Alternative Proteolytic Processing of Mouse Mammary Tumor Virus Superantigens

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Mouse mammary tumor viruses express a superantigen essential for their life cycle. It has been proposed that viral superantigens (vSags) require processing by prohormone convertases (PCs) for activity. We now observe, using a panel of mutant forms of potential PC cleavage sites and in vitro cleavage assays, that only the CS1 (position 68 to 71) and CS2 (position 169 to 172) sites are utilized by furin and PC5. Other members of the convertase family that are expressed in lymphocytes are not endowed with this activity. Furthermore, mutant forms of two different viral superantigens, vSag7 and vSag9, which completely abrogated in vitro processing by convertases, were efficient in functional presentation to responsive T-cell hybridomas. This effect was observed in both endogenous presentation and paracrine transfer of the vSag. Processing by convertases thus appears not to be essential for vSag function. Finally, we have identified the purified endosomal protease cathepsin L as another protease that is able to cleave convertase mutant vSag in vitro, yielding fragments similar to those detected in vivo, thus suggesting that proteases other than convertases are involved in the activation of vSags.
sensitive to mutations, resulting in intracellular retention and degradation with loss of biological activity (25). Transfection of vSag7 into furin-deficient CHO cells resulted in much-reduced presentation, but some residual vSag activity remained (27). This activity could be abrogated by treatment with the arginine-specific protease inhibitor leupeptin (27). This inhibitor is inefficient toward convertases (28, 30), raising the possibility that only certain convertases can cleave vSags. Furthermore, we show that while convertases can cleave vSags at the two conserved endoprotease sites, this processing is not essential for functional activity. Finally, alternate proteases such as cathepsin L can substitute for convertases to process vSags to fragments of sizes similar to those detected in vivo.

**MATERIALS AND METHODS**

**Cell lines, transfections, and T-cell stimulation assays.** DAP-DR1 cells are DAP-3 murine fibroblasts transfected with the human DR1 class II molecule (22). CH12, a murine B-cell lymphoma line expressing I-Ek was grown as described previously (8, 30). Enzymes were produced as follows. BSC40 monolayers at 70 to 80% confluence were washed three times with phosphate-buffered saline (PBS), and recombinant vaccinia virus infections (16) were carried out for 30 min at room temperature. Cells were incubated at 37°C for 18 h.

**Presentation.** In order to assess the potential involvement of each of the convertases in the medium, since they can provide a source of convertases in the medium, we expressed a vSag7-responsive hybridoma Kmls 13.11 cells were tested with vSags by different MHC class II-positive antigen-presenting cells (APCs) to their responsive hybridoma cells bearing specific Vβ elements (20, 45). Furin, PACE4, PC5, and PC7 are broadly distributed convertases responsible for processing of proproteins along the constitutive secretory pathway (8, 30, 42). In order to assess the potential involvement of each of these convertases in vSag activation, their expression was verified by RT-PCR in cells used in our vSag functional presentation assays. Positive controls included Art-20 cells, which express all of the known convertases except PC5, and Art-20-PC5 cells, which are Art-20 cells transfected with mouse PC5 (8). vSag7-responsive hybridoma Kmls 13.11 cells were tested since they can provide a source of convertases in the medium, given the knowledge that both furin and PC7 can recycle between the cell surface and Golgi (28, 42). Hence, it was plausible that vSag processing could occur in trans at the APC surface. Figure 1 shows that cells used for vSag presentation

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**TABLE 1. Oligonucleotides used for mutagenesis and RT-PCR analysis**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>5′ CS1..................GGTTCTGTCGCTCCATGGACCTCCATCC</td>
<td></td>
</tr>
<tr>
<td>3′ CS1..................GCTTGGGAGATGCAGGACGAGAA</td>
<td></td>
</tr>
<tr>
<td>5′ CS2..................GAAAGAGACAAAGCAGCCGCTCCCTCC</td>
<td></td>
</tr>
<tr>
<td>3′ CS2..................AGCTCGGGTTGCACCGTTCTGATTTTTT</td>
<td></td>
</tr>
<tr>
<td>5′ CSX..................GAAGGGGAAAAAGTGGTGTGC</td>
<td></td>
</tr>
<tr>
<td>3′ CSX..................GAACACACACTCTTTTTCTTTTC</td>
<td></td>
</tr>
<tr>
<td>vSagHis ..................CTTTGCTGGGCTACTGCGGGAGATGGC</td>
<td></td>
</tr>
<tr>
<td>Furin ..............GGACACAGCTTTCTTGGTCGA</td>
<td></td>
</tr>
<tr>
<td>PACE4 ..................GCATAGAAGAAATCACCAGAA</td>
<td></td>
</tr>
<tr>
<td>PACE4 ..................TTGATGCAATCAGGACGAGAAC</td>
<td></td>
</tr>
<tr>
<td>PCC ..............GTGGTGCCACTGCTGCAATGGTGAGA</td>
<td></td>
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<tr>
<td>PCC ..............TTGTGCTGGCTGTGGCTTCCAC</td>
<td></td>
</tr>
<tr>
<td>PCC ..............CCCCATGATGAGGAGAGATG</td>
<td></td>
</tr>
<tr>
<td>PCC ..............AAAGGAGATCCCTTCTTCA</td>
<td></td>
</tr>
</tbody>
</table>

**Recombinant convertase production.** Recombinant vaccinia virus constructs for convertase production have been described previously (8, 30). Enzymes were produced as follows. BSC40 monolayers at 70 to 80% confluence were washed three times with phosphate-buffered saline (PBS), and recombinant vaccinia virus infections were carried out for 30 min at room temperature. Cells were incubated at 37°C for 18 h. Culture supernatants were centrifuged to remove debris, concentrated 20-fold on Centricron-30 cartridges (Millipore Corp., Bedford, Mass.), and stored at -20°C in 40% glycerol until use. Enzymatic activity was determined by cleavage of the fluorogenic peptide substrate pERTRK-MCA (Peptides International, Louisville, Ky.), and fluorescence was monitored on an LS50B spectrofluorometer (Perkin-Elmer Corp., Norwalk, Conn.).

**In vitro transcription-translation and cleavage assays.** In vitro transcription and translation of vSag7 were carried out using an Escherichia coli S30 system (Promega Corp., Madison, Wis.). In brief, 50-μl reaction mixtures containing 1 μg of DNA and 20 μCi of [35S]methionine (1,200 Ci/mmol; New England Nuclear) were incubated at 37°C for 60 min. Products were batch purified using 20 μl of Ni-nitroliotriacetic acid agarose (Qiagen Inc., Mississauga, Ontario, Canada) and eluted with 50 μl of 1× PBS-200 mM imidazole. For in vitro cleavage with convertases, 10 μl of purified vSag7 was incubated overnight with 1 μ of each convertase in 50 mM Tris·HCl (pH 7.0)–2 mM CaCl2–0.1 mM 2-mercaptoethanol–0.01% Triton X-100. For cathepsin L cleavage, the imidazole was removed by overnight dialysis against 1× PBS on Slide-A-Leez microdialysis cassettes with a 10-KDa cutoff (Pierce Chemical Co., Rockford, Ill.). Ten microliters of labeled vSag7 was incubated with 10 ng of cathepsin L (Calbiochem, La Jolla, Calif.) in 85 mM sodium acetate–15 mM acetic acid–1 mM EDTA–2 mM dithiothreitol (pH 5.5) at 25°C (34). Products were fractionated by sodium dodecyl sulfate (SDS)–15% polyacrylamide gel electrophoresis (PAGE), transferred to polyvinylidene difluoride (PVDF) membranes (Roche Diagnostics), and exposed overnight on PhosphorImager screens (Molecular Dynamics, Sunnyvale, Calif.).

**RESULTS**

**Only furin and PC7 are expressed in the cells used for vSag presentation.** We have previously reported presentation of vSags by different MHC class II-positive antigen-presenting cells (APCs) to their responsive hybridoma cells bearing specific Vβ elements (20, 45). Furin, PACE4, PC5, and PC7 are broadly distributed convertases responsible for processing of proproteins along the constitutive secretory pathway (8, 30, 42). In order to assess the potential involvement of each of these convertases in vSag activation, their expression was verified by RT-PCR in cells used in our vSag functional presentation assays. Positive controls included Art-20 cells, which express all of the known convertases except PC5, and Art-20-PC5 cells, which are Art-20 cells transfected with mouse PC5 (8). vSag7-responsive hybridoma Kmls 13.11 cells were tested since they can provide a source of convertases in the medium, given the knowledge that both furin and PC7 can recycle between the cell surface and Golgi (28, 42). Hence, it was plausible that vSag processing could occur in trans at the APC surface. Figure 1 shows that cells used for vSag presentation...
are approximate amino acid numbers. TM, transmembrane region. At the bottom DAP-DR1; 5, Kmls 13.11. The values on the left are molecular sizes in base pairs. 

FIG. 1. RT-PCR analysis of convertase expression. Using 100 ng of reverse-transcribed total cellular RNA, convertase expression was analyzed by PCR using the primers listed in Table 1. Lanes: 1, ArT-20-PCS; 2, ArT-20; 3, CH12; 4, DAP-DR1; 5, Kmls 13.11. The values on the left are molecular sizes in base pairs.

(DAP-DR1 and CH12) and the vSag7-responsive hybridoma cells only express furin and PC5. Given that the ArT-20-PCS cells have been transfected with PC5 and express nonphysiological levels of PC5 (8), we cannot rule out PC5 expression below the limit of detection of the RT-PCR assay used.

Generation and biochemical characterization of vSag cleavage site mutants. The structure of vSags and the nomenclature used for convertase-processing sites are shown in Fig. 2. While the CS1 (68 to 71) and CS2 (169 to 172) endoprotease sites are conserved in most vSags, the CSX (194 and 195) site is not present in all MMTV sequences (4) and lacks the P4 arginine required for furin-like convertase recognition (29, 42). These convertase cleavage sites were subjected to in vitro mutagenesis (Fig. 2) for biochemical characterization and functional presentation to T cells. The two R→S mutations introduced at CS2 were chosen because a similar change present in the insulin receptor results in extreme insulin resistance (54). This mutated protein is refractory to furin cleavage but can still bind insulin, indicating that protein structure is preserved (54). All of the mutants referred to in this paper are identified by the mutated residues unless otherwise indicated (e.g., in CS12, the CS1 and CS2 sites are mutated while CSX is not).

Biochemical characterization of vSags has always proven difficult due to the low protein levels (12, 49, 50). In order to characterize the exact cleavage sites utilized by the different convertases and demonstrate that the mutations introduced abrogated convertase processing, we resorted to an in vitro approach. The extracellular portion of the mutants was cloned into DAP-DR1, a murine fibroblastic cell line transfected with the DR1 class II molecule (22), and vSag7 was stably transfected in CH12, a murine B-cell lymphoma line. The mutant vSag9s were not introduced into CH12, as these cells express endogenous vSag9 (43). The efficiency of the WT and vSag cleavage site mutants was tested in functional presentation to responsive T-cell hybridomas. Two different hybridoma lines were used: Kmls 13.11, which is responsive to vSag7 but not to vSag9, and VJ5#11, which is responsive to vSag9 but not to vSag7. T-cell hybridoma stimulation was assayed by monitoring IL-2 production. The optimal APC-to-hybridoma cell ratio for presentation of WT vSags was determined to be 1 to 3 (data not shown). Figure 4 clearly shows that neither the CS1 nor the CS2 single mutations had a major effect on functional presentation of vSag9 in DAP-DR1 cells (Fig. 4A) with levels of IL-2 production by the T-cell hybridoma comparable to those obtained following stimulation with transfectedants expressing WT molecules. Similar results were obtained with vSag7 in two different cell lines: DAP-DR1 (Fig. 4B) and CH12 (Fig. 4C). To eliminate the possibility that cleavage at either one of the free convertase sites was activating vSag7 or vSag9, the CS12 double mutants were tested and stimulation was comparable to that obtained with the WT molecules at the optimal ratio for stimulation (Fig. 4A, B, and C).

Mutants encompassing all putative convertase-processing sites can still be presented to T cells. Biochemical analysis using B cells transfected with vSag7 has revealed a 16-kDa fragment that might correspond to processing at CSX (position 194 and 195) (49, 50). This raised the possibility that the presentation seen with the CS12 double mutants might be attributed to cleavage at the CSX position. However, the CSX site is not conserved in all MMTV isolates (4) and lacks a P4 arginine found in typical convertase sites (33), arguing that convertase-mediated processing at this position would be unlikely. Indeed, our data show that this position is not cleaved in vitro by either furin, PC5, or PC7 (Fig. 3E), showing that this motif is not a convertase substrate. To rule out the possibility that the presentation observed with vSag7 and vSag9 CS12 double mutants was due to cleavage at the CSX position, a nonclassical convertase site, it was mutated to a site previously shown to be uncleavable (5). A representative experiment is illustrated in Fig. 5A and shows that DAP-DR1 cells expressing vSag7 CS12X triple mutants, after transient transfection, were only 30% less efficient than the WT in endogenous pre-
sentation assays. This effect was observed at all effector-to-target cell ratios.

It is known that vSags can be transferred from class II$^\text{2}$ to class II$^\text{1}$ cells both in vivo (47, 48) and in vitro (10). Clearly, vSags must be cleaved for such a transfer to occur, so we used the transfer assay, as outlined in Materials and Methods, to verify whether the CS12X triple mutant could still be shed in vitro. Figure 5B shows a representative experiment for transfer presentation between DAP cells transiently transfected with the vSag7 CS12X triple mutant and BJAB. Similar to what was observed with endogenous presentation (Fig. 5A), the CS12X triple mutant was 30% less efficient than the WT in transfer presentation (Fig. 5B). The presentation seen in the transfer assay shows that vSag processing must occur, either because the mutations introduced did not abrogate convertase processing or alternate proteases can cleave vSags. Since the biochemical analysis shows that the mutations introduced abrogate convertase cleavage (Fig. 3E) and the functional data show that convertase mutants can still stimulate T cells, it is clear that convertase processing is not required for vSag activity. The stimulation observed in the transfer assay with the CS12X triple mutant (Fig. 5B) suggests that alternate proteases could be involved in vSag processing.

**Cathepsin L can cleave vSag7 into discrete fragments.** Given that the convertase site mutations abrogate in vitro processing of vSag7 and the mutant molecules are still efficient in functional presentation, we investigated whether other proteases might cleave vSag7. Localization studies have shown that vSags are present in the MIIC endosomal compartments enriched in H2-M and MHC class II molecules (12). Cathepsins are the most abundant lysosomal and endosomal proteases responsible for antigen generation and invariant chain (Ii) degradation. In addition, they are present in MIIC compartments (2, 6). Thus, we investigated whether cathepsins could cleave vSag7. Cathepsin L is a ubiquitously expressed cysteine protease that has trypsin activity (2, 6) and, hence, would be expected to cleave at the dibasic motif conserved among vSags (4). Furthermore, it is inhibited by leupeptin (2), which was previously shown by Mix and Winslow to inhibit the residual vSag activity observed with vSag cleavage mutants. The radio-labeled WT vSag7 and the CS12X triple mutant were subjected to in vitro cleavage with purified cathepsin L, and a fragment of about 27 kDa appeared after cathepsin L digestion (Fig. 6). This 27-kDa fragment should result from cleavage between the CS1 and CS2 sites. Interestingly, a predominant 27-kDa vSag COOH fragment has been previously detected biochemically (12, 26, 49, 50). It is also clear from Fig. 6 that the convertase site mutations did not abrogate cathepsin L processing, raising the possibility that the efficient presentation observed with convertase site mutants might be due to alternate processing by cathepsin L or other related lysosomal or endosomal enzymes.

**DISCUSSION**

We have provided a rigorous analysis of the processing requirements of vSags using two complementary approaches: first, by performing in vitro processing of the cleavage site mutants by recombinant convertases to determine the exact sites utilized by each convertase (Fig. 3); second, by studying the functional presentation of two different vSags using two different types of APCs, including B lymphocytes, the natural host cells for vSag presentation (Fig. 4 and 5). Given that all of the convertase site mutants tested were efficient in functional presentation, alterations of vSag structure can be easily ruled out.
It is evident from the results presented here that cleavage at the CS1 proximal convertase site is not required for vSag activity, in agreement with results obtained by other groups (49, 50). Furthermore, exogenous MMTV-SIM has superantigenic activity (24) but possesses a CS1 site lacking the canonical P4 arginine required for convertase recognition. Given the high level of phylogenetic conservation of this position, it appears likely that this region serves a function different from the one relevant to superantigenic activity.

It is clear from our in vitro cleavage assays that the dibasic CSX site is not a substrate for convertases (Fig. 3E). This confirms and extends a previous report that showed that furin could not cleave at the CSX position (37). The 16-kDa COOH-terminal fragment previously detected biochemically (49, 50) and assumed to arise from cleavage at the CSX position would thus be derived from cleavage by another protease. This is supported by our data showing that the CS12X triple mutant, which cannot be cleaved by convertases, can stimulate T cells in the transfer assay (Fig. 5B), where cleavage is expected to be required.

The data presented here show that convertase-mediated processing at the CS2 site is not essential for presentation of vSag9 and vSag9 to T cells. While the CS2 mutants were efficient in functional presentation (Fig. 4), the mutations introduced completely abrogated convertase cleavage (Fig. 3E). This is in sharp contrast to a report suggesting that processing at CS2 is essential for presentation (37). It is likely that the mutations introduced in that study (RKRR → GEEF) have altered the structure of the superantigen, leading to intracellular retention or degradation since this mutant was not expressed at the cell surface (37). In support of this possibility is a report that showed that several different point mutations abolished...
vSag cell surface expression and presentation because of retention in the endoplasmic reticulum (25). Processing at the CS2 position appears to occur in vivo, given that the 18-kDa fragment detected by Western blot analysis corresponds to cleavage at that position (49, 50), and Fig. 3C shows that an 18-kDa fragment is generated by both furin and PC5. However, the presence of such a fragment does not prove that processing at that position is required for vSag activity.

Using a mutant furin-deficient CHO cell line transfected with vSag7 and the murine MHC class II molecule IEd, Mix and Winslow (27) have shown that reintroduction of furin could increase vSag presentation to T cells, arguing that furin participates in generating active vSag. Nevertheless, the residual vSag activity in these furin-deficient cells could only be inhibited by leupeptin (27), an inhibitor inefficient toward the subtilase-type convertases (28, 30). This suggested that alternate proteases might contribute to the activation of vSags. Recently, more conservative mutations of the putative CS2 and CSX sites have been introduced by inserting sequences naturally occurring in MMTV isolates (51). Using WT CHO cells as APCs, these authors showed that removal of the CS2 site (while keeping a WT CSX site) abolished presentation and that cell surface expression was readily detectable (51). The discrepancy between these reports proposing an essential requirement for convertase-mediated vSag processing and ours might be due to differences in the cell lines used. While cathepsins are ubiquitously expressed (38), their function in antigen processing is tissue dependent (32).

We do not exclude the possibility that convertase-mediated processing generates the active superantigen, but we propose that alternative proteases like cathepsins can substitute for this set of proteases. Alternatively, it is possible that dibasic sequence-specific convertases might activate a cell surface enzyme which is critical for cleavage of vSag. Possible candidates include surface mammalian proteins containing a disintegrin and metalloprotease domains (ADAMs) (52), such as ADAM-17 (tumor necrosis factor alpha-converting enzyme) (3) and ADAM-10 (Kuzbanian) (40). Another argument for alternate processing resides in experiments aimed at biochemical characterization of vSags. The major vSag COOH-terminal cleavage product detected in B cells, after N-glycanase treatment to remove glycosylation, has a molecular mass of 27 kDa (12, 26, 49, 50). This 27-kDa COOH-terminal product would correspond to cleavage between the CS1 and CS2 positions. Given that in vitro processing by convertases was not observed in this region, alternate proteases must be involved in vSag processing.

Little is known about vSag intracellular trafficking, mainly because of major technical difficulties in detecting the protein. The protein appears to be highly unstable (19) and targeted for degradation in the endoplasmic reticulum if mutations are introduced (25) or glycosylation is perturbed (26). It has been proposed that vSags and MHC class II molecules might interact in the endoplasmic reticulum and traffic together to the cell surface (49, 50). Such a hypothesis was also supported by the fact that increasing MHC class II levels has a much more profound effect on T-cell stimulation than increasing vSag levels (23). However, it has been reported that vSags traffic independently of MHC class II molecules and traffic together to the cell surface (49, 50). The presence of such a fragment does not prove that processing at that position is required for vSag activity.

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