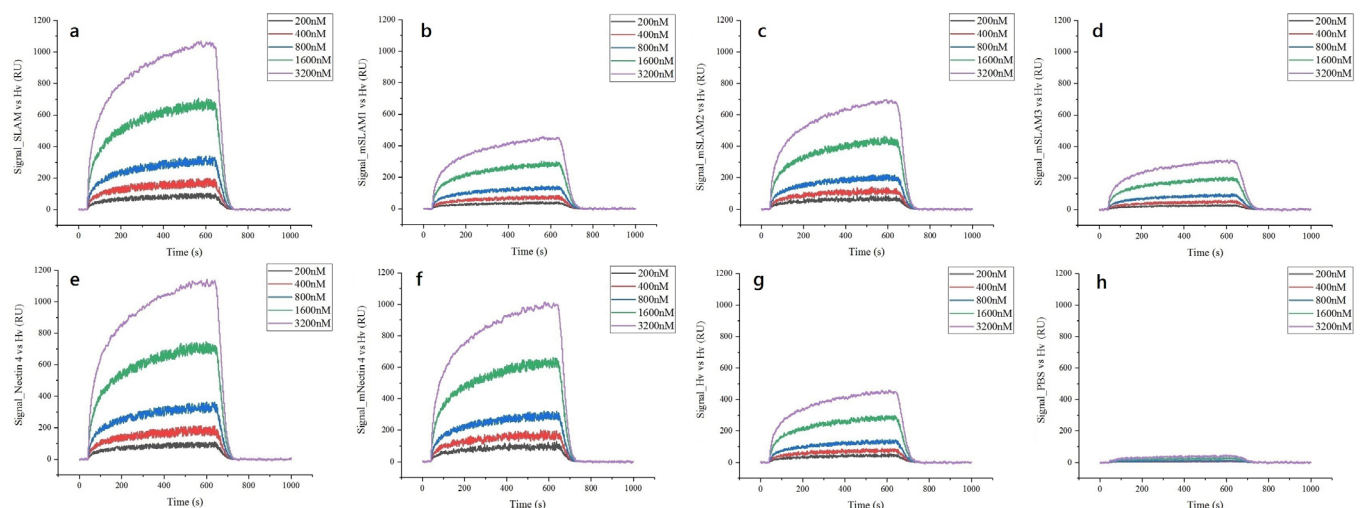


Fig. 3. SPR sensorgrams of the immobilized Hv–HA binding to the analytes at different concentrations (200, 400, 800, 1600 and 3200 nM). (a) SLAM–Myc. (b) mSLAM1–Myc. (c) mSLAM2–Myc. (d) mSLAM3–Myc. (e) Nectin 4–Myc. (f) mNectin 4–Myc. (g) Hv–HA. (h) PBS.

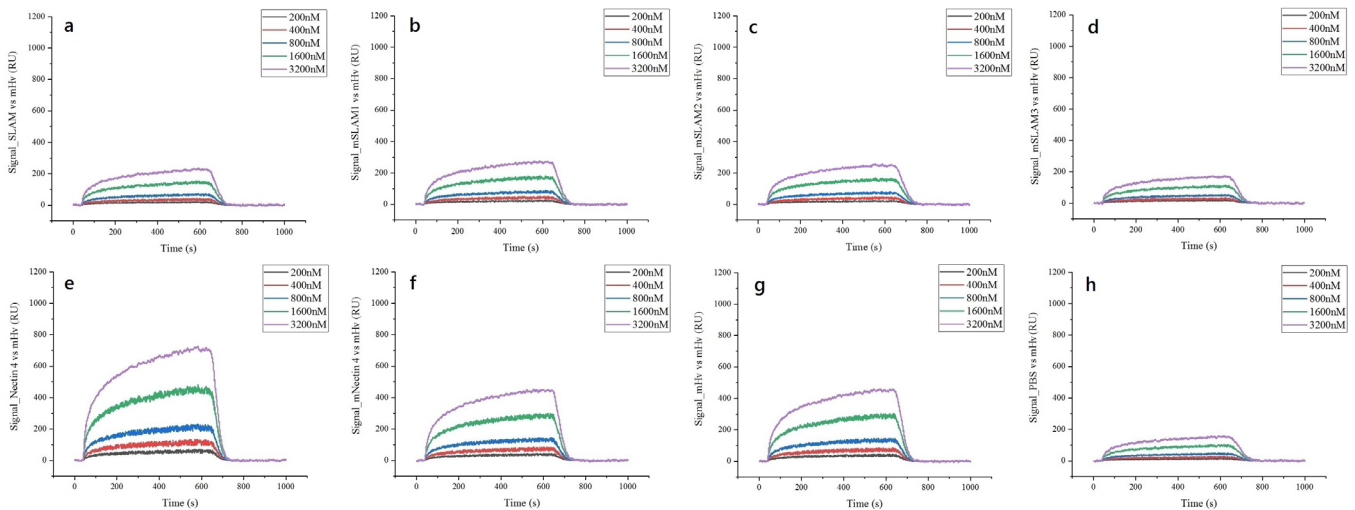


Fig. 4. SPR sensorgrams of the immobilized mHv-HA binding to the analytes at different concentrations (200, 400, 800, 1600 and 3200 nM). (a) SLAM-Myc. (b) mSLAM1-Myc. (c) mSLAM2-Myc. (d) mSLAM3-Myc. (e) Nectin 4-Myc. (f) mNectin 4-Myc. (g) mHv-HA. (h) PBS.

$\beta 5$ and is involved in binding to receptors [12, 16]. Although the key sites of the MV H interfaces that bind to three receptors in the $\beta 4$ – $\beta 5$ groove overlap somewhat, there are still considerable differences among the residues and structures of the MV H–receptor complexes [11–13, 41]. Several amino acid residues around the hydrophobic pocket in the $\beta 4$ (residues 448–507) and $\beta 5$ (residues 524–556) strand regions are essential for binding to the respective receptors. The PPRV Hv–shSLAM binding interface was consistent with that of the MV H–marmoset SLAM (maSLAM) complex [12, 16]. In this study, based on the virological and predictive reports, we constructed the WT and mutants of PPRV H and two

receptors [14–16, 28, 29, 31]. Then we investigated the role of mutated amino acids residues in the PPRV H–receptor interactions by SPRi and co-IP.

The results of SPRi were consistent with those of co-IP in the present study. The PPRV H–mSLAM3 complex actually lost binding affinity, and the K_D value was far greater than that for Hv–SLAM, showing an approximately 10^4 -fold difference. The Hv protein and mSLAM3 could not be coprecipitated. This indicated that the mutated amino acids (H62A, L64N, K76A, K78A, E123A and H130A) in mSLAM3 play a vital role in the PPRV H–SLAM interaction,

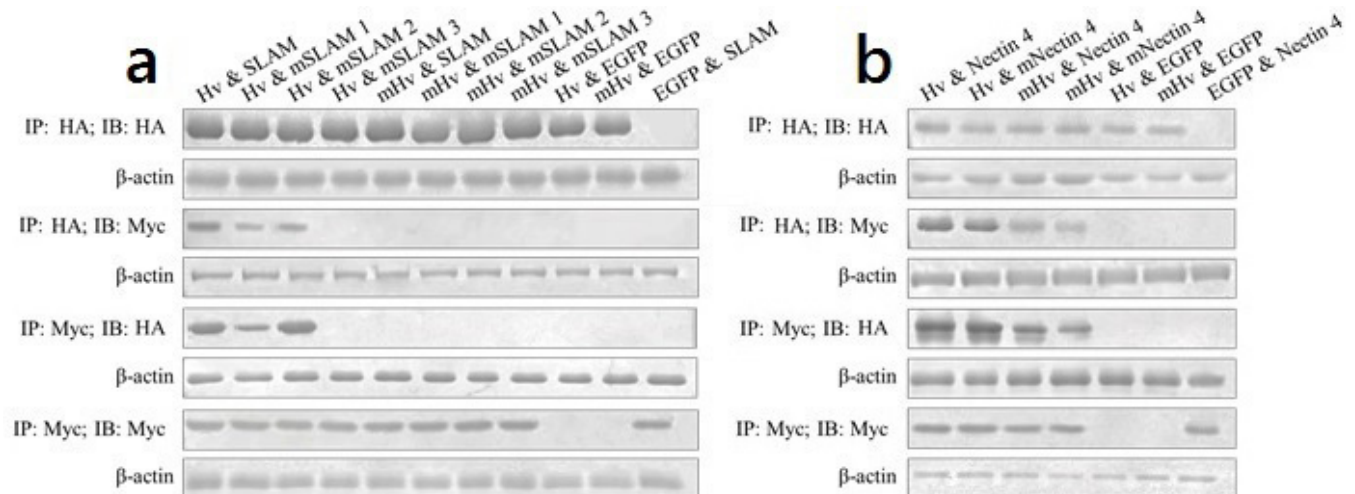


Fig. 5. Identification of the interaction between PPRV H and receptors by co-IP assay (a) The interaction of PPRV H with SLAM or its three mutants in CHO cells. (b) The interaction of PPRV H with Nectin 4 or mNectin 4 in CHO cells.

Table 2. The calibrated ratios of PPRV H interaction with SLAM and site-directed mutants determined by co-IP

Group	IP: Anti-HA	IP: Anti-HA	IP: Anti-Myc	IP: Anti-Myc
	IB: Anti-HA	IB: Anti-Myc	IB: Anti-HA	IB: Anti-Myc
Hv and SLAM	100.00%	100.00%	100.00%	100.00%
Hv and mSLAM1	97.84%	55.07%	36.99%	83.78%
Hv and mSLAM2	93.88%	89.50%	59.29%	88.22%
Hv and mSLAM3	104.68%	0.00%	0.00%	108.74%
mHv and SLAM	91.97%	0.00%	0.00%	118.61%
mHv and mSLAM1	92.71%	0.00%	0.00%	113.50%
mHv and mSLAM2	90.67%	0.00%	0.00%	120.19%
mHv and mSLAM3	93.83%	0.00%	0.00%	117.74%
Hv and EGFP	90.07%	0.00%	0.00%	0.00%
mHv and EGFP	90.49%	0.00%	0.00%	0.00%
EGFP and SLAM	0.00%	0.00%	0.00%	101.59%

which is consistent with the molecular simulation results [12]. It should be noted that the mSLAM1 protein bound to PPRV Hv with a very weak intensity, and the coprecipitation ability of Hv–mSLAM1 interaction was remarkably decreased to 36.99% of the WT level. The suggestion that the mutated amino acids (I61P, H62A, I210P A211R, S226A and R227A) in mSLAM1 have a crucial role in PPRV H binding to SLAM is in agreement with previous findings [14, 29–31]. Computational analysis showed that K77 and E123 in human and canine SLAM play pivotal roles in binding to the H protein of MV and CDV [16, 42, 43]. The two residues are known to be well conserved among the reported mammalian SLAMs. Nine amino acid residues in PPRV H (R389, L464, I498, R503, R533, Y541, Y543, F552 and Y553) were previously predicted to play pivotal roles in binding to SLAM [12]. Among these residues, four (R503, R533, Y543 and F552) were predicted to have and did show strong attractive interactions with SLAM [12, 16]. It should be noted that the amino acids at position 503 of PPRV

H and MV H proteins are different, being arginine and propeller acid, respectively. Furthermore, the virological experiments verified that the R533 of MV H is essential for binding to SLAM [44, 45]. The present study showed the mHv protein lost binding activity to the WT and mutated SLAM, and the interaction of mHv protein with SLAM was not detected by co-IP. Based on the results, we confirmed that amino acids at positions R389, L464, I498, R503, R533, Y541, Y543, F552 and Y553 of PPRV H protein and I61, H62, L64, K76, K78, E123, H130, I210, A211, S226 and R227 in caprine SLAM determine the specificity of the PPRV H–SLAM interaction. The key residues of caprine SLAM are located at the region of 21 sites that are shared by hosts susceptible to the specific morbillivirus species [28]. The apparent affinity of Hv–mSLAM2 interaction was similar to that of Hv–SLAM. It would be interesting to experimentally assess residues M67, E69, D73, K77, R85, F119 and S121 of caprine SLAM and R191, D505, D507, D530 and R556 of PPRV H protein.

Table 3. The calibrated ratios of PPRV H interaction with Nectin 4 and mNectin 4 determined by co-IP

Group	IP: Anti-HA	IP: Anti-HA	IP: Anti-Myc	IP: Anti-Myc
	IB: Anti-HA	IB: Anti-Myc	IB: Anti-HA	IB: Anti-Myc
Hv and Nectin 4	100.00%	100.00%	100.00%	100.00%
Hv and mNectin 4	90.30%	86.18%	70.50%	100.52%
mHv and Nectin 4	87.49%	34.33%	51.94%	90.66%
mHv and mNectin 4	92.84%	21.57%	30.36%	128.04%
Hv and EGFP	95.08%	0.00%	0.00%	0.00%
mHv and EGFP	101.47%	0.00%	0.00%	0.00%
EGFP and Nectin 4	0.00%	0.00%	0.00%	94.33%

Unfortunately, the key amino acid residues of the PPRV Hv–Nectin 4 interaction were not obtained in the present study. The PPRV Hv protein showed a strong attractive affinity ($K_D=1.79\times10^{-6}$ M) with the mutant mNectin 4; the coprecipitation ability remained above 70.5% of the WT level. The mutated sites of mNectin 4 were determined according to the predicted amino acids of PPRV H–Nectin 4 interaction by our research team (unpublished data). The mutated residues of caprine Nectin 4 were completely inconsistent with the predicted sites of human Nectin 4 interaction with MV H protein [16]. We could not rule out the possibility that this result was caused by the inaccuracy of mutant amino acids in the caprine Nectin 4 protein. The PPRV mHv protein bound to Nectin 4 with medium intensity ($K_D=2.11\times10^{-5}$ M) and 51.94% of the WT coprecipitation level or less. However, three residues of PPRV Hv, L464, Y541 and Y543, were the key amino acids of the MV H–Nectin 4 interaction [11, 16, 40]. The mutation at position 543 of MV H could result in the loss of binding activity with Nectin 4 [41, 46]. It is suggested that the mutated residues L464, Y541 and Y543 of PPRV Hv protein also play critical roles for interaction with caprine Nectin 4. The next step is to study the key amino acids involved in the interaction between PPRV H protein and caprine Nectin 4 further.

All in all, the amino acid residues R389, L464, I498, R503, R533, Y541, Y543, F552 and Y553 of PPRV H protein and I61, H62, L64, K76, K78, E123, H130, I210, A211, S226 and R227 in caprine SLAM were found to determine the specificity of the PPRV H–SLAM interaction. Although the key residues of PPRV H protein interacting with Nectin 4 were not confirmed, the present results lay a foundation for further study. Further along, the identified amino acids residues may be useful targets for developing peptide-based vaccines and inhibitors (antiviral drugs) against PPR.

METHODS

Plasmids and reagents

The recombinant plasmids pET30a-Hv (GenBank accession no. X74443), pET30a-SLAM (GenBank accession no. DQ228869) and pET30a-Nectin 4 (GenBank accession no. XP_004002729) and the vectors pCMV-Myc and pCMV-HA were provided by the Lanzhou Veterinary Research Institute of the Chinese Academy of Agricultural Sciences and were used to construct eukaryotic expressing plasmids. *Escherichia coli* DH5 α , T4 DNA ligase and all restriction enzymes were purchased from TaKaRa (PR China). QIAprep spin Miniprep was from Qiagen. DMEM and F12K were purchased from HyClone (USA). Lipofectamine 3000 was from Invitrogen. Foetal bovine serum (FBS) was purchased from Gibco BRL Life Tech. The Pierce BCA Protein Assay kit and the Pierce Protein A/G Magnetic Beads kit were purchased from Thermo Fisher Scientific (USA). The microarray chips were products of Betterways, Inc. Biotin and streptavidin were purchased from Sigma (USA). Anti-HA, anti-Myc and anti- β -actin monoclonal antibody were purchased from Abcam (USA). The HRP-coupled secondary antibodies were products of

Table 4. The site-directed mutants

WT amino Acids→mutated amino acids				
mSLAM1	mSLAM2	mSLAM3	mNectin4	mHv
61: I→P	64: L→N	62: H→A	58: G→R	389: R→A
62: H→A	67: M→N	64: L→N	63: Q→A	464: L→N
210: I→P	69: E→A	76: K→A	63: Q→A	498: I→P
211: A→R	73: D→A	78: K→A	84: K→A	503: R→A
226: S→A	85: R→A	123: E→A	131: F→N	533: R→A
227: R→A	119: F→N	130: H→A	132: P→A	541: Y→N
	121: S→A		136: F→N	543: Y→N
	130: H→A			552: F→N
				553: Y→N

Bioss (PR China) and the DAB HRP colour development kit was a Beyotime product (PR China).

Construction of eukaryotic expression vectors

The mutants mHv, mSLAM1, mSLAM2, mSLAM3 and mNectin 4 were generated based on a backbone of PPRV H (Nigeria 75/1), capra hircus SLAM (DQ228869) and ovis aries Nectin-4 (XP_004002729) protein in combination with the virological and predictive reports [11, 12, 14, 29–31, 40]. The site-directed mutants are shown in Table 4. Hv and mHv were introduced into pCMV-HA plasmid with HA and His tags; receptors and mutants were cloned into pCMV-Myc plasmid with Myc and His tags. The clones were verified by double digestion with corresponding restriction enzymes and sequencing.

Cell culture and transfection

HEK293 cells and CHO-K1 were obtained from the Shanghai Institutes for Biological Sciences (SIBS, PR China). HEK293 cells were grown in DMEM and supplemented with 10% FBS and 1% penicillin/streptomycin (Thermo Scientific, USA). CHO-K1 cells were cultured in F12K supplemented with 5% FBS, 100 U ml⁻¹ penicillin and 100 U ml⁻¹ streptomycin at 37 °C in a humidified 5% CO₂ incubator.

For the production of recombinant proteins, HEK293 cells were transiently transfected with plasmids using Lipofectamine 3000 according to the manufacturer's instructions. The cells were cultured with 293 expression medium in a disposable flask (Thermo Fisher Scientific, USA) that was shaken at 110 r.p.m. using a rotary shaker for 6 d at 37 °C in a humidified 5% CO₂ incubator.

For immunoprecipitation, CHO cells were seeded into six-well plates to confluence. The different combinations of Hv–HA, mHv–HA, SLAM–Myc, Nectin 4–Myc, mSLAM1–Myc, mSLAM2–Myc, mSLAM3–Myc, mNectin 4–Myc or pcDNA3.1–EGFP were co-transfected using Lipofectamine 3000 as instructed by the manufacturer. Forty-eight hours

after transfection, the cells were lysed and subjected to immunoprecipitation.

Protein purification

The culture medium of transfected HEK293 cells was harvested and centrifuged, and then the supernatant was filtered through a 0.22 µm membrane filter (Merck Millipore, Germany). The expressed proteins were purified through dialysis (25 mM Tris, 150 mM NaCl, pH8.0) and Ni-IDA chromatography (Detai Biologics, PR China). The purified proteins were separated by SDS-PAGE and then analysed by Western blot, and the protein concentration was estimated using the Pierce BCA Protein Assay kit. The protein samples were stored in PBS at –80 °C.

Surface plasmon resonance imaging

Binding assays were performed using the Berthold bScreen LB 991 Label-free Microarray System (Berthold Technologies, Germany) according to the manufacturer's instructions. All of the reactions were performed at 4 °C.

The fresh microarray chips were treated according to the standard operating procedure provided by the manufacturer. The microarray chips used in this experiment were coated with a 47.5 nm thick gold layer and received photo-cross-linker chemical modification. The purified samples and controls, diluted to print concentration in PBS buffer, were printed onto the chip surface with a Biodot AD-1520 Array Printer (BioDot, Inc., USA). Each sample was printed on four parallel chips. The biotin (positive control) and DMSO (negative control) interaction with streptavidin were used as system controls for the measurement of specific signals.

The printed chips were dried by a stream of nitrogen to evaporate the solvent in the sample dots. Subsequently, the sensor chips were quickly transferred to a UV spectroirradiator for a photo-cross-linking reaction. Then, the chips were exposed to UV irradiation in a UV chamber (Amersham Life Science, USA). The irradiation programme was as follows: 100 µW cm⁻², 2 min; pause 2 min; 100 µW cm⁻², 2 min; pause 2 min; 25 µW cm⁻², 15 min. The chips were subsequently rinsed with dimethylformamide, ethanol and distilled water for 15 min to remove non-specifically adsorbed compounds and were dried under a stream of nitrogen. Two more sensor chips were prepared as replicates. The chips were inserted into the SPRi instrument for measurement.

All protein samples were injected at a rate of 0.5 µL s⁻¹. PBS (pH=7.0) was used as both an analyte and running buffer. Additionally, the solvent (PBS) for proteins was also tested. At the end of the affinity test, PBS was crosswise tested with all proteins on the chip as the background noise control.

During the SPRi test, the surface was first primed three times with HBS-EP running buffer [containing 10 mM HEPES, pH 7.0, 150 mM NaCl, 3 mM EDTA and 0.005% (v/v) of P20 surfactant] at a rate of 2 µL s⁻¹ for 40 s and one time with running buffer (1×PBS) at a rate of 2 µL s⁻¹ for 40 s. The eight proteins flowed as analytes were diluted separately

with PBS to five different concentrations (200, 400, 800, 1600 and 3200 nM). A solution of 10 mM glycine-HCl (pH 2.0) was used to regenerate the surface at a rate of 2 µL s⁻¹. Each cycle of sample analysis consisted of a 600 s association phase, a 360 s dissociation phase and a 300 s regeneration phase. The raw sensorgrams and affinity parameters of the binding process of ligands and analytes were recorded in real time. The response unit (RU) of surface resonance was compared to determine the different binding affinities between each sample dot. The interaction parameters, the association rate constant (k_a/k_{on}), the dissociation rate constant (k_d/k_{off}), and the equilibrium dissociation constant (K_D , k_d/k_a), were processed and analysed using the data analysis software of the bScreen LB 991 unlabelled microarray system according to a single-site binding model (1:1 Langmuir binding) with mass transfer limitations for binding kinetics determination. All binding curves were normalized by subtracting the background signal.

Co-immunoprecipitation assay

At 48 h post-transfection, cells were harvested and washed twice with ice-cold PBS (pH 7.4) and then lysed with RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) for 30 min on ice. The lysates were centrifuged at 12000 r.p.m. for 30 min at 4 °C and the supernatants were immunoprecipitated using a Pierce Protein A/G Magnetic Beads kit (Thermo Fisher scientific). Rabbit anti-HA and mouse anti-Myc tag monoclonal antibodies (Abcam) were covalently linked to protein A/G magnetic beads by disuccinimidyl suberate (DSS; Thermo Fisher), respectively. Co-IPs with the antibody-coupled resin were performed at 4 °C overnight. The following day, the beads were washed and the bound protein was eluted. The elution fractions were separated on SDS-PAGE for Western blot analysis.

Western blot and data analysis

Samples were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were incubated with anti-HA, anti-Myc, or anti-β-actin, followed by appropriate HRP-conjugated secondary antibodies. The signals were visualized using the DAB HRP colour development kit. The relative expression of proteins of co-IP was analysed quantitatively using an Arrayit SpotLight Fluorescence Scanner and the AlphaView SA system (version 3.2.4.0, Cell Biosciences, Inc.)

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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