

Brief Definitive Report**Proteolytic Processing Is Required for Viral Superantigen Activity**By Chae Gyu Park,[‡] Mi-Young Jung,^{*} Yongwon Choi,^{*‡} and Gary M. Winslow[§]

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Summary

The mouse mammary tumor virus-7 superantigen (vSAG7) is proteolytically processed in B cells at as many as three positions. Proteolytic processing appears to be important for superantigen activity because a processed form of vSAG7 was predominant among those forms that were found to bind to major histocompatibility complex class II molecules. To determine the functional significance of proteolytic processing, a mutation was introduced in vSAG7 at one of the sites where proteolytic cleavage is thought to take place in B cells. Elimination of the putative processing site at position 171 abrogated detectable vSAG7 surface expression in B cells, indicating that proteolytic processing is required for vSAG7 function. Coexpression in insect cells of vSAG7 and furin, a proprotein-processing enzyme, also demonstrated that furin could process vSAG7 at position 171.

Superantigens bind to MHC class II molecules and stimulate T cells that bear specific V β s as part of their TCR (1, 2). They are encoded by bacteria and by mouse mammary tumor viruses (MMTVs). The soluble bacterial superantigens have been well characterized at the biochemical and cellular levels, but the biochemical nature of the MMTV superantigens (vSAGs) has been obscure. Biochemical studies of the superantigen encoded by MMTV7 (vSAG7) have indicated that vSAG7 is detected on the cell surface in a proteolytically processed form (3). Proteolytic processing is thought to occur in the Golgi region or in a post-Golgi compartment and yields a product(s) that is (are) cleaved at as many as three positions (4). At least one processed form of the protein has been shown to bind to MHC class II molecules (4). It has not been determined, however, if processing is required for normal vSAG7 function, and the exact positions in the protein where the proteolytic processing occurs are unknown.

vSAG7 is among a number of viral glycoproteins and cellular protein precursors that undergo proteolytic processing. Proteolytic cleavage of the precursor proteins commonly occurs at sites characterized by paired basic residues and is thought to result from the action of any of a family of mammalian subtilisin-like serine proteases (also known as proprotein convertases [PCs]) (5, 6). In several cases, proteolytic processing has been shown to be required for protein activity (7–10).

Analysis of the vSAG7 amino acid sequence suggested the presence of three possible basic endoprotease recognition sites at residues 68–71 (RARR in single-letter amino acid code), 168–171 (RKRR), and 190–194 (KEGKR). The approximate

molecular weights of the vSAG7 proteolytic products that have been detected using SDS-PAGE are consistent with those predicted to be generated if cleavage occurred at or near these putative processing sites (4). Although a vSAG7 product, probably processed at residues at 168–171, predominated among the forms found to be associated with class II I-A^k (4), it has not been determined if proteolytic processing of vSAG7 is required for presentation to T cells.

To determine the importance of residues 168–171 for proteolytic cleavage and vSAG7 function, amino acid substitutions were introduced at this position in vSAG7 using site-directed mutagenesis, and the effects of the substitutions on vSAG7 function were examined at the cellular and biochemical levels. In this study, we show that proteolytic cleavage was required for vSAG7 surface expression in B cells and presentation to T cells. In addition, the furin endoprotease, which recognizes the amino acid motif RXX/RR, is shown to be a candidate vSAG7-processing endoprotease.

Materials and Methods

Mutagenesis and Plasmids. To generate each mutant *sag7* gene, the *sag7* gene was amplified by PCR with mutagenic primers, as shown in Fig. 1 B. For the *sag7*^{m171} mutation, two different PCR products of the *sag7* gene were amplified in separate reactions. One reaction used the 5' m171 primer (5'-GAAAATGGAGAGGAG-TTCTCAACCGCA-3') and primer B (5'-GGGGATCCCTAA-AAGGGATCGAAGCCGA-3'), and the other reaction used the 3' m171 primer (5'-TGCGGTGAGAACTCCTCTCCATTTTC-3') and primer A (5'-GGGAATTCTCGAGATGCCGCGCCTGCAG-

3'). The two products obtained in the first PCR were joined together in the second round of the PCR, as described previously (11), with primers A and B, to generate the complete *sag7*^{m171} gene (Fig. 1 B). The *sag7*^{m194} mutation was generated in a similar manner as *sag7*^{m171}, except that different mutagenic primers were used: the 5' m194 primer (5'-AGAAGGAGAATTCAGTGTG-3') and the 3' m194 primer (5'-CACACTGAATTCCTCTCT-3'). The PCR products from each of the second reactions were cloned and sequenced before any further manipulation.

Constructs encoding soluble forms of vSAG7 (BvSAG7) were generated by PCR using the primer (5'-GGGAATTCAGATGGCAATCGGTCTCCTGTGCTGTGCAGCCTTGTCTCTCCTGTGGGCAGGTCAGTGAATGCTGGTGTCACTCAGGGTGTGGTGTTCGGTTCGTGCTCGCAGGGCT-3') and primer B, described above, using the wild-type or mutant *sag7* genes as templates. The soluble forms lack the cytoplasmic domain and transmembrane portion of vSAG7. The soluble forms were created by fusion in frame to the human TCR V β 13 signal peptide (MAIGLLCCAALSLWAGPVNA↓GVTQ; ↓ = signal peptide cleavage site; 12). After verifying the sequences of each PCR product, the genes encoding the soluble forms of vSAG7 were cloned into a baculovirus expression vector pBACp10pH (derived from pAcUW51; PharMingen, San Diego, CA) (13) to generate the plasmids pBsag7, pBsag7^{m171}, and pBsag7^{m194}. The human furin (also known as PACE) cDNA was isolated from pMT3, kindly provided by Dr. A. Rehemtulla (Howard Hughes Medical Institute, University of Michigan Medical Center, Ann Arbor, MI), and ligated into pBACp10pH to create the plasmid pBAC-furin (14).

Cell Culture, Transfections, and Flow Cytometric Analysis. The culture of B cell lymphoma CH12.1 and the T cell hybridomas have been described previously (3, 11, 15). *Spodoptera frugiperda* (Sf9) cells were cultured in Grace's media (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FCS, 100 μ g/ml penicillin G, 200 μ g/ml streptomycin, and 0.25 μ g/ml amphotericin B. To generate B cell lines expressing vSAGs, the wild-type and *sag7*^{m171} genes were inserted into pH β Apr-1-neo and transfected by electroporation into the B cell lymphoma CH12.1, as described previously (11). The transfectants were also examined for their ability to stimulate the T cell hybridomas by measuring lymphokine production, as described previously (3). Flow cytometry was performed on a FACScan[®] (Becton Dickinson & Co., Mountain View, CA) flow cytometer, as described previously (3, 4).

Production of vSAG7 and Furin in Insect Cells. Recombinant baculoviruses were produced by transfecting the transfer plasmids pBsag7, pBsag7^{m171}, pBsag7^{m194}, and pBAC-furin with rescue viral DNAs into Sf9 cells using a transfection kit (BaculoGold[™]; PharMingen), following conditions as described by the manufacturer. For protein production, Sf9 cells were infected with cloned viruses and cultured for 3 d before preparing the supernatants or cell lysates. Furin enzyme activity from the membrane fractions was assayed as described previously (16, 17).

Biochemical Characterization. Cell lysate preparation, affinity chromatography, endoglycosidase treatment, SDS-PAGE, and immunoblotting were performed as described previously (3, 4). Purification of vSAG7 bound to class II I-A^k was performed as described previously (4).

Results and Discussion

Proteolytic Processing Is Required for vSAG7 Presentation. The three putative processing sites in vSAG7 have been defined previously as the transmembrane (residues 68–71; RARR), proximal (168–171; RKRR), and distal (190–194; KEGKR)

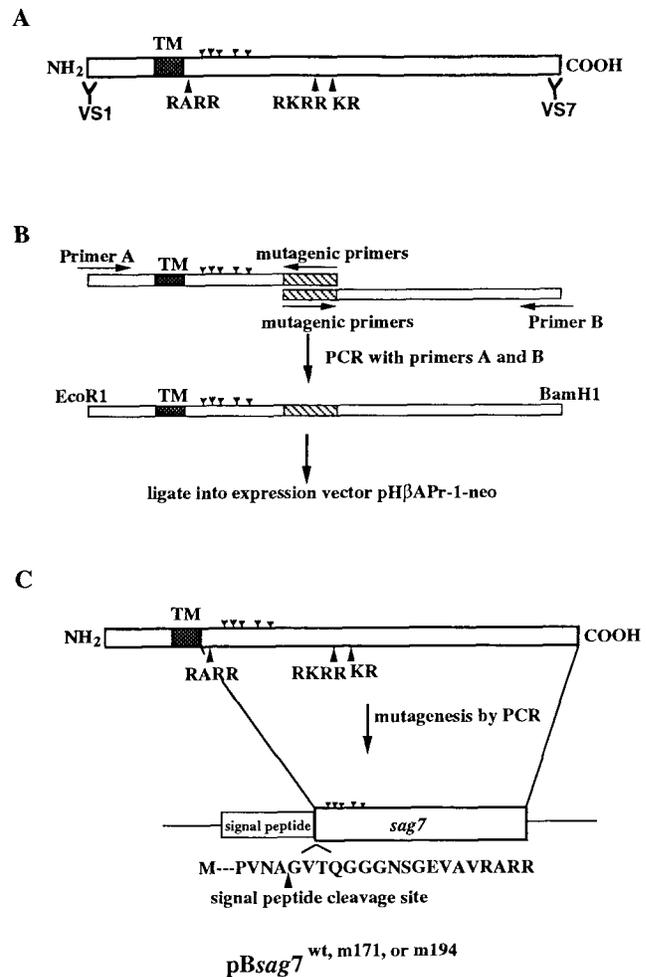


Figure 1. vSAG7 protein expression vectors. (A) Schematic of vSAG7 illustrating the antibody binding sites (VS1, VS7), the transmembrane region (TM), putative proteolytic processing sites (RARR, RKRR, KR), and the sites of N-linked glycosylation (branches). (B) Mutant *sag7* genes were generated using PCR, as shown and as described in Materials and Methods. Arrows, regions of primer-template hybridization; stippling, region of hybridization of mutagenic oligonucleotides. (C) Strategy used for construction of baculovirus-vSAG7 expression vectors. A portion of the amino acid sequence of the signal peptide and the position of the signal peptide cleavage site are shown.

cleavage sites (5, 6) (Fig. 1 A). In particular, it has been suggested that proteolytic cleavage at the proximal site may be required for the binding of vSAG7 to MHC class II molecules (4). To directly test if proximal cleavage site-dependent processing was required for vSAG7 presentation to T cells, amino acid substitutions were made at the proximal site by site-directed mutagenesis. The substitutions introduced acidic and hydrophobic residues in place of the tetrabasic sequence (RKRR to GEEF). The nonconservative substitutions were predicted to prevent processing by any of the known PCs (5, 6).

The mutant *sag7* gene, *sag7*^{m171}, was introduced into an expression vector under the control of the human β -actin promoter (Fig. 1 B) (18) and transfected into the B cell lymphoma CH12.1 (15) to generate the cell line CH12.1/S7^{m171}. Surface expression of the wild-type and mutant forms of

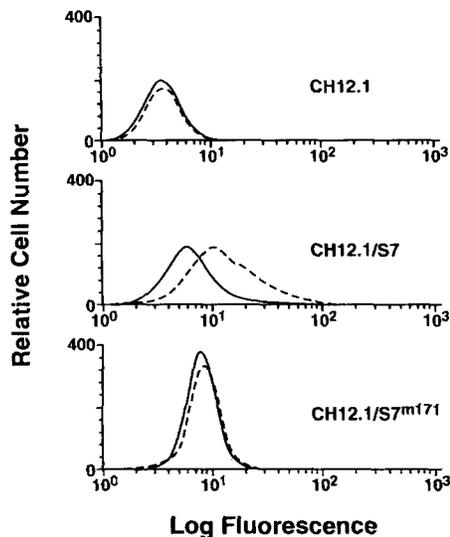


Figure 2. Mutant vSAG7s are not detected on the cell surface. The surface expression of vSAG7 on cell lines, CH12.1, and the transfectants was analyzed with VS7 antibody in the absence (dotted line) or presence (solid line) of 1 mg/ml of the p7 competitor peptide as described previously (11). The levels of surface expression of class II MHC proteins in the transfectants were not significantly different (data not shown).

vSAG7 was analyzed by flow cytometry using the monoclonal antibody VS7, which recognizes the COOH terminus of vSAG7 (3). CH12.1/S7, the cell line transfected with the wild type *sag7* gene, showed a significant level of vSAG7 surface expression, as previously reported (11). In contrast, no surface expression of vSAG7 was observed on CH12.1/S7^{m171} (Fig. 2).

T cell recognition is more sensitive than flow cytometry for assay of vSAG7 expression on B cells, so the transfectants were also tested for their ability to stimulate appropriate T cell hybridomas. CH12.1/S7 stimulated T cell hybridomas, which expressed the vSAG7-responsive V β elements V β 6 and V β 8.1, but these same hybridomas were not stimulated by

CH12.1/S7^{m171} (data not shown). The data suggest that the mutant vSAG7 was not transported to the surface, or was nonfunctional. The absence of detectable surface expression of vSAG7^{m171} in these cells was probably not caused by a failure in protein production because a significant amount of intracellular protein was detected (Fig. 3). Therefore, proteolytic processing appeared to be critical for vSAG7 presentation to T cells and/or for proper surface expression.

To determine if the vSAG7^{m171} underwent any proteolytic processing, the protein was partially purified and analyzed biochemically. In experiments using CH12.1/S7, VS7 detected several forms of vSAG7, including a precursor protein (gpPr45^{sag}), and two COOH-terminal proteolytic cleavage products, previously defined as p18 and p16 (Fig. 3) (4). It was suggested that p18 and p16 originated as a result of proteolytic processing at the proximal and distal vSAG7 cleavage sites, respectively. In affinity-purified preparations of vSAG7^{m171}, gpPr45^{sag} was detected, but only a single COOH-terminal cleavage product was apparent (Fig. 3 *a*, lane 2). Comparison of the vSAG7^{m171} with wild-type vSAG7 revealed that the cleavage product detected in preparations of CH12.1/S7^{m171} protein corresponded to the cleavage product p16, indicating that the m171 substitution eliminated the production of p18 in B cells (Fig. 3 *b*, lanes 1 and 2). Therefore, p18 in vSAG7 originated most likely after proteolytic processing at the proximal cleavage site, as has been previously suggested (4).

It has been demonstrated previously that p18, but not p16, binds to the class II molecule I-A^k (4). To further support the conclusion that the vSAG7^{m171} proteolytic cleavage product was indeed p16, the migration of vSAG7^{m171} was compared with vSAG7 products that were found to bind to I-A^k (Fig. 3 *b*). The data in Fig. 3 *b* demonstrate that the vSAG7^{m171} cleavage product does not comigrate with the vSAG7 cleavage product, p18, that was found to bind to I-A^k.

The Transmembrane and Proximal Proteolytic Processing Sites Are Substrates for the Endoprotease Furin. The basic amino acid proteolytic cleavage sites that have been described in

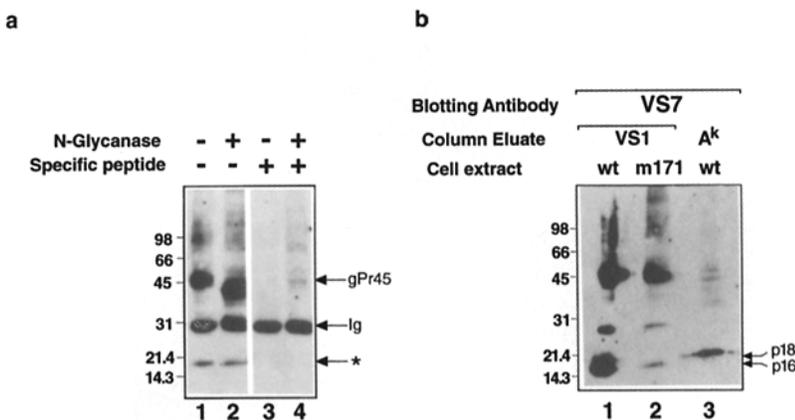


Figure 3. The m171 substitution eliminated proteolytic processing at the proximal cleavage site. (a) vSAG7^{m171} was purified by affinity chromatography, treated with N-glycanase (+), or left untreated (-), as indicated, electrophoresed in a 8–20% SDS-polyacrylamide gradient gel, transferred to blotting membranes, and detected with VS7 in the presence (+) or absence (-) of the p7 competitor peptide. gpPr45 is the full-length high mannose (endo H-sensitive) form of the vSAG7 (4). *The COOH-terminal vSAG7^{m171} cleavage product. Ig, immunoglobulin light chain eluted from the affinity column and detected by the secondary antibodies. (b) Cell lysates from CH12.1/S7 (wt) or CH12.1/S7^{m171} (m171) were chromatographed on affinity columns containing antibodies directed against vSAG7 (VS1) or I-A^k (17/227) as described previously (4). The column eluates were examined by Western analysis using VS7. The positions of the wild type proteolytic cleavage products (p18 and p16) are indicated. Molecular mass markers, in kilodaltons, are indicated at the left of each gel.

vSAG7 are potential substrates for PCs. One PC, furin, has been detected in most mammalian cell types, including thymus and spleen (19, 20), unlike other PCs, which are expressed primarily in neuroendocrine tissues (21, 22). Therefore, experiments were performed to determine if furin could act as a vSAG7-processing endoprotease.

A soluble form of vSAG7 (BvSAG7) was engineered by truncation of the cytoplasmic and transmembrane regions and by introduction of a signal peptide to facilitate secretion. BvSAG7 was expressed in Sf9 cells after infection with recombinant baculoviruses containing the *Bsag7* gene (Fig. 1 C). BvSAG7 was detected in the culture medium by Western analysis using the VS7 antibody. BvSAG7 was secreted from Sf9 cells in three forms: an *N*-glycosylated major product of 36 kD (gp36), a minor *N*-glycosylated product of 33 kD (gp33), and a nonglycosylated proteolytic product, p18 (Fig. 4, lanes 1 and 5). The *N*-glycosylation status of the products were determined using *N*-glycanase (data not shown).

Similar results were obtained in parallel experiments using recombinant baculoviruses that expressed a form of BvSAG7 that contained a peptide epitope at the NH₂ terminus of the secreted protein. The peptide allowed BvSAG7 to be recognized by the mAb 12CA5 (23). Among the three secreted protein products recognized with VS7 antibody, only the major protein product, gp36, was also recognized by 12CA5 antibody (Park, C. G., M. Y. Jung, and Y. Choi, unpublished data). These data indicated that the major product (gp36) was the full-length BvSAG7 protein and the other proteins were proteolytic processing products.

The p18 secreted by the Sf9 cells was similar in size to the COOH-terminal cleavage product of vSAG7 detected in B

cells, and gp33 was hypothesized to originate from proteolytic cleavage at the RARR sequence found at residues 15–18 of the recombinant protein, BvSAG7 (Fig. 1 C). The data suggested that Sf9 cells contain an endogenous furinlike activity, as has been suggested (24), and that BvSAG7 protein can undergo limited proteolytic processing in insect cells.

BvSAG7 was also treated with membrane fractions prepared from Sf9 cells expressing the human furin gene. Under these conditions, the amount of the proteolytic cleavage products p18 and gp33 increased to two- to threefold relative to the full-length gp36 protein (Fig. 4). Similar results were obtained when BvSAG7 was coexpressed with furin in Sf9 cells (Fig. 4, lanes 5–8). Therefore, BvSAG7 was a substrate for furin. The enhanced production of gp33 upon treatment with the furin-containing membrane fractions also suggested that the putative transmembrane cleavage site (RARR) was a substrate for the furin endoprotease.

To identify the COOH-terminal cleavage site recognized by human furin, soluble forms of vSAG7^{m171} (BvSAG7^{m171}) and a second mutant protein, BvSAG7^{m194}, were also expressed in Sf9 cells. BvSAG7^{m194} contains substitutions at residues 193–194 (KR changed to EF) that were predicted to eliminate the distal putative proteolytic processing site. Because the distal processing site did not appear to be recognized by furin in insect cells, it was not possible to examine the effect of the amino acid substitutions on processing at this site. However, BvSAG7^{m194} migrated in a manner similar to BvSAG7, and coexpression with furin greatly enhanced production of the COOH-terminal cleavage product p18 (Fig. 4, lanes 7 and 8). In contrast, p18 was not generated as a proteolytic cleavage product of BvSAG7^{m171}, either in the presence or absence of furin (Fig. 4, lanes 3 and 4 and lanes 9 and 10).

These data suggested that in Sf9 cells, p18 was generated by proteolytic processing at the proximal cleavage site (RKRR) of vSAG7, as in B cells, and this site was a substrate for furin. These results are consistent with the earlier interpretation that vSAG processing occurs in the Golgi region or a post-Golgi compartment (4), where furin has been shown to be localized (25). Furthermore, the results also indicate that the putative distal processing site was not a substrate for furin. This suggests that a processing enzyme other than furin may generate the COOH-terminal cleavage product p16, that the distal site is not accessible to furin in BvSAG7, or that the specificity of furin may differ between insect and B cells. The analyses have not localized the precise site of the distal cleavage event used to generate p16.

The data presented here show that proteolytic processing is required for T cell stimulation and/or surface expression of vSAG7 in B cells. Because full-length vSAG7 has not been unambiguously detected on the cell surface of an APC, it is unknown if vSAG7 will activate T cells in the absence of proteolytic processing. Nevertheless, the data presented here demonstrate that proteolytic processing is a requisite event for viral superantigen presentation by APC.

Earlier work suggested that proteolytic processing at the proximal endoprotease site may enhance binding of vSAG7 to class II MHC (4). vSAG7^{m171} was not proteolytically

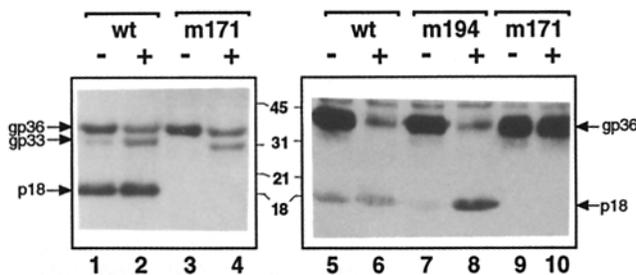


Figure 4. Baculovirus expression of vSAG7 and proteolytic cleavage by the endoprotease furin. (Left) Culture supernatants from Sf9 cells that had been infected with recombinant baculoviruses carrying *Bsag7* or *Bsag7*^{m171} were incubated with membrane preparations obtained from either control baculovirus-infected Sf9 cells (-) or from Sf9 cells infected with viruses carrying the mammalian furin gene (+). The supernatants were electrophoresed in a 8–20% SDS-polyacrylamide gradient gel, transferred to blotting membranes, and detected using VS7. (Right) Sf9 cells were infected with baculoviruses expressing the wild-type or mutant BvSAG7 alone (-) or coinfecting with baculoviruses carrying the mammalian furin gene (+). Culture supernatants were electrophoresed in a 10–20% SDS-polyacrylamide gradient gel, and vSAG7 proteins were detected by Western analysis using VS7. gp36, full length BvSAG7; gp33, BvSAG7 proteolytic cleavage product generated by processing at the putative transmembrane processing site; p18, BvSAG7 product generated by processing at the proximal site. The relative amounts of three protein products, gp36, gp33, and p18, were quantified with the enhanced laser densitometer (Ultrascan XL; LKB Instruments, Bromma, Sweden) and described in the Materials and Methods.

processed to p18, so presumably it does not bind, or binds weakly to class II. Therefore, one possible explanation for the failure to observe surface expression of vSAG7^{m171} is that

class II binding is a prerequisite for efficient surface expression of vSAG in B cells, as has been suggested (3).

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