Direct Evidence for the Role of COOH Terminus of Mouse Mammary Tumor Virus Superantigen in Determining T Cell Receptor Vβ Specificity

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Summary

It has recently been shown that open reading frames in the 3' long terminal repeats of mouse mammary tumor viruses encode superantigens. These viral superantigens (vSAGs) stimulate most T cells expressing appropriate Vβs almost regardless of the rest of the variable components of the T cell receptors (TCR) expressed by those cells. vSAGs produce a type II integral membrane protein with a nonessential short cytoplasmic domain and a large glycosylated extracellular COOH-terminal domain, which is predicted to interact with major histocompatibility complex class II molecules and the TCR. The transmembrane region of vSAG also has an internal positively charged lysine residue of unknown significance. A set of chimeric and mutant vSAG genes has been used in transfection experiments to show that only the extreme COOH-terminal portion of vSAGs determine their TCR Vβ specificities, and to show that the lysine residue in the transmembrane domain is not essential for the function of vSAG.

Superantigens (SAGs), combined with MHC class II molecules, interact with a high proportion of TCR-α/β (1, 2). This high frequency of interaction occurs because SAG–MHC complexes stimulate virtually all the T cells bearing particular Vβ elements almost irrespective of other variable components (Vα, Jα, and DβJβ) in TCR (3–7). The recognition of SAG–MHC complexes by TCR is markedly different from the recognition of conventional peptide antigens, which require the contribution of both TCR α and β chains (8–11). The unusual way in which SAG–MHC complexes interact with TCR has been partly explained by recent experiments showing that SAGs interact with the TCR primarily through the hypervariable region 4 (HVR4), well away from TCR-α and the third hypervariable region (12–14).

Most murine endogenous SAGs are now known to be encoded by the open reading frames (orf) in the 3' LTR of mouse mammary tumor viruses (MMTV) (15–22). For example, Mls-1 antigen which stimulates T cells bearing Vβ 6, 7, 8.1, and 9, has been shown to be encoded by the orf in the 3' LTR of endogenous MMTV-7 (17, 23). MMTV-1, -3, -6, and -13 encode SAGs that stimulate T cells expressing Vβ3 elements (22). The orf of different MMTVs are now termed viral superantigens (vSAGs) (17, 18, 22–24). For example, Mls-1 is denoted as vSAG-7 because the SAG is encoded by MMTV-7.

It has recently been shown that vSAGs encode 45-kD glycoproteins (23, 25, 26). The data provided by in vitro trans-

lation experiments suggested that the vSAG produces a type II membrane protein with a large glycosylated extracellular COOH-terminal domain and a small, nonessential, intracellular NH₂-terminal cytoplasmic domain (25, 26). With the use of mAb specific for the COOH-terminal portion of vSAG-7, it has been directly shown that vSAG-7 indeed produces a membrane glycoprotein with an extracellular COOH-terminal domain (23). In addition, it has been shown that vSAGs are produced as 45-kD transmembrane precursor glycoproteins that are proteolytically processed to yield 18.5-kD surface proteins (23). However, it is still not clear whether this processed 18.5-kD polypeptide is sufficient for the function of SAG, and whether the proteolytic cleavage of the protein is essential for its function.

The extensive sequence analysis of vSAG genes has shown that different vSAGs have very similar protein sequences with only 10–15% variation (16–18, 22). It is interesting that most sequence differences are located in two regions: polymorphic region I from amino acid residue 174 to 198, and region II from amino acid residue 288 to the COOH-terminus (see Fig. 1) (16–18, 22). For example, the amino acid residues in these two regions of vSAG-1 (Vβ3 associated) are very different from those of vSAG-7 (Vβ 6, 7, 8.1, and 9 associated). However, the sequences of polymorphic regions I and II of vSAGs-1, -3, -5, and -13, which interact with TCR Vβ3, are all identical. This has led to the suggestion that these V regions are contributing to the TCR specificity of different vSAGs. An-

other unusual feature of this protein is the positively charged lysine residue in the transmembrane region (see Fig. 1). By analogy to the transmembrane region of TCR α and β chains, it has been suggested that the lysine residue in the transmembrane region of the vSAG may contribute to the function of this protein (26, 27).

To test the importance of polymorphic regions I and II of vSAG, and the importance of the lysine residue in the transmembrane region for the superantigenic property of vSAG, we have constructed various chimeric and mutant vSAG genes and studied the properties of their protein products. We report here that only polymorphic region II is important in determining the TCR Vβ specificity of the vSAG, and that the lysine residue in the transmembrane region is not essential for the function of this protein as a superantigen.

Materials and Methods

Construction of Chimeric and Mutant vSAG Genes. The vSAG-1 and vSAG-7 genes have been described previously (22, 23). Chimeric vSAG-ln7c and -7nlc series of genes were constructed by restriction enzyme digestion and ligation of pTZ18R-vSAG-1 and -7 (22, 23). The vSAG-7/KL was constructed by PCR-mediated, site-directed mutagenesis as described previously (13, 14).

The primer set of 5'-ATACTCATTCTCTGCTGCCTCCTT-GGCATA-3' (5' primer) and 5'-TATGCCAAGGAGGCAGCAGAG-AATGAGTAT-3' (3' primer) was used to replace the lysine residue to leucine residue. The mutant vSAGs were amplified with a set of primers (5'-GGGAATTCTCGAGATGCCGCGCCTGCAG-3' and 5'-GGGGATCCTCTAGAGGGAACCG-3'), cloned into pTZ18R (Pharmacia Fine Chemicals, Piscataway, NJ) or pBluescript KS+ (Stratagene, La Jolla, CA), sequenced, and ligated into the mammalian expression vector pHbAPr-1 as previously described (28).

Transfection of vSAG Genes. Wild-type vSAGs and their derivative genes were linearized with PvuI and electroporated into the B cell lymphoma, CH12.1, using a Gene Pulser Transfection Apparatus (Bio-Rad Laboratories, Cambridge, MA) at 240 V, 500 μF. The transfectants were selected by growth in G418 (700 μg/ml) and were screened for expression of transfected genes by Northern blot analysis or flow cytometric analysis using biotinylated VS7 mAb as previously described (23).

*Assay of vSAG Function.* CH12.1 transfectants were screened

for their ability to stimulate the T cell hybridomas KMls-8 (Vβ6+) and 5KC-73.8 (Vβ3+) because the vSAG-1 and vSAG-7 are known to stimulate mouse T cells bearing these Vβs, respectively. The T cell hybridoma KOX15-8.3 (Vβ15+) was used as negative control (18). The stimulation of T hybridomas was assayed by lymphokine production as previously described (29).

Results and Discussion

Regions of vSAG Involved in Determining TCR Vβ Specificity. The sequence analysis of vSAGs revealed two regions that are highly polymorphic among vSAGs (Fig. 1) (16–18, 22). For example, vSAG-7 and vSAG-1 have 46 amino acid residue differences, of which eight residues are located in the polymorphic region I and 31 residues in the polymorphic region II (Fig. 1). Based on the sequence comparison of different vSAGs that have same the TCR Vβ specificity, it was suggested that polymorphic region I and/or II would determine the TCR Vβ specificity of a given vSAG (16–18, 22). To test this hypothesis directly, we have constructed several chimeric vSAGs from vSAG-1 and vSAG-7, Vβ3 and Vβ6 associated, respectively. The predicted amino acid sequences of vSAG-1 and vSAG-7 genes are shown in Fig. 1.

There are three convenient restriction enzyme sites (BsmI, StuI, and PpuMI) at the same location in vSAG-1 and vSAG-7 (Fig. 1). Therefore, these restriction enzymes were used to generate chimeric vSAG genes, vSAG-ln7c, in which the gene fragment 5' to a restriction enzyme site was from vSAG-1 and the gene fragment 3' to a restriction enzyme site was from vSAG-7 (Fig. 2). These chimeric vSAG genes (named vSAG-ln7c-Bsm, -Stu, and -Ppu, Fig. 2) were cloned into the mammalian expression vector, pHβAPr-1, and transfected into MHC class II–expressing CH12.1 lymphoma cells by electroporation. Transfectants were assayed for stimulation of T cell hybridomas expressing Vβ3 and Vβ6, the targets of vSAG-1 and vSAG-7, respectively.

The results of representative experiments are illustrated in Fig. 3. As previously shown, the untransfected cell line, CH12.1, failed to stimulate KMls-8 (Vβ6) and 5KC-73.4 (Vβ3) (30, 31). The transfectants expressing vSAG-1,
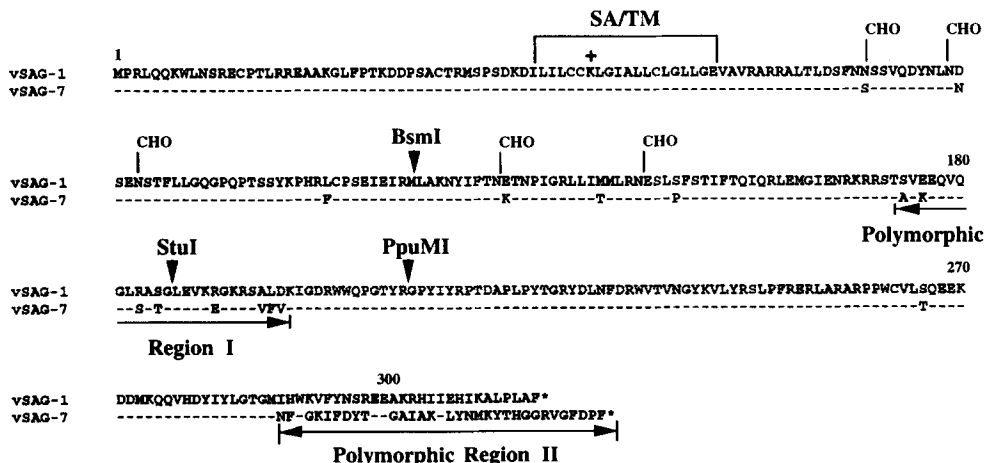
Figure 1. Sequences of vSAG-1 and vSAG-7. The protein sequence of vSAG-7 (17) is shown aligned with that of vSAG-1 (22). (*SA/TM*) Putative signal anchor/transmembrane region; (*CHO*) N-linked glycosylation sites. (+) The positive lysine residue.
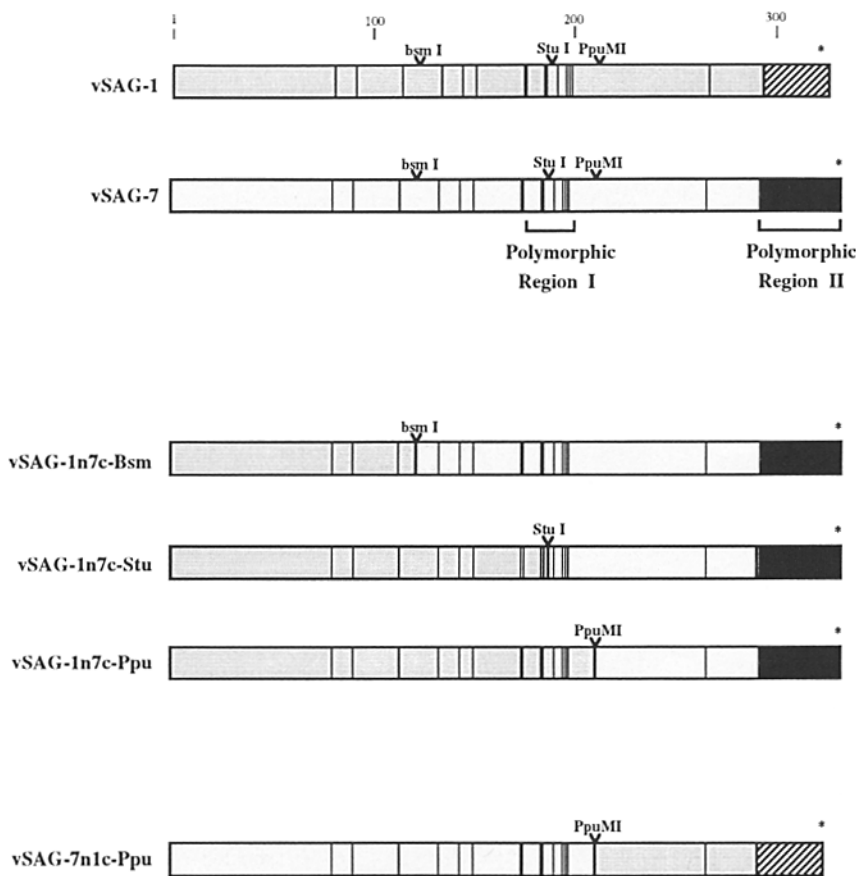
Figure 2. Construction of chimeric vSAG genes. The positions of restriction enzyme sites are indicated. (*) Termination codon. (*Shaded boxes*) Portion from vSAG-1. (*Solid vertical lines*) Differences in amino acid residues between vSAG-1 and vSAG-7.
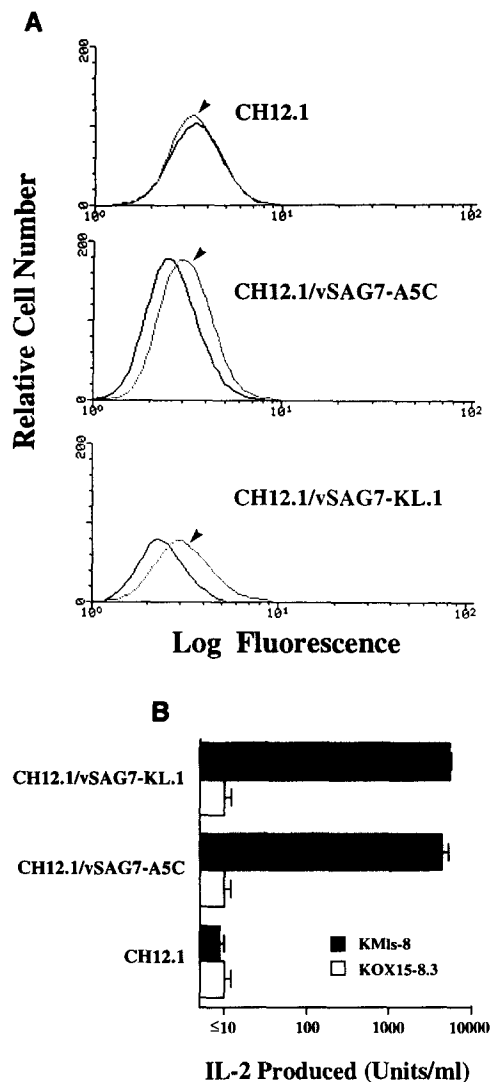
CH12.1/vSAG1-1, stimulated 5KC-73.4. Likewise, the transfectants expressing vSAG-7, CH12.1/vSAG7-A5C, stimulated KMls-8 (23). All the transfectants expressing chimeric vSAG-ln7c-Bsm, -Stu, and -Ppu (CH12.1/Bsm-1N7C.5, CH12.1/Stu-1N7C.9, and CH12.1/Ppu-1N7C.10, respectively) stimulated KMls-8, but failed to stimulate 5KC-73.8. A $V\beta15^+$ T cell hybridoma, KOX15-8.3, was not stimulated by any of the B cell lines, as expected. Since vSAG-ln7c-Ppu is essentially identical to vSAG-1 except in polymorphic region II, the data provide direct evidence that the polymorphic region II of the vSAG-7 is sufficient to determine the $V\beta$ specificity of vSAG-7. These data also suggest that polymorphic region I is not involved in determining TCR $V\beta$ specificity of vSAG.

Reciprocal experiments have also been done in this study, using the chimeric vSAG, vSAG-7nlc-Ppu, in which the gene fragment 3' to the PpuI site in vSAG-1 is replaced with that in vSAG-7. The transfectants expressing the chimeric vSAG-7nlc-Ppu gene, CH12.1/Ppu-7N1C.1, stimulated 5KC-73.8 but not KMls-8, which is consistent with the data described above (Fig. 3).

Is the Positively Charged Lysine Residue in the Transmembrane Region of the vSAG Essential for Its Function? All the MMTV encoded vSAGs known to date have an internal positively charged lysine residue in the transmembrane region (Fig. 1) (25, 26). By analogy to the transmembrane region of TCR α and β chains, it has been proposed previously that the ly-
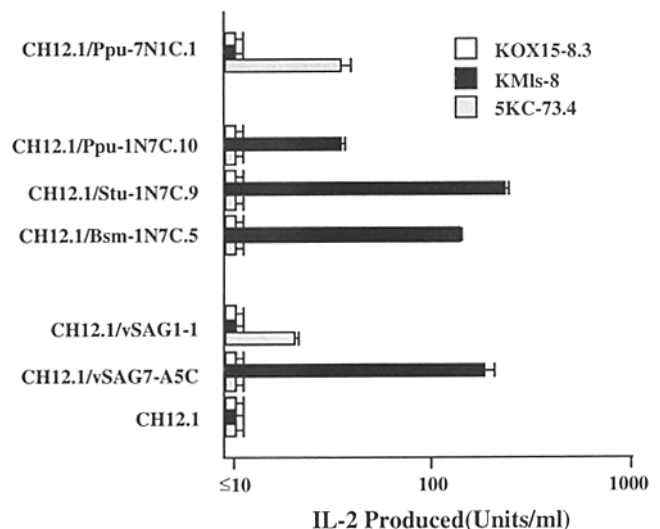
Figure 3. The importance of polymorphic region II in determining TCR $V\beta$ specificity. CH12.1 cells transfected with different vSAG genes were tested for their ability to stimulate various T cell hybridomas, KMls-8 ($V\beta8.1$), 5KC-73.8 ($V\beta3$), and KOX15-8.3 ($V\beta15$) as described previously (18, 30, 31). Stimulation was assayed 24 h later by levels of secreted lymphokines in the supernatants.

A

CH12.1

CH12.1/vSAG7-A5C

CH12.1/vSAG7-KL.1

Log Fluorescence

Relative Cell Number

B

CH12.1/vSAG7-KL.1

CH12.1/vSAG7-A5C

CH12.1

■ KMls-8
□ KOX15-8.3

≤10   100   1000   10000

IL-2 Produced (Units/ml)

Figure 4. (*A*) Surface expression of vSAG-7 on cell lines. The cell lines CH12.1, CH12.1/vSAG7-A5C, and CH12.1/vSAG7-KL.1 were analyzed with VS7 antibody. (*Solid line*) Flow cytometric histogram in the presence of peptides; (*broken line with arrowheads*) flow cytometric histogram in the absence of peptides. (*B*) Effect of amino acid substitution lysine to leucine in the transmembrane region on the superantigenic activity of vSAG-7. The transfectants expressing similar levels of vSAG on the cell surface were tested for their ability to stimulate T cell hybridomas, KMls-8 (Vβ8.1).

sine residue of vSAGs may have an important role in the function of SAG, such as in the interaction of SAG with class II molecules (25, 26).

To test this hypothesis, we have generated a mutant vSAG-7 by changing the lysine residue to a leucine by site-directed mutagenesis. This mutant vSAG, vSAG-7/KL, was ligated into a mammalian expression vector and transfected into CH12.1 cells as described above. The transfectants with similar surface vSAG-7 expression were selected and tested for their ability to stimulate T cells. Representative experiments are shown in Fig. 4. The transfectant, CH12.1/vSAG7-KL.1, expressing the mutant vSAG-7, stimulated KMls-8 as efficiently as CH12.1/vSAG7-A5C, which expresses wild-type vSAG-7.

These data suggest that the internal positively charged lysine residue in the transmembrane region of vSAG-7 is not essential for its function. Since the transfectants expressing similar surface levels of wild-type and mutant vSAG stimulated KMls-8 with approximately the same efficiency, it is also unlikely that the lysine residue is involved in the interaction of vSAG antigen with MHC class II molecules.

The experiments in this study have conclusively shown that only polymorphic region II at the extreme COOH-terminus of vSAGs determines the TCR Vβ specificity of a given vSAG. Polymorphic region I of vSAG is not involved in determining the TCR Vβ specificity. However, possible involvement of polymorphic region I for the function of vSAG is not excluded as yet. Since chimeric vSAGs of different polymorphic regions I and II produce functional proteins, it seems unlikely that polymorphic region I interacts directly with amino acid residues in polymorphic region II which interact with the Vβ element of TCR. It has been noticed for some time that different vSAGs show variable degrees of stimulation of target T cells in mixed lymphocyte reactions, even though all vSAGs are very efficient in clonal deletion of target T cells during T cell development in the thymus (1-7). Therefore, it is possible that polymorphic region I may be involved in presentation of vSAG to T cells, either by affecting the interaction of vSAGs with MHC class II molecules or the transport of vSAG to the cell surface.

By making a mutant vSAG-7 lacking an internal positively charged residue in the transmembrane region, we have shown that the lysine residue at position 51 is not important for the surface expression of vSAG. Since the mutant vSAG stimulated target T cells as efficiently as the wild-type vSAG, it is unlikely that the lysine residue is involved in the association of vSAG with MHC class II molecules, which is a prerequisite for the stimulation of T cells by vSAG.

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