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Identification of Amino Acid Residues Involved in the Interaction between Measles Virus Haemagglutin (MVH) and Its Human Cell Receptor (Signaling Lymphocyte Activation Molecule, SLAM)

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Signaling lymphocyte activation molecule (SLAM; also known as CD150) is a newly identified cellular receptor for measles virus (MV). The interaction between MV Haemagglutin (MVH) and SLAM is an initial step for MV entry. We have identified several novel SLAM binding sites at residues S429, T436 and H437 of MVH protein and MVH mutants in these residues dramatically decrease the ability to interaction with the cell surface SLAM and fail to co-precipitation with SLAM in vivo as well as malfunction in syncytium formation. At the same time, K58, S59 and H61 of SLAM was also identified to be critical for MVH and SLAM binding. Further, these residues may be useful targets for the development of measles therapy.

Keywords: Co-precipitation, Hemagglutinin, Measles virus, Signaling lymphocyte activation molecule

Introduction

Measles virus (MV), a member of the Morbillilivirus genus in the Paramyxoviridae family in the Mononegavirales order, cause Measles disease, which is responsible for 1 million infants death each year all over the world (Masse *et al.*, 2002). The high morbidity and mortality is mostly due to the secondary infections caused by the Measles virus induced immunosuppression (Griffin, 2001). Notwithstanding the inoculability of the live vaccine, measles virus still belongs to the ten of the most dangerous pathogens worldwide.

MV envelope is comprised of two glycoprotein, fusion (MVF) protein and MVH, which can interaction with the cell surface receptor to initiate infection (Wild and Buckland, 1995). The host rang of measles virus is limited between human and certain large primates (Masse *et al.*, 2002). Laboratory MV initiates infections of host cells through CD46 (membrane cofactor protein 46) ubiquitously expressed on human nucleated cells (Dorig *et al.*, 1993; Naniche *et al.*, 1993; Gerlier *et al.*, 1995). And wide type MV exclusively uses SLAM, a novel receptor expressed on the dentritic cell, activated B and T lymphocyte as receptor (Tatsuo *et al.*, 2000; Erlenhoefer *et al.*, 2001; Hsu *et al.*, 2001).

SLAM, a CD2 member of the immunoglublin superfamily, is a type I transmembrane protein that has a N-terminal V domain followed with a C2 domain, a transmembran segment and cytoplastic tail (Cocks *et al.*, 1995; Sidorenko and Clark, 2003). SLAM is an unusual molecule in that it acts both as a signaling membrane receptor and as a soluble and membrane-bound growth promoting molecule. Membrane-bound and soluble SLAM induce proliferation and Ig synthesis by activated human B Cells and soluble SLAM enhances production of IgM, IgG, and IgA by activated B cells (Punnonen *et al.*, 1997). SLAM interacts physically with SLAM-associated protein in T and natural killer cells, which correlate with the X-linked lymphoproliferative syndrome, to block a competing interaction between SLAM and Src homology 2 domain-containing tyrosine phosphatase (Siminovitch and Neel, 1998; Feng, 1999; Li *et al.*, 2003). Since SLAM is an important molecule in signaling and immunity, SLAM-dependent cell entry may be directly related to pathogenesis.

There are a lot of reports about the residues responsible for MVH-CD46 binding: MVH residue Y481 is important in attaching to CD46 (Bartz *et al.*, 1996; Lecouturier *et al.*, 1996; Hsu *et al.*, 1998; Schneider *et al.*, 2002); a MVH mutant possessing a five alanines replaced 473-to-477 region loses the hemadsorption function which has been considered as a native property of MV (Shibahara *et al.*, 1994, Lecouturier *et al.*, 1996); the S546G substitution has been shown to increase hemadsorption and CD46 binding (Buckland, 1999). Unlike CD46, the research on residues of MVH responsible for

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MVH-SLAM binding is rather limited. Since the identification of SLAM as a receptor of wide-type MV in 2000, there are several residues on MVH has been found to be responsible for MVH-SLAM binding. The existence of overlapping sites but distinct interacting surfaces for the two receptors on MVH was recently proposed by Santiago et al. (Santiago *et al.*, 2002). Nicolas Masse reported that some residues on MVH such as D505, D507, D530 and R533 participate in MVH-SLAM binding (Masse *et al.*, 2004).

To find potential binding sites of MVH and SLAM, amino acids 27-135 of SLAM was used to screen a 10-mer phage display peptide library in our previous work (Hu *et al.*, 2004). After 4 rounds of screening and sequence analysis, the deduced amino acid sequence of screened peptides SGFDPLITHA and SDWDPLFTHK showed highly homologous with amino acid 429-438 of MVH (SGFGPLITHG) (Hu *et al.*, 2004). Moreover peptides SGFDPLITHA and SDWDPLFTHK specifically inhibited the binding of MVH to SLAM and further inhibit MV infection, which suggests that amino acid 429-438 in MVH protein is functionally involved in receptor binding and may constitute part of the receptor-binding determinants on MVH protein (Hu *et al.*, 2004). At the same time, purified soluble MVH protein was used to screen a 15-mer random peptide library and we got the peptide MSKNIHFVVTLG WSL which was highly homogenous to the peptide of SLAM 56-70 MNKSIHIVVTMAKSL. This result was consistent with the report that V domain (amino acid 1-138) of human SLAM is necessary and sufficient to interact with the MVH protein and allow MV entry (Ono *et al.*, 2001). To further determine the importance of sequence SGFGPLITHG of MVH protein and MNKSIHIVVTMAKSL of SLAM during the interaction between MVH and SLAM, site-directed mutagenesis in MVH and SLAM were carried out based on the software ANTHEPROT 4.3 analysis and generated five mutants: MVH (S429A), MVH (T436A/H437A), SLAM (K58A/S59A), SLAM (H61A) and SLAM (K68A/S69A) in this work. And a series of experiments was done to study the effects of these mutants on binding between MVH and SLAM.

Materials and Methods

Cell plasmids and antibody. B95-8 cells, an Epstein-Barr virus transformed human lymphoblastoid cell line, and CHO (Chinese hamster ovary) were kindly presented by Dr. Zhen in Chinese Typical Culture Center, Wuhan University, were cultured in 1640 medium with 10% heat-inactivated newborn calf serum.

The construction of plasmids pCDNA3.1-h for expressing MVH, pHM6-*f* for expressing MVF and pEGFP-slam for expressing SLAM had been described before (Zhang *et al.*, 2005).

The expression and purification of soluble MVH protein was described previously (Hu *et al.*, 2004).

The monoclonal antibody specific for SLAM were purchased from the Santa. Cruz Company. And we injected the soluble MVH protein into the rabbit to get the antiserum against MVH protein. FITC-conjugated goat anti-mouse antibody was purchased from the SanYing Company (Wuhan city, China).

PCR site-directed mutagenesis. The primers were designed according to the sites needed to mutant and the length of these primers is about 30-40 amino acids.

Based on QuikChange Site-Directed Mutagenesis Kit the PCR was carried out using pcDNA3.1-h and pEGFP-slam as templates (Stratagene). Then the products were digested by DpnI at 37°C for 2 h to wipe off the template DNA. Finally the products were sequenced and five correct mutants, MVH (S429A), MVH (T436A/H437A), SLAM (K58A/S59A), SLAM (H61A) and SLAM (K68A/S69A), were obtained.

Flow cytometric analysis. B95-8 Cells were transfected with MVH (S429A), MVH (T436A/H437A), wild type pcDNA3.1-h and pcDNA3.1 separately. 48 hours later the cells were incubated with mouse anti SLAM antibody (in 100ul PBS containing 0.4°C BSA and 0.01 M NaN_3)for 1 hour at 4°C. Cells were gently washed twice with ice-cold PBS and incubated with a 1 : 200 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin in 100 μl PBS at 4°C for 1 hour. After twice washes, cells were fixed in 1% paraformaldehyde and flow cytometric analysis was performed on Beckman-Coulter XL-MCL (Beckman Coulter, Fullerton, CA, USA).

Tansfection and immunoprecipate (IP). Plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Briefly, 2 μl of Lipofectamine 2000 was diluted in 50 μl of 1640 culture medium, mixed, and incubated at room temperature for 5 min. Meanwhile, plasmid DNAs were diluted in 50 μl of 1640 culture medium. The two solutions were softly mixed and incubated for 20 min before they were added to the cells seeded in 24 well plates. Cells were lysed with the Mammalian Protein Extraction Reagent (Pierce co-immuno-precipitation kit) 48 h posttransfection and separated into two parts evenly. One part of the lysate was incubated with rabbit anti-MVH antibody, whereas the other part was incubated with mouse anti SLAM antibody for for 2 hours at 4°C. Then Immobilized G was added respectively and rotated for another 2 hours at 4°C followed by three washes with washing buffer (10 mM Tris-CHCl, pH 7.4, 150 mM NaCl, 0.1% SDS, and 1 mM EDTA). The bound proteins were eluted with elution buffer (10mM Tris-CHCl, pH 7.4, 2% SDS, 0.3 M NaCl, and 1 mM EDTA). The Western blotting was carried out with anti-MVH or anti-SLAM for detection.

Cell fusion assay. B95-8 cells were seeded on 6-well plate and co-tranfected with MVH (S429A) and MVF, MVH (T436A/H437A) and MVF, MVH and MVF respectively. After 48 h the cells were analyzed under an inverted light microscope for the presence of syncytium.

Results

The S429, T438 and H437 of MVH protein is critical for the interaction between MVH and SLAM. In order to clearly

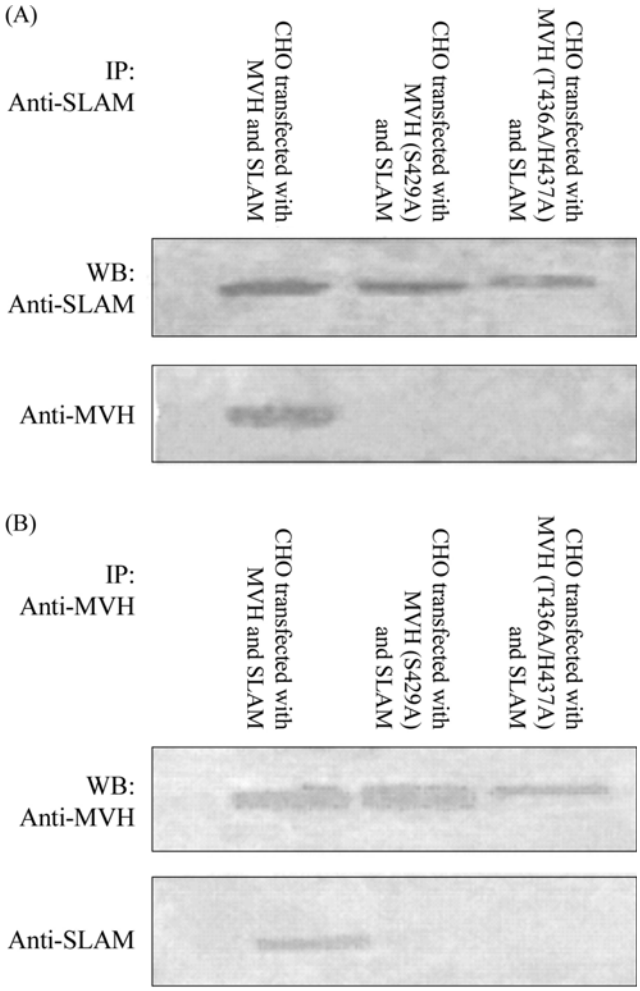

Fig. 1. (A) inmunoprecipitation of SLAM-MVH complex with anti-SLAM. Cell lysate was incubated with protein G coupled with anti-SLAM antibody. Cell lysate was detected by anti-SLAM to insure the expression of SLAM. Proteins from the immunoprecipited fractions were detected by anti-MVH. (B) inmunoprecipitation of SLAM-MVH complex with anti-MVH. Cell lysate was incubated with protein G coupled with anti-MVH antibody. Cell lysate was detected by anti-MVH to insure the expression of MVH. Proteins from the immunoprecipited fractions were detected by anti-SLAM.

investigate if both MVH (S429A) and MVH (T436A/H437A) have the ability to interaction with SLAM in *vivo*, MVH (S429A), MVH (T436A/H437A) and parent MVH were co-transfeced with SLAM respectively into CHO cells, and then immunoprecipitation and western blot were carried out. Western blot analysis revealed that when the immuno-precipitation were carried out with mouse anti-SLAM antibody, SLAM can only be precipitated with nature MVH and was incapable to precipitate with both MVH (S429A) and MVH (T436A/H437A) (Fig.1A). Similarly, when the immuno-precipitation was performed with rabbit anti-MVH antibody, only wild type MVH can precipitate with SLAM and neither MVH (S429A) nor MVH (T436A/H437A) can co-precipitate with SLAM (Fig.1B). Meanwhile expression of SLAM and three different kinds of MVH protein was identified using anti-SLAM antibody and anti-MVH antibody respectively. These results indicated that the MVH mutants, MVH (S429A) and MVH (T436A/H437A), lost the ability to bind with SLAM. It reveals that S429, T436 and H437 of MVH protein are essential for SLAM-MVH interaction.

The MVH (S429A) and MVH (T436A/H437A) obviously decrease the ability to regulate the cell surface SLAM compared with wild type MVH. It has been reported that the cell surface SLAM can be regulated by measles virus infection and so was the transient expression of the MVH protein of measles virus (Welstead *et al.*, 2004). Obviously, the expression of SLAM on the surface of MV infected and MVH protein transient expressing cells is the outgrowth of the interaction between MVH and SLAM. Thus, detection of surface SLAM expression can be used as an indirect evidence for MVH and SLAM binding. According to this we analyzed the influence of the two mutants on cell surface SLAM expression using flow cytometric analysis. The results indicate that cell surface SLAM of B95-8 cells transfected with MVH is 39% compared with that of the non-transfected B95-8 cells (Fig. 2A-3), while surface SLAM expressing values of B95-8 cells transfected with MVH (S429A) and MVH (T436A/H437A) are 57.1% and 69.7% respectively (Fig. 2A-5, 4). At the same time, surface SLAM expressing value of B95-8 cells transfeced with pcDNA3.1 is 83.6% (Fig. 2A-6). Values of non-transfected B95-8 cells as positive control and non-transfected and non-labelled B95-8 cells as negative control are 97.2% and 0.99% respectively (Fig. 2A-1, 2). According to the results, a histogram was created (Fig. 2B), from which we can clearly see that MVH mutants have much less effect on the expression of cell surface SLAM than wild type MVH. Therefore, we concluded that S429, T436 and H437 of MVH play an important role in the interaction between MVH and SLAM.

The MVH (S429A) and MVH (T436A/H437A) of MVH protein drastically inhibit SLMA-dependent syncytium formation in B95-8 cells. It has been well-know that MV infection and co-expression of MVH and MVF can cause host cell fusion which leads to the formation of syncytium (Masse *et al.*, 2002). In order to make out if the MVH mutants still have the ability to form syncytium, we co-express MVH, MVH (S429A), and MVH (T436A/H437A) respectively with MVF in B95-8 cells. When MVH (S429A) and MVH (T436A/H437A) were respectively co-transfeced with MVF in B95-8 cells, cell fusion was not available (Fig. 3-3 and Fig. 3-4). But wild type MVH protein co-expressed with MVF giving an obvious fusion phenomena in B95-8 cells (Fig. 3-2). Untransfected B95-8 cells and B95-8 cells transfected with pcDNA3.1 used as control, also have no syncytium formation (Fig. 3-1, Fig. 3-5). Thus, we verified that the S429, T436 and

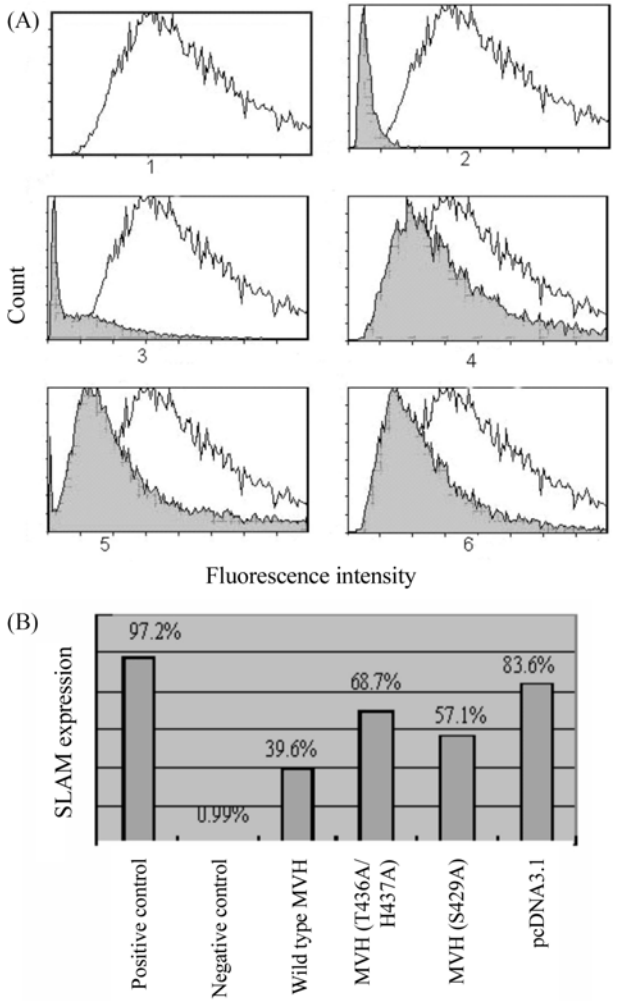

Fig. 2. (A) expression of SLAM on the surface of B95-8 cells in the presence of MVH, MVH (S429A), and MVH (T436A/H437A). 1. FITC-labelled B95-8 cells as positive control 2. unlabelled B95-8 cells as negative control; 3. B95-8 cells transfected with MVH; 4. B95-8 cells transfected with MVH (T436A/H437A); 5. B95-8 cells transfected with MVH (S429A); 6. B95-8 cells transfected with vector pcDNA3.1. (B) SLAM cell surface expression. Each column represents the percent of SLAM expression.

H437 of MVH are important in syncytium formation.

The H61 K58 and S59 of SLAM are critical in MVH and SLAM interaction, while the K68 and S69 have little influence on the interaction between MVH and SLAM. To verify the importance of SLAM amino acid H61, K58, S59, K68 and S69 in the binding between MVH and SLAM, SLAM (K58A/S59A), SLAM (H61A) and SLAM (K68A/S69A) were co-tranfected with MVH into Hela cells respectively. At the same time, Hela cells co-transfected with wild type SLAM and MVH was used as positive control. After 48 h, cells were harvested and lysed, and then immunoprecipitation and western blot were carried out. Western blot analysis revealed that when the immuno-

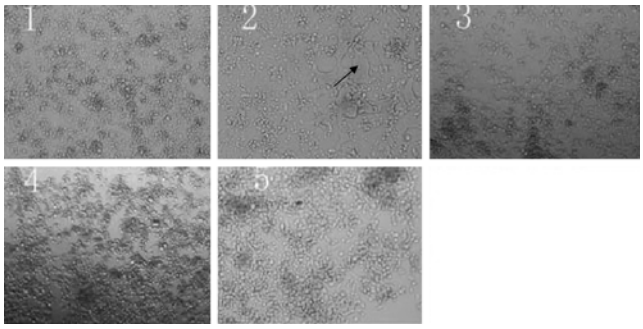

Fig. 3. Lose of syncytium formation by co-expressing MVH (S429A) with MVF (3) and MVH (T436A/H437A) with MVF (4). MVH and MVF co-expressed B95-8 cell (2), the arrow indicate syncytium. Untransfected B95-8 cells (1) and B95-8 cells transfected with pcDNA3.1(5) as control. Fusion is observed under a microscope 24 to 48 h after co-transfection.

precipitation was carried out with rabbit anti-MVH antibody, MVH can only be co-precipitated with wild type SLAM and SLAM (K68A/S69A), while it was almost incapable to co-precipitate with both SLAM (K58A/S59A) and SLAM (H61A) (Fig. 4A). Similarly, when the immunoprecipitation was performed with mouse anti-SLAM antibody, wild type SLAM and SLAM (K68A/S69A) can co-precipitate with MVH, while SLAM (K58A/S59A) and SLAM (H61A) hardly can co-precipitate with SLAM (Fig. 4B). Meanwhile, expression of MVH and four different kinds of SLAM protein was identified using anti-MVH antibody and anti-SLAM antibody respectively. Thus, the H61, K58 and S59 of SLAM are critical in MVH and SLAM interaction, while the K68 and S69 have little influence on the interaction between MVH and SLAM.

Discussion

Since MV initiates infection of host cells with the attachment of MVH to cell surface receptors SLAM and CD46, abolishment of the binding between MVH and cell receptors has been a main target to MV therapy. CD46 as the first receptor for MV has been found to have a number of residues responsible for binding with MVH. Yet, SLAM, the novel MV receptor, has been regarded as a more universal receptor and both wild and laboratory MV strains can use it as the receptor. So a great number of researches have been focused on the interaction between SLAM and MVH that is considered to be a splendid outlook both in mechanism of MV entry and treatment.

In previous research, we got two peptides which can inhibit MV infection effectively using the phage display assay (Hu *et al*., 2004). Now to further explore the potential sites involved in interaction between MVH and SLAM, we mutate the MVH protein residues S429, T436 and H437 in the 429-C438 (SGFGPLITHG) region of MVH according to the ANTHEPROT

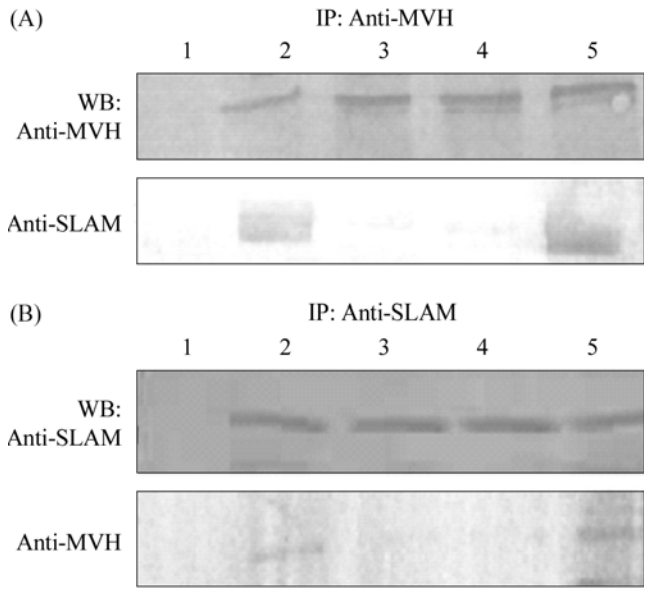

Fig. 4. (A) Inmunoprecipitation of SLAM-MVH complex with anti-MVH. 1.The cell lysates combining with the Protein G in the absence of anti-MVH were detected by anti-SLAM indicated the negative control 2. Cells co-expressing wild type SLAM and MVH. 3. Cells co-expressing SLAM (K58A/S59A) and MVH. 4. Cells co-expressing SLAM (H61A) and MVH. 5. Cells co-expressing SLAM (K68A/S69A) and MVH. Cell lysates were detected by anti-MVH to insure the expression of MVH. (B) Inmunoprecipitation of SLAM-MVH complex with anti-SLAM. 1. The cell lysates combining with the Protein G in the absence of anti-SLAM were detected by anti-MVH indicated the negative control 2. Cells co-expressing wild type SLAM and MVH. 3. Cells co-expressing SLAM (K58A/S59A) and MVH. 4. Cells co-expressing SLAM (H61A) and MVH. 5. Cells co-expressing SLAM (K58A/S59A) and MVH. Cell lysates were detected by anti-SLAM to insure the expression of MVH.

4.3 software analysis. After co-expression of the MVH mutants and MVF protein in B95-8 cells, we found that they dramatically decrease the ability to interact with the cell surface SLAM and fail to co-precipitation with SLAM in *vivo.* In addition, MVH (S429A) and MVH (T436A/H437A) indicate malfunction in syncytium formation. These two mutants showed slight ability to interact with the cellular receptor SLAM compared with the wild type MVH. These results indicated that S429, T436 and H437 of MVH protein played important roles in the binding to SLAM, which are the possible mechanisms for the two peptides with the ability to inhibit MV infection (Hu *et al.*, 2004).

Recently, surface plasmon resonance had been applied to monitor the binding of CD46 and SLAM to MVH protein using a BIAcore instrument. It demonstrated that the binding sites of two receptors in MVH protein were of an overlapping nature, which is coincident with the receptor competition binding experiments (Santiago *et al.*, 2002). In the three-dimensional surface representation of the structural model of MVH, three residues (D505, D507 and R533) align the rim on one side of the cavity on the top surface of the MVH globular head and form the basis of a single continuous site that overlaps with the 546-548-549 CD46 binding site (Masse *et al.*, 2004). In the revised structural model's 3D surface representation, Y481 which shown to be essential for the MVH-CD46 binding phenotype (Cocks *et al.*, 1995; Griffin, 2001) actually contacts Y524, a residue whose mutation has an effect on both CD46 and SLAM binding (Masse *et al.*, 2004). S429, T436 and H437 of MVH protein involved in SLAM binding are adjacent with Y481 and 524 to 526 residues according to 3D model (Masse *et al.*, 2004) and all these sites are a part of an extruded-loop structure of MVH protein (Alkhatib and Briedis, 1986; Erlengoefer *et al.*, 2001). So we deduced that S429, T436 and H437 may overlap with Y481 and 524 to 526 CD46 binding residues, which affected MVH-SLAM binding together.

At the same time, we screen a 15-mer random peptide phage library using soluble MVH protein and a peptide MSKNIHFVVTLGWSL was obtained which is homologous with amino acids 56-70 (MNKSIHIVVTMAKSL) of SLAM protein (date not show). The result is consistent with the conclusion that V domain is critical for SLAM to act as a receptor for MV (Ono *et al.*, 2001). In 2003, Ohno *et al.* reported that exchange of residues at positions 58-67 of human SLAM allows mouse SLAM, which can not used as a MV receptor, to act efficiently as a receptor for MV. In this research, we investigated five amino acids on SLAM and found that three of them, the K58, S59 and H61, are important in the interaction between MVH and SLAM. Meanwhile the K68 and S69 of SLAM are unrelated to the interaction between MVH and SLAM. It is reported that H61 and its adjacent residues at positions 60 and 63 are critical for SLAM to act as a receptor for MV (Ohno *et al.*, 2003). We assumed that these amino acids may be included in a large functional domain on SLAM to interact with MVH or significant in maintaining the tertiary structure of SLAM to act as a MV receptor. More important, it is possible that the 429-438 Region of MVH and 56-70 region of SLAM were all involved in the subtle interaction structure of these two proteins because we got the homologous peptides using the bi-directional phage display assays. Moreover, the results seemed to get logical consistent in both sides. However, the elaborated interaction structure of MVH and SLAM is deserved to be explored further in the crystallization analysis. Anyway, it would be an exciting progress if we could get the subtle interaction structure. It is important to learn the mechanism of MV infection and develop the novel vaccine to prevent its infection.

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