Histidine at position 61 and its adjacent amino acid residues are critical for the ability of SLAM (CD150) to act as a cellular receptor for measles virus

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INTRODUCTION

Measles virus (MV), a member of the genus Morbillivirus, family Paramyxoviridae, causes measles, a highly contagious acute disease characterized by fever, cough, conjunctivitis and a generalized maculopapular rash. Despite the availability of effective live vaccines, measles remains a major cause of childhood mortality, especially in developing countries, due mainly to secondary and opportunistic infections caused by MV-induced immunosuppression (Griffin, 2001).

MV is a non-segmented, negative-stranded RNA virus with two envelope glycoproteins, the haemagglutinin (H) and fusion (F) proteins, which mediate receptor binding and membrane fusion, respectively (Griffin, 2001). CD46 was shown to be a cellular receptor for the Edmonston and Halle laboratory strains of MV (Dorig et al., 1993; Naniche et al., 1993) as well as some wild-type strains of MV (Manchester et al., 2000; Schneider et al., 2002). CD46 is a complement regulatory molecule that is expressed on all nucleated cells in humans (Liszewski et al., 1991). On the other hand, wild-type MV strains isolated in the marmoset B cell line B95a or human B cell lines were found usually to use a molecule other than CD46 as a cellular receptor (Hsu et al., 1998; Lecouturier et al., 1996; Tanaka et al., 1998; Tatsuo et al., 2000a). Recently, we have shown that human signalling lymphocyte activation molecule (SLAM, also known as CD150) acts as a cellular receptor for both laboratory and wild-type strains of MV (Tatsuo et al., 2000b). Subsequent studies established that SLAM is the principal cellular receptor for MV (Erlenhofer et al., 2001, 2002; Hsu et al., 2001; Ono et al., 2001a; Yanagi et al., 2002). Other morbilliviruses, such as canine distemper virus (CDV) and rinderpest virus, were found also to use SLAM as cellular receptors (Tatsuo et al., 2001). The MV Edmonston strain can use both SLAM and CD46 as receptors (Tatsuo et al., 2001). The MV Edmonston strain was shown to bind both SLAM and CD46, with SLAM binding more tightly to the H protein than CD46, with a lower dissociation rate.

SLAM is a type 1 membrane protein that comprises two highly glycosylated immunoglobulin superfamily domains, V and C2, a transmembrane segment and a cytoplasmic tail (Cocks et al., 1995; Sidorenko & Clark, 1993, 2003). The cytoplasmic domain contains three tyrosine residues
surrounded by SH2 domain-binding sequences, where SLAM associates with the SLAM-associated protein, protein tyrosine phosphatase SHP-2, and inositol phosphatase SHIP (Sayos et al., 1998; Shlapatska et al., 2001). SLAM is expressed on activated T and B cells, immature thymocytes, memory T cells (Aversa et al., 1997; Cocks et al., 1995; Sidorenko & Clark, 1993), activated monocytes (Minagawa et al., 2001) and mature dendritic cells (Bleharski et al., 2001; Kruse et al., 2001; Ohgimoto et al., 2001; Polacino et al., 1996), and is reported to be a self-ligand (Mavaddat et al., 2000). It acts as a co-stimulatory molecule in lymphocyte activation and modulates interferon-γ production (Aversa et al., 1997; Cocks et al., 1995; Latour et al., 2001; Punnonen et al., 1997; Sidorenko & Clark, 1993).

Mouse SLAM (mSLAM) has about 60% identity at the amino acid level to human SLAM (hSLAM) (Castro et al., 1999) and hardly acts as a receptor for MV (Ono et al., 2001b), partly accounting for the non-susceptibility of mice to MV infection. Various fusion proteins containing the V domain of hSLAM are able to act as receptors for MV, whereas a chimeric molecule containing the mouse V domain and the human C2, transmembrane and cytoplasmic domains barely functions as a receptor for MV. The cytoplasmic domain of hSLAM is dispensable for receptor function. Furthermore, the soluble molecules possessing the V domain of hSLAM bind to cells expressing the MV H protein but not to cells expressing irrelevant envelope proteins. Thus, the V domain of hSLAM is necessary and sufficient to interact with the MV H protein and allow MV entry (Ono et al., 2001b).

In this study, we attempted to map the region(s) within the V domain that is responsible for the difference in receptor function between hSLAM and mSLAM. We found that three amino acid residues at positions 60, 61 and 63 of hSLAM are critical for receptor function. Substitutions at these positions with the human-type residues allowed mSLAM to efficiently act as a receptor for MV, while introduction of changes at these positions compromised the ability of hSLAM to act as a receptor.

**METHODS**

**Cells and viruses.** Derivations of cell lines used in this study have been described elsewhere (Kobune et al., 1990; Tatsuo et al., 2000a). Chinese hamster ovary (CHO) and B95a cells were grown in RPMI 1640 medium supplemented with 7 and 10 % heat-inactivated FBS, respectively. 293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 7.5 % heat-inactivated FBS. Growth medium was supplemented with 0.15 % sodium bicarbonate. The vesicular stomatitis virus (VSV) pseudotype was prepared by infecting with VSVAG*G (bearing the VSV envelope G protein) 293T cells, which had been transfected with the appropriate expression plasmids encoding envelope proteins as described previously (Takada et al., 1997; Tatsuo et al., 2000a, 2001). VSVAG*KAHF had the H protein of the wild-type MV strain KA and the F protein of the Edmonston strain. VSVAG*CDVHF had the H protein of a wild CDV strain and the F protein of the Onderstepoort strain. The wild CDV strain was isolated in Vero cells expressing DogSLAMtag (Tatsuo et al., 2001; F. Seki and Y. Yanagi, unpublished results). The wild-type KA strain of MV was grown and titrated as reported elsewhere (Tatsuo et al., 2000a).

**Construction of expression plasmids.** Isolation of hSLAM and mSLAM cDNAs has been described previously (Ono et al., 2001b; Tatsuo et al., 2000b). In order to remove the 5′-untranslated region and leader, sequences following codons encoding amino acid positions 22 and 24 were amplified from hSLAM and mSLAM cDNAs, respectively. Those cDNAs were then subcloned behind the sequences encoding the immunoglobulin κ leader and 17 amino acid residues containing the influenza virus haemagglutinin (HA) epitope of the expression vector pDisplay (Invitrogen). The fragment containing the immunoglobulin leader sequence, HA epitope and hSLAM or mSLAM cDNA was subcloned into pCAGGS (Niwa et al., 1991), producing pCAGHA-hSLAM and pCAGHA-mSLAM. Plasmids encoding chimeric molecules were constructed from pCAGHA-hSLAM and pCAGHA-mSLAM by gene splicing using overlap extension (SOEing), as described in detail elsewhere (Ono et al., 2001b; Vallejo et al., 1995). The PCR products encoding chimeric molecules were subcloned into pCAGGS. Site-directed mutagenesis was also performed in a similar manner with primers in which mutations were introduced. All constructs were verified by DNA sequencing. The expression plasmid encoding dog SLAM (pCAGDogSLAMtag) has been described previously (Tatsuo et al., 2001).

**Immunofluorescence staining.** CHO cells were plated in 6-well plates and transiently transfected with expression plasmids using Lipofectamine Plus reagent (Invitrogen). At 24 h after transfection, these transfected cells were stained with mouse anti-HA monoclonal antibody 12CA5 (Boehringer Mannheim) or mouse anti-hSLAM monoclonal antibody IPO-3 (Kamiyama Biomedical) and then with FITC-labelled secondary antibody. Stained cells were analysed on a FACScan machine (Becton-Dickinson). Dead cells that stained positively with propidium iodide were excluded from analysis.

**Virus infection of cells.** CHO cells were plated in 96-well plates and transiently transfected with 50 ng plasmid DNA using Lipofectamine Plus reagent. At 24 h after transfection, these cells were infected with 50 μl of the serially diluted VSVAG*-KAHF or VSVAG*-CDVHF. Infectious titres of the pseudotypes were determined by counting the number of GFP-expressing cells and are expressed as infectious units (IU) ml⁻¹. In other experiments, transfected CHO cells were infected with the wild-type KA strain of MV and examined for cytopathic effect under a light microscope at 24 h after infection.

**RESULTS**

**Studies with human/mouse chimeric molecules revealed a region in the V domain of SLAM that is critical for receptor function**

We have shown previously that the V domain of hSLAM is necessary and sufficient for its function as a cellular receptor for MV (Ono et al., 2001b). In order to define further the critical region(s) within the V domain, we constructed various plasmids encoding chimeric molecules on the mSLAM backbone in which parts of the V domain were replaced with corresponding sequences from hSLAM (Fig. 1). Plasmids were designed such that the molecules produced had the influenza virus HA tag at the N terminus. Parental hSLAM and mSLAM were tagged in a similar manner with the influenza virus HA epitope (HA-hSLAM and HA-mSLAM). CHO cells were transiently transfected...
Critical residues for MV receptor SLAM

Fig. 1. V domain structures of human/mouse chimeric SLAM proteins after cleavage of the leader sequence and their receptor function as determined by the VSV pseudotype assay. Thick and thin lines indicate sequences derived from hSLAM and mSLAM, respectively. The influenza virus HA tag at the N terminus is indicated by a filled oval. Chimeric molecules are based on HA-mSLAM and therefore have C2, transmembrane and cytoplasmic domains derived from mSLAM. We considered a chimeric molecule as having MV receptor function if it gave more than one-tenth of the infectivity titre with HA-hSLAM.

with these expression plasmids and cell surface expression of chimeric molecules was examined by staining with anti-HA antibody and FITC-labelled secondary antibody at 24 h after transfection. All mSLAM-based chimeric molecules studied exhibited comparable levels of cell surface expression (Fig. 2, top row and data not shown). These transfected cells were then infected with the VSV pseudotype bearing the H protein of the wild-type KA strain and the F protein of the Edmonton strain (VSVΔG*KAHF) and infectivity titres were determined by counting the number of GFP-expressing cells at 24 h after infection. The infectivity titre of VSVΔG*KAHF was \(10^{4.8} - 10^{5.2} \text{ IU ml}^{-1}\) on CHO cells expressing HA-hSLAM and was about 2 logs lower (\(10^{2.7} - 10^{3.1} \text{ IU ml}^{-1}\)) on CHO cells expressing HA-mSLAM. In the following experiments, we considered a chimeric molecule as having MV receptor function if it gave more than one-tenth of the infectivity titre with HA-hSLAM.

We first tested two chimeric molecules, m(h23-67) and m(h68–139). The molecule designated m(h23-67) was a chimera based on HA-mSLAM, whose amino acid residues at positions 23–67 were replaced with the corresponding human sequences. Note that the amino acid positions used throughout this report are those of hSLAM because mouse V domain sequences do not align perfectly with human sequences due to an insertion at residue 52 of mSLAM. All chimeric and mutant molecules were designated in the same way. Replacement of either the N- or the C-terminal portion of the V domain was not sufficient to provide mSLAM with a significant receptor activity.

We then extended the replacement with the human sequences toward the C-terminal part of the V domain. m(h23-117) gave an infectivity titre comparable to that with HA-hSLAM, whereas m(h23-97) did not have receptor function. Although this result suggested that critical amino acid residues were present at positions 98–117 of hSLAM, m(h98–117) did not have receptor function, like m(h68–139), which contained these residues. Thus, we thought that in addition to the residues at positions 98–117, another region(s) might be required also. Therefore, we examined three chimeric molecules possessing non-continuous segments from hSLAM, m(h23-47, 68–139), m(h23-57, 68–139) and m(h23-47, 58–139). Among them, only m(h23-47, 58–139) gave almost the same infectivity titre as HA-hSLAM. Thus, we suspected that amino acid residues both at positions 58–67 and at positions 98–117 of hSLAM were necessary for receptor function. In fact, m(h58–67, 98–117) showed good receptor activity. Unexpectedly, m(h58–67) also gave nearly the same infectivity titre as HA-hSLAM, despite the absence of the human sequences at positions 98–117.

In order to understand these inconsistent results better, we produced three more chimeric molecules, m(h58–117) showed MV receptor function but m(h58–97) and m(h23-67, 98–117) did not. Taken together, these data indicate that when mSLAM-based chimeric molecules have human sequences at positions 58–67 or 58–117, they have MV receptor function. However, if they have human sequences encompassing positions 23–67 or 58–97, they do not exhibit receptor function, despite the presence of human sequences at positions 58–67.

A few amino acid changes conferred receptor function on mSLAM

Since replacement of amino acid residues at positions 58–67 with the human sequences conferred a good receptor function on mSLAM, we concentrated our study on this region. There are three amino acid differences in positions 58–67 between hSLAM and mSLAM (Table 1). Therefore, m(h58–67) can be called m(h60, 61, 63). Table 1 also lists the sequences of SLAMs from other species in this region. In order to determine which amino acid residue is important for receptor function, we mutated these three residues individually in HA-mSLAM, producing m(h60), m(h61) and m(h63). The surface expressions of these molecules were confirmed on CHO cells transiently transfected with the expression plasmids (Fig. 2). Cells were then infected with VSVΔG*KAHF and infectivity titres were determined. Among these three constructs, m(h61) gave over 10-fold higher titre than HA-mSLAM, but m(h60) and m(h63) did not (Fig. 3). Since m(h61) did not give as high a titre as m(h60, 61, 63), we also prepared two other constructs...
containing two amino acid substitutions, including position 61, m(h60, 61) and m(h61, 63) (Fig. 2). They gave slightly higher titres than m(h61) (Fig. 3).

We also assessed receptor function of mutant molecules by infecting transfected cells with MV. As expected, CHO cells expressing the mutant SLAMs that had receptor function as determined by the VSV pseudotype assay developed syncytia, whereas CHO cells expressing the mutants without receptor function did not (Fig. 4 and data not shown).

m(h23–57, 68–139), which has the V domain of hSLAM with the mouse-type sequences at positions 60, 61 and 63, did not show receptor function (Fig. 1) nor did it allow cells to develop syncytia (Fig. 4). To prove further the importance of these amino acid residues in receptor function of hSLAM, we produced mutants based on HA-hSLAM,

**Fig. 2.** Cell surface expression of chimeric SLAM proteins. CHO cells were transfected with expression plasmids encoding the indicated molecules. At 24 h after transfection, cells were stained with anti-influenza virus HA epitope monoclonal antibody 12CA5, followed by FITC-labelled secondary antibody. Solid lines indicate the staining pattern of CHO cells transfected with the plasmid encoding hSLAM without the influenza virus HA tag, used as a negative control. m(h58–67) can be called m(h60, 61, 63).
Table 1. Amino acid sequences of SLAMs from different species

Amino acid residues at positions 58–67 of hSLAM are compared with those at corresponding positions of SLAMs from other species. Identity with the human sequence is indicated as ‘–’.

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h(m61), h(m63) and h(m61, 63). These chimeras have substitutions at position 61, at position 63 and at positions 61 and 63 with the mouse-type residues, respectively. Cell surface expression levels of these mutants were comparable among them but not as high as other chimeric and mutant molecules (Fig. 2). While h(m63) gave a reduced but significant titre, h(m61) and h(m61, 63) showed only the same level of infectivity as HA-mSLAM (Fig. 3).

Residues at positions 60, 61 and 63 are also important for the function of SLAM as a receptor for CDV

CDV, like MV, is a member of the genus Morbillivirus and uses dog SLAM as a cellular receptor (Tatsuo et al., 2001). In order to examine whether the residues at positions 60, 61 and 63 are also critical for the function of SLAM as a receptor for CDV, we transiently transfected CHO cells with expression plasmids encoding various SLAM proteins and

infected them with the VSV pseudotype bearing the H protein of a wild CDV strain and the F protein of the Onderstepoort strain (VSVΔG*-CDVHF). It showed a high infectivity titre ($7 \times 10^5$ IU ml$^{-1}$) on CHO cells expressing dog SLAM but a very low titre ($1 \times 10^3$ IU ml$^{-1}$) on CHO cells expressing HA-hSLAM (Fig. 5). Human and dog SLAMs have the same residues at positions 60 and 61 but have different residues at position 63: valine in hSLAM and leucine in dog SLAM. We tested CDV receptor function of hSLAM whose position 63 was changed to leucine, as seen in dog SLAM. VSVΔG*-CDVHF showed about a 10-fold higher titre on CHO cells expressing h(m63) (which is essentially HA-hSLAM except leucine at position 63) than it
did on CHO cells expressing HA-hSLAM. The results were more impressive with HA-mSLAM and its mutant whose positions 60 and 61 were changed to the dog-type sequences (isoleucine and histidine, respectively). When transfected cells were infected with VSVAG*-CDVHF, m(h60, 61), which shares the same residues at positions 60, 61 and 63 with dog SLAM, conferred almost a 100-fold higher titre on the cells compared with HA-mSLAM.

DISCUSSION

We have previously shown that hSLAM, but hardly mSLAM, acts as a cellular receptor for MV (Ono et al., 2001b; Tatsuo et al., 2000b). In this study we attempted to map amino acid residues in SLAM that are critical for its receptor function by making chimeric molecules between hSLAM and mSLAM. In the first set of experiments, we showed that the exchange of the residues at positions 58–67 allowed mSLAM to act efficiently as a receptor for MV. However, another region(s) also appeared to influence receptor function because some chimeric molecules containing the human sequences in this region, such as m(h23–67), m(h23–97) and m(h58–97), did not act as a receptor, indicating that the presence of the human sequences at positions 58–67 alone is not sufficient for receptor function. Nevertheless, we concentrated our analysis on this region in the present study, since m(h58–67) showed almost the same receptor activity as HA-hSLAM.

Among 10 amino acid residues located at positions 58–67, there are three differences between hSLAM and mSLAM. hSLAM has isoleucine, histidine and valine at positions 60, 61 and 63, respectively, whereas mSLAM has valine, arginine and leucine at the corresponding positions. Substitution of arginine at position 61 with histidine enabled mSLAM to act as a receptor for MV, while converse substitution at position 61 compromised the receptor function of hSLAM. Thus, histidine at position 61 appeared to be critical for SLAM to act as a receptor for MV. Although substitution at position 60 with isoleucine or at position 63 with valine alone did not increase receptor activity of mSLAM very much, combined substitutions with histidine at position 61 and one or both of these residues provided higher infectivity titres than that with a single substitution at position 61 with histidine. Thus, isoleucine at position 60 and valine at position 63 also appear to contribute to receptor function of hSLAM.

Besides hSLAM and mSLAM, the predicted amino acid sequences of dog, cow and marmoset orthologues have been reported (Tatsuo et al., 2000b, 2001; Tatsuo & Yanagi, 2002). SLAMs from these five different species have about 60–70% identity at the amino acid level to one another, except human and marmoset SLAMs, which have 86% identity (Castro et al., 1999; Tatsuo et al., 2001; Tatsuo & Yanagi, 2002). The sequences at positions 58–67 are well conserved among these species (Table 1). Among the three residues implicated in receptor function in the present study, isoleucine 60 and histidine 61 are conserved in human, marmoset, dog and cow SLAMs. Interestingly, SLAMs from these species have been shown to act as receptors for MV, as determined by the VSV pseudotype assay and by the development of syncytia after MV infection (Tatsuo et al., 2000b, 2001). Furthermore, the VSV pseudotype assay demonstrated that when residues at positions 60, 61 and 63 were made the same as those in dog SLAM, both hSLAM and mSLAM became to act more efficiently as a receptor for CDV. These observations also support the contention that residues at these three positions are critical for receptor function of SLAM.

Since the tertiary structure of SLAM has not been determined, we cannot locate exactly these critical residues on SLAM. Analysis using Mac Vector 7.0 (Oxford Molecular) predicted that the region around positions 58–67 of hSLAM assumes the β-sheet by Chou–Fasman program and the α-helix by Robson–Garnier program. The corresponding region of mSLAM was predicted to take the β-sheet by both programs. Substitutions at this region did or did not cause alteration of its secondary structure as predicted by these programs. However, we could not find a consistent relationship between the predicted secondary structure and MV receptor function.

Are these residues directly involved in binding to the H protein of MV? In order to answer this question, we synthesized several peptides (12–23 residues in length) containing sequences around these residues, hoping that they may serve as a short soluble receptor (Knauss & Young, 2002). We examined whether they bound to cells expressing the MV H protein. We also tested whether the peptides could block MV infection of susceptible cells or the binding of soluble hSLAM to cells expressing the MV H protein. In either experiment, we have not been able to obtain evidence that the short peptides containing these residues bind to the MV H protein (data not shown). Thus, these residues may not directly interact with the H protein and instead be involved in keeping the proper tertiary structure of SLAM. Alternatively, it is also possible that several peptides we have synthesized thus far could not form accurate tertiary structures so that they failed to interact with the H protein. Without more detailed structural information, we cannot decide between these possibilities at this moment.

In this study, we have examined only the residues different between human and mouse molecules. In order to thoroughly map the residues critical for receptor function, we have to systematically introduce mutations in hSLAM and eventually determine the structure of the MV H protein–SLAM complex. As mentioned above, our present study has already implicated some regions other than positions 58–67 in influencing receptor function of SLAM. Nonetheless, our finding is the beginning to an understanding of the interaction between the MV H protein and SLAM. It is hoped that these studies will lead eventually to the development of compounds that can inhibit MV infection by blocking their interaction.
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