SLAM EXPRESSION IS NOT DOWNREGULATED BY MEASLES VIRUS INFECTION

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Summary. – It has been reported that the signaling lymphocyte-activation molecule (SLAM), the second receptor of Measles virus (MV) on the cell surface appears to be downregulated by MV infection or by expression of MV hemagglutinin (H) (Tanaka *et al*., 2002; Welsteadt *et al*., 2004). The aim of this study was to analyze this phenomenon in more detail using a Chinese vaccine strain (S191) of MV. Flow cytometry confirmed apparent downregulation of SLAM in the virus-infected cells and in the cells transfected with a plasmid expressing viral H. Moreover, a similar effect was obtained by incubation of the cells with UV-inactivated virus or soluble viral H. Real-time quantitative PCR showed that the SLAM mRNA level remained stable during the virus infection, and Western blot analysis demonstrated that the SLAM content of total membrane proteins did not change change after the virus infection. Thus we conclude that SLAM expression is stable during the MV infection and that its apparent downregulation reported earlier and confirmed also in this study was just the result of masking of the antibody recognition sites on SLAM with MV H during the flow cytometry assay.

Key words: Measles virus; SLAM; hemagglutinin; downregulation; expression; flow cytometry; real-time PCR; Western blot analysis

Introduction

MV (the species *Measles* virus, the genus *Morbillivirus,* the subfamily *Paramyxovirinae*, the family *Paramyxoviridae*) contains a single-stranded negative-sense genomic RNA. The latter encodes two envelope glycoproteins, H and fusion (F) protein which interact with the cell surface receptors to mediate virus entry (Griffin and Bellini, 1996). CD46 was identified as a cellular receptor for vaccine strains of MV such as the Edmonston and HaLe (Dorig *et al*., 1993; Naniche *et al*., 1993a), while most wild-type MV strains preferentially use the immune cell-specific protein SLAM (also known as CDw150) as receptor (Erlenhoefer *et al*., 2001; Hsu *et al*., 2001; Ono *et al*., 2001a; Tatsuo *et al*., 2004; Li *et al*., 1999). Since three morbilliviruses, MV, Canine distemper virus, and Rinderpest virus enter cells through SLAM and are immunosuppressive (Tatsuo *et al*., 2001), the SLAM-dependent cell entry may play a great role in viral pathogenesis (Vongpunsawad *et al*., 2004). SLAM is a type I transmembrane protein whose N-terminal V domain is necessary and sufficient for interaction with MV H, while the remaining domains, C2, TM, and CY are not required for the infection (Ono *et al*., 2001b). SLAM belongs to the CD2 subset of the Ig superfamily and is expressed on the surface of a proportion of non-infected and Epstein-Barr virus (EBV)-transformed B cells, activated T cells, memory T cells, T cell clones, and immature thymocytes (Erlenhoefer *et al*., 2001). SLAM interacts with a SLAM-associated protein (SAP) in T and NK cells, which form a SLAM/SAP receptor-adaptor complex, one of the few cell surface-signaling units controlling the terminal differentiation of the cells into Th1

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Abbreviations: EBV = Epstein-Barr virus; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; H = hemagglutinin; MV = Measles virus; MAbs = monoclonal antibodies; p.i. = post infection; SAP = SLAM-associated protein; SLAM = signaling lymphocyte-activation molecule membrane

and Th2 ones (Wang *et al*., 2004). Ligation of SLAM also induces IFN-γ production in CD41 T cell clones and Ig production in activated B cells (Cocks *et al*., 1995; Punnonen *et al*., 1997; Aversa *et al*., 1997a). Thus SLAM may be involved in expanding Th0/Th1 immune responses (Aversa *et al*., 1997b).

A downregulation of receptors following virus infection has been reported for several viruses including Human immunodeficiency virus 1 (Klatzmann *et al*., 1984) and Human herpesvirus 6 (Santoro *et al*., 1999). Naniche *et al*. (1993a) have reported that the cell surface CD46 expression is downregulated after MV infection and that MV H alone is sufficient in this respect (Naniche *et al*., 1993b). However, there have appeared also some contradictory reports. For example, cell surface CD46 was proved to be downregulated in the cells infected with vaccine strains or Vero cell-isolates but not with wild-type strains of MV isolated in marmoset B95a or human B cell lines (Lecouturier *et al*., 1996; Schneider-Schaulies *et al*., 1995a,b). Furthermore, CD46 expression was not downregulated by the infection with MV strain S191 both on mRNA and protein level (Hu *et al*., 2004). Previous results proving the downregulation of cell surface CD46 expression might result from the fact that the antibody recognition sites on CD46 were masked by the interaction between HA and CD46 and could not be detected by flow cytometry (Hu *et al*., 2004a).

As for the modulation of SLAM expression by MV infection, Welstead *et al*. (2004) have used Montefiore 89 and Edmonston strains of MV to infect marmoset and human B cell lines. The flow cytometry using SLAM-specific monoclonal antibodies (MAbs) A12 and IPO-3 revealed a substantial decrease of cell surface SLAM expression for both virus-cell systems. There was also the evidence that MV H, when either expressed in cells or incubated with them, induced a downregulation of cell surface SLAM expression (Tanaka *et al*., 2002). Welstead *et al*. (2004) have proposed two possible mechanisms by which either intracellular interaction between HA and SLAM in ER or a receptor-mediated binding to HA at host cell surface could lead to the SLAM downregulation during MV infection. However, the MAbs IPO-3 and A12 were confirmed to recognize the V domain of SLAM, which is regarded as the binding site of MV H (Ono *et al*., 2001b).

Based on these observations and proposed mechanisms, we regard the estimation of modulation of cell surface SLAM expression using the IPO-3 and A12 MAbs in flow cytometry as inappropriate. In this study, we infected B95-8 cells naturally expressing SLAM with MV S191 strain and assayed the cell surface SLAM expression by flow cytometry. Besides infectious virus we also tested UV-inactivated virus and soluble H for their effects on SLAM level. Moreover, the effect of transient H expression in the cells on SLAM level and the SLAM level in total membrane proteins of virus-infected cells were assayed.

Materials and Methods

Cells, plasmid, virus and antibodies. Marmoset B95-8 cell line, purchased from China Typical Culture Center, Wuhan University, Wuhan, P.R. China, was cultured in the Medium 1640 with 10% of heat-inactivated FCS. MV S191 vaccine strain was grown and titrated in Vero cells. The pCDNA3.1-h plasmid expressing MV H has been described earlier (Zhang *et al*., 2005). MAbs specific to SLAM (IPO-3) and Na^+/K^+-ATPase, respectively, were purchased from Santa Cruz Company, USA. A FITC-conjugated goat anti-mouse Ig was purchased from Beijing ZhongShan Biotechnology Co. Ltd, P.R. China). Soluble MV H was a gift from Dr. Hu, Wuhan University, Wuhan, P.R. China (Hu *et al*., 2004b). A FITC-conjugated goat anti-mouse antibody was purchased from SanYing Company, Wuhan, P.R. China.

Flow cytometry. B95-8 cells in 6-well plates were (i) infected with MV at a multiplicity of infection of 5 for 48 hrs, (ii) incubated with UV-inactivated MV for 48 hrs, (iii) transfected with pCDNA3.1-h for 48 hrs, or (iv) incubated with soluble MV H for 1 hr. The cells not treated in either way represented a positive control. The cells were harvested and incubated for 1 hr at 4°C with 100 µl of 1:500 dilution of the mouse anti-SLAM MAb IPO-3 containing 0.4% BSA. The cells were washed twice with PBS and incubated with a 1:200 dilution of FITC-conjugated goat anti-mouse Ig in 100 µl of PBS at 4°C for 1 hr. After 2-fold washing, flow cytometry was performed using a Beckman-Coulter XL-MCL flow cytometer (Beckman Coulter, USA). The assay made without of the incubation with IPO-3 represented a negative control.

Quantitative real-time PCR was performed with Sybr Green I fluorescent dye (Ueno *et al*., 2002). To obtain cDNA total cellular RNA was extracted and subjected to reverse transcription in duplicate. The reaction mixture (20 µl) consisted of 0.2 µl of cDNA, 0.4 µmoles of each primer, 25 mmol/l $MgCl_2$, 2 U of Taq DNA polymerase, SYBR Green I and a reaction buffer (all from Roche Molecular Biochemicals, Germany). The following primers synthesized by ShangHai Sangon Biotech Co. (P.R. China) were employed: 5'-GCGGAATTCTCATTGGCTGATGGATC-3' (forward, nt 115–140) and 5'-AGGTGGTCCAGATAGA ACTTGTAGCG 3' (reverse, nt 1349–1374) for SLAM, 5'-GCCTCAGCACCAACCTAGATGTAA-3' (forward, nt 249–272) and 5'-GGATTGACCTCTGATTGTAGTGGG-3' (reverse, nt 1334–1357) for HA, and 5'-CATGGAG AAGGCTGGGGCTC-3' (forward, nt 414–433) and 5'-CACTGACACGTTGGCAGTGG-3' (reverse, nt 483–502) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The reaction was carried out in a Rotor-Gene 3000 Real-Time Amplication Operator with a pre-incubation at 95°C for 10 mins followed by 35 cycles of 95°C/30 secs

(denaturation), 61°C/30 secs (annealing), and 72°C/30 secs (extension). Fluorescence was determined at the end of every extension phase. Finally, the PCR products were analyzed by agarose gel electrophoresis.

Quantitative analysis of PCR products. To determine the copy numbers of SLAM and HA mRNAs through standard curves the recombinant plasmids pEGFP-slam and pcDNA-h were used, respectively. A housekeeping gene, GAPDH was employed to standardize total RNA.

The observed C_T value of a target, defined as the cycle number at which the fluorescence significantly exceeded the background, was used for determination of the corresponding copy number of a target using a RG3000 Version 4.6 software (Corbett Research).

Western blot analysis. Cell membrane proteins were extracted using the Membrane Protein Extraction Kit (Shenzhen Jingmei Biotech Co., Ltd, P.R. China) according to the manufacturer's instructions. B95-8 cells infected with MV were harvested at 0, 12, 24, and 36 hrs post infection (p.i.), washed twice with PBS and three times with a membrane extraction buffer (0.25 mol/l saccharose, 10 mmol/l triethanolamine, and 10 mmol/l acetic acid pH 7.4), resuspended in 1 ml of the membrane extraction buffer supplemented with 1 mmol/l EDTA, and processed in a Dounce homogenizer. The cell homogenate was centrifuged at 1,000 rpm for 5 mins to pellet nucleoli and the supernatant was clarified at 4,000 rpm for 10 mins. The entire procedure was performed on ice and the above steps were repeated three times. Finally, the membrane extract was subjected to SDS-PAGE and the proteins were blotted onto a polyvinylidene difluoride membrane (Millipore). The blots were blocked with 5% skim milk in TBS pH 8.0 for 1.5 hrs at room temperature, incubated with a rabbit anti-Na^+/K^+-ATPase and mouse anti-SLAM (IPO-3) antibodies, respectively, for 1 hr, washed 3 times with TBS, incubated with an AP-conjugated secondary antibody, and analyzed by enhanced chemiluminescence (Pierce) To determine the amount of SLAM in each sample, the intensity of the respective bands was assessed by densitometry scanning usingthe NIH Image Program. Ratios of SLAM or Na^+/K^+-ATPase for 12, 24 and 36 hrs p.i. were relative to 0 hr p.i. Na^+/K^+-ATPase was used for standardization of the concetration of total membrane proteins.

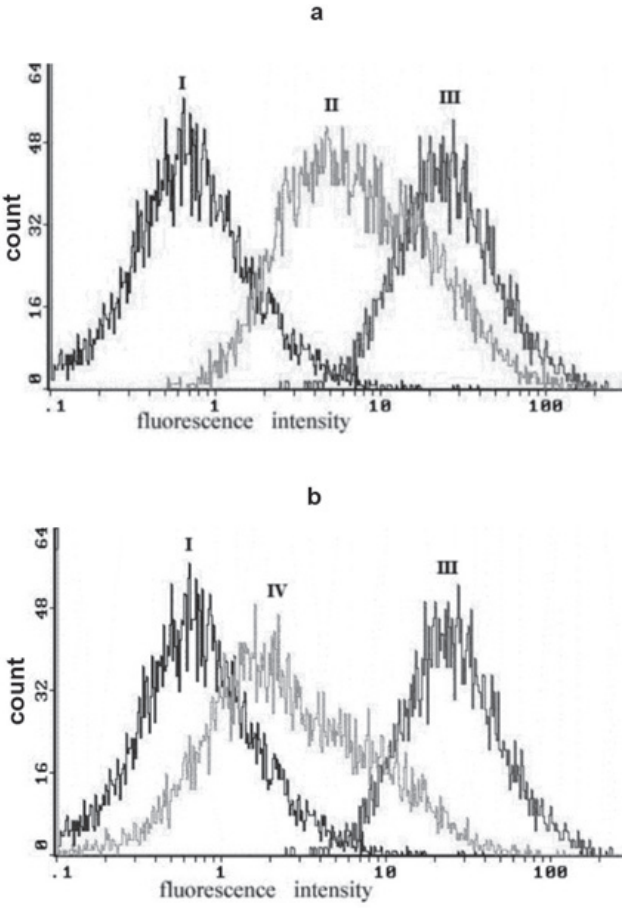

Fig. 1

Cell surface SLAM expression in the cells transfected with a plasmid expressing MV H (a) and in the cells incubated with soluble MV H (b)

B95-8 cells, flow cytometry. (a): The cells transfected with the plasmid pCDNA3.1-h expressing H (II). (b): The cells incubated with soluble H (IV). The cells assayed for SLAM in the absence of IPO-3 (negative control) (I); the cells neither transfected with pCDNA3.1-h nor incubated with H (positive control) (III).

Results

Binding of MV H to SLAM is sufficient to reduce cell surface SLAM expression

To investigate the effect of MV H on cell surface SLAM expression B95-8 cells were transfected with pCDNA3.1-h expressing MV H or incubated with soluble H and monitored for the cell surface SLAM expression by flow cytometry. The results showed that SLAM surface expression in the cells transfected with pCDNA3.1-h or incubated with soluble H was strongly reduced compared with controls (Figs. 1a and 1b). So we suggest that the apparent downregulation of SLAM expression by H could be just caused by the binding of MV H to SLAM.

To further verify this assumption B95-8 cells were incubated with different concentrations of HA (5, 10 and 15 μg/ml) for 1 hr and the cell surface SLAM expression was assayed by flow cytometry. The results showed that the cell surface SLAM expression decreased with increasing

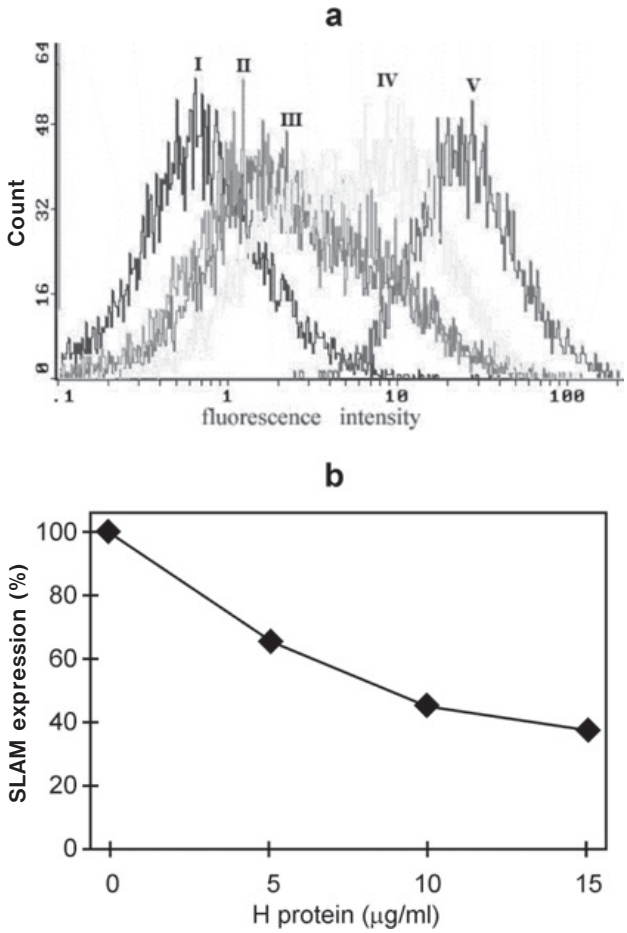

Fig. 2

Cell surface SLAM expressionin the cells incubated with different concentrations of soluble MV H

B95-8 cells, flow cytometry. (a): The cells incubated with 15, 10, 5, and 0 μg/ml H, respectively (II-V); the cells assayed for SLAM in the absence of IPO-3 (negative control) (I); (b): The values from Fig. 3a used for construction of the curve.

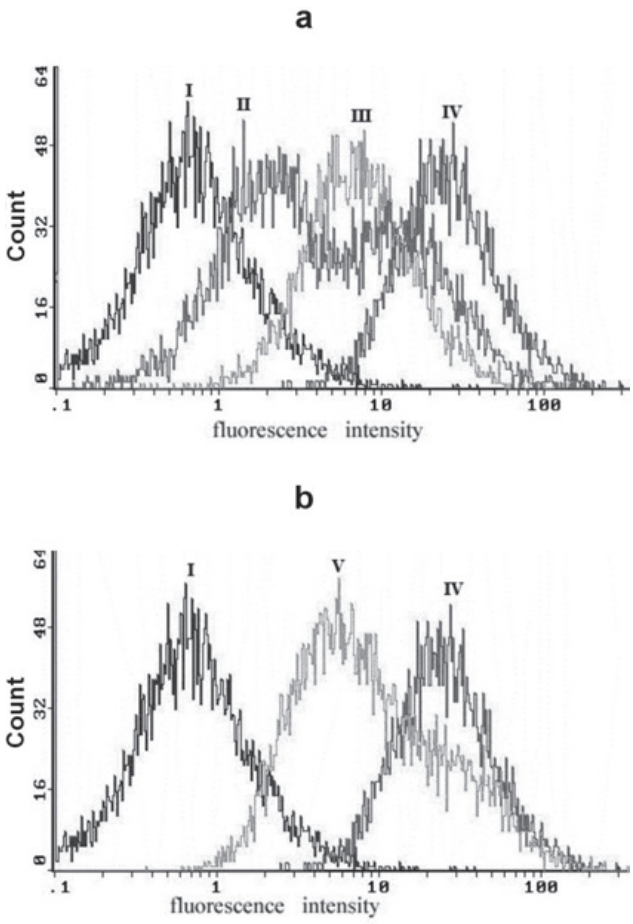

Fig. 3

Cell surface SLAM expression in the cells incubated with infectious (a) and UV-inactivated MV (b)

B95-8 cells, flow cytometry. (a): The cells infected with MV and analyzed at 6 hrs (III) and 48 hrs (II) p.i. (b): The cells incubated with UV-inactivated MV (V). The cells neither infected nor incubated with UV-inactivated virus (positive control) (IV). The cells assayed for SLAM in the absence of IPO-3 (negative control) (I).

concentration of HA (Fig. 2), indicating that mere binding of H to SLAM was enough to cause the decrease of cell surface SLAM. We suggest that the recognition sites for the IPO-3 antibody on SLAM were blocked by the binding of H to SLAM, which resulted in the apparent downregulation of SLAM expression observed by flow cytometry.

MV infection is not involved in the regulation of cell surface SLAM expression

Since the binding of soluble MV H to SLAM decreased the cell surface SLAM expression as determined by flow cytometry (Fig. 2b), infectious and UV-inactivated MV were used to determine whether MV infection is essential for the downregulation of the cell surface SLAM expression. B95-8 cells were infected with MV (Fig. 3a) and incubated with UV-inactivated MV (Fig. 3b), respectively, and the cell surface SLAM expression was assayed by flow cytometry at 6 hrs and 48 hrs p.i. The results showed that a decrease of cell surface SLAM expression was observed in both the virus-infected cells and the cells incubated with inactivated virus. They demonstrated that the virus infection was irrelevant for the downregulation of cell surface SLAM expression and that mere binding of HA to SLAM could cause the effect. Interestingly, the cell surface SLAM level in virus-infected cells at 48 hrs p.i. was lower than that in

Table 1. Copy numbers of SLAM and GAPGH mRNAs and relative copy number of SLAM/GAPDH mRNA

Hrs p.i.	Copy number of SLAM mRNA	Copy number of GAPDH mRNA mRNA	Relative copy number of SLAM/GAPDH
0	21677	95499	0.227
12	597	2659	0.224
24	1117	49317	0.225
48	4083	18621	0.219

Average values from three experiments.

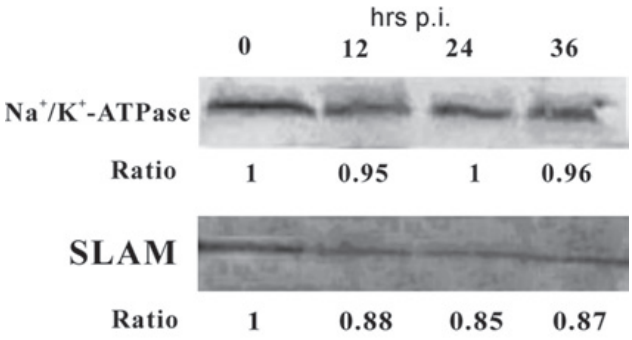

Fig. 4

Western blot analysis of SLAM content of membrane proteins of MV-infected cells

The SLAM content (relative to that at 0 hr p.i.) of the membrane proteins of B95-8 cells infected with MV for 0, 12, 24, and 36 hrs p.i. was assayed. Na^+/K^+-ATPase was used for standardization of the concentration of total membrane proteins. The ratios indicated at the bottom of lanes were determined by densitometry scanning of the blots.

the cells incubated with UV-inactivated virus. This could be caused by the newly synthesized HA present in the cytoplasm which could either bind to cytoplasmic SLAM and thus prevent its transport to cell membrane or bind to cell membrane SLAM and thus block its binding sites for the IPO-3 MAb.

SLAM mRNA is stable during MV infection

To investigate whether the SLAM mRNA level is stable during MV infection quantitative real-time PCR was performed. B95-8 cells infected with MV for 0, 12, 24, and 48 hrs, respectively, were analyzed for SLAM, GAPDH and H mRNAs. The latter was used as a control of the virus infection. From the Ct values obtained by real-time PCR the copy-numbers of SLAM and GAPDH mRNAs as well as the relative SLAM/GAPDH mRNA copy number were determined (Table 1). The relative SLAM/GAPDH copy number represented the ratio of the copy number of SLAM to that of GAPDH. Relative copy numbers for samples from different times p.i. showed almost no differences. Thus the quantitative real-time PCR revealed that the SLAM expression in B95-8 cells was stable during MV infection and there was no regulation of SLAM expression on mRNA level.

SLAM is not internalized during MV infection

Because the IPO-3 antibody used in assaying SLAM by flow cytometry recognized an epitope on the V domain of SLAM, which is also responsible for the binding of MV H, such an assay was not appropriate for cell surface SLAM expression. To prove whether the cell surface SLAM is internalized during MeV infection or not, total membrane proteins of B95-8 cells infected with MV were extracted at 0, 12, 24, and 36 hrs p.i., respectively, and assayed for SLAM by Western blot analysis (Fig. 4). The Na^+/K^+-ATPase whose level is unaffected by MV infection (data not shown) was used as internal standard. The results showed that the cell surface SLAM level did not change significantly as compared with Na^+/K^+-ATPase, hence SLAM was not internalized during the virus infection.

Discussion

In this study, the level of SLAM mRNA and protein in B95-8 cells during MV infection was investigated. The results of quantitative real-time RT-PCR revealed that the SLAM mRNA level remained stable during the infection. This result was inconsistent with previous ones stating that the cell surface SLAM expression was downregulated during MV infection (Tanaka *et al*., 2002; Welstead *et al*., 2004).

To prove that the apparent downregulation of cell surface SLAM expression during MV infection determined by flow cytometry was really related to virus infection we compared the effects of infectious and UV-inactivated virus on the cell surface SLAM expression in B95-8 cells using the same method. As the results showed a reduced SLAM expression in both cases, they indicated that it was just an interaction between MV H and SLAM that resulted in the reduction of cell surface SLAM expression, and that the virus infection can be definitely excluded from involvement in the regulation of cell surface SLAM expression. This interpretation was supported also by the finding of a decrease in cell surface SLAM following either the incubation of the cells with soluble H or transfection of the cells with a plasmid expressing H. Interestingly, the higher was the HA concentration the lower level of cell surface SLAM was found. Therefore it is proposed that the antibody recognition sites on SLAM could be masked by the binding of H and

such a masking would results in the apparent downregulation when assayed by flow cytometry using the IPO-3 antibody to SLAM. This could also explain why the level of cell surface SLAM at 48 hrs in the virus-infected cells was lower than that in the cells incubated with UV-inactivated virus. Namely, it is likely that newly synthesized HA was transported to cell surface and bound to SLAM so that it masked its recognition sites for the IPO-3 antibody. It has also been reported that a specific interaction between SLAM and H in the ER prevents the export of newly synthesized SLAM from the ER to the cell surface (Welstead *et al*., 2004). Hence it is very likely that the newly synthesized H in the cells following MV infection could bind to the newly synthesized cytoplasmic SLAM and thus prevent its transport to cell membrane.

It has been reported that not only the MV infection but also the expression of MV H alone results in a downregulation of cell surface SLAM expression (Tanaka *et al*., 2002; Welsteadt *et al.,* 2004). All these results were obtained by flow cytometry using the IPO-3 antibody to SLAM for detection of cell surface SLAM. It has also been reported that IPO-3 inhibits the development of CPE in MV-infected cells (Tangye *et al*., 2001). Therefore is likely that IPO-3 inhibits MV infection either by direct blocking of the MV-binding sites on the V domain of SLAM or by steric hindrance (Erlenhoefer *et al*., 2001), and that when H binds to the SLAM V domain there is hardly place for binding also IPO-3. Thus the result of a reduced level of cell surface SLAM obtained using this antibody and interpreted as a downregulation does not exclude the possibility that the epitope on SLAM for IPO-3 was masked by MV H.

It has been widely accepted that MV infection is initiated by an interaction between H and SLAM (Erlenhoefer *et al*., 2001; Tatsuo *et al*., 2000). There is an explanation of the mechanism of CD46 downregulation by MV infection, according to which CD46 is internalized (Naniche *et al*., 1993b). To investigate whether a similar receptor-mediated endocytosis occurs in SLAM-mediated MV infection, the SLAM content in membrane proteins of B95-8 cells infected with MV was assayed by Western blot analysis. The result confirmed that there was no obvious modulation of cell surface SLAM during virus infection and that SLAM was not internalized.

On the basis of the above analysis we bring forward a hypothesis that the apparent downregulation of cell surface SLAM expression observed by the flow cytometry using the IPO-3 antibody could result from the masking of the IPO-3 recognition sites on SLAM by MV H. Since SLAM can associate with SAP, which is an important molecule of the signaling path way, the interaction between SLAM and MV H may effectively initiate the signaling pathway to prevent a superinfection of the host cell. Much work is still needed to offer a better understanding of the complicated nature of the signaling network.

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