Brief Definitive Report

Proteolytic Processing Activates a Viral Superantigen

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Summary

Mouse mammary tumor virus (MMTV) superantigens (vSAg) undergo proteolytic processing at residues that have been demonstrated in vitro to be recognition sites for the endoprotease furin. To examine the role of furin in the presentation of vSAg7 to T cells, the vSAg7 and class II MHC IE^k genes were introduced into Chinese Hamster Ovary (CHO) cells (furin-positive) and into a furin-negative CHO variant (FD11). Both transfected cell lines efficiently presented peptide antigen and bacterial superantigens to T cell hybridomas. However, while the furin-positive cells presented vSAg7 well, the furin-negative cells presented poorly. Transient transfection of the furin-negative cells with an expression plasmid containing the *furin* gene restored the ability to present vSAg7 efficiently. The marginal presentation of vSAg7 observed using the furin-negative transfectants was eliminated after culture with the protease inhibitor leupeptin, suggesting that one or more endoproteases other than furin have a detectable but limited capacity to proteolytically activate vSAg7. Biochemical analyses revealed that vSAg7 was largely unprocessed in the absence of furin. Thus, viral superantigens, unlike bacterial superantigens, require proteolytic processing to activate T cells.

Superantigens interact with class II MHC proteins and stimulate T cells largely on the basis of their specificity for particular beta chain variable regions (Vβ) of the T cell receptor (1). Unlike conventional class II MHC peptide antigens, which are generated by proteolysis, superantigens are not thought to require proteolytic processing for presentation to T cells. Although this lack of processing appears to be characteristic of bacterial superantigens, MMTV superantigens (vSAg) have been detected on the surface of APC in a proteolytically processed form (2, 3). Proteolytic processing of vSAg7 has been observed to occur at as many as three positions that correspond to the locations of basic amino acid motifs that are recognition sequences for a family of mammalian processing endoproteases known as convertases (4–6). Convertases typically cleave after sequences containing the amino acid motif RXK/RR, and are responsible for the processing of cellular and viral precursor proteins in both the constitutive and regulated secretory pathways (7–10).

Phylogenetic comparison of vSAgs has revealed a high degree of conservation of the putative proteolytic processing sites (11, 12), and has suggested that processing is important for activity. Mutagenesis of the convertase recognition motif at position 168-171 in vSAg7 blocked the generation of one processing product (13). The mutated vSAg7 was not expressed at the cell surface, however, so it was not determined whether the unprocessed vSAg could recognize T cells had it been expressed on the cell surface.

To clarify the role of proteolytic processing, vSAg7 presentation was examined using APC that were deficient for the convertase furin. Furin is a candidate superantigen convertase because it is expressed ubiquitously, unlike most convertases (14). In addition, furin has been shown to process vSAg7 in vitro at two of the three candidate processing sites (13). To examine the role of furin in vSAg7 activation, the vSAg7 and class II IE^k genes were introduced into CHO cells, and into a furin-negative variant, FD11 (15). vSAg7 presentation was greatly reduced in the furin-negative cell line, and the residual activity was eliminated by the addition of the protease inhibitor leupeptin. Biochemical analyses revealed that vSAg7 was largely unprocessed in the absence of furin. Thus, the data confirm that proteolytic processing is essential for vSAg7 presentation, and implicate furin as a candidate in vivo vSAg convertase.

Materials and Methods

Biochemicals. The moth cytochrome c (mcc 88-103) peptide was synthesized at the Wadsworth Laboratories. Staphylococcal enterotoxin A (SEA) was from Sigma Chemical Co. (St. Louis, MO).

Plasmids and Transfections. The vSAg7 expression plasmid pSRαvSAg7 was generated by cloning the EcoRI-BamHI frag-

ment from pMtv7 c-2 (a gift of Dr. Y. Choi, Rockefeller University) into the plasmid pSRαpuro (a gift of Dr. F. Denis, Institut de Recherches Cliniques de Montréal, Montréal, QP, Canada). The $IE^k\alpha$ chain expression plasmid pEXVEα has been described (16). The $IE^k\beta$ chain expression plasmid phBATr-1-neo was a gift of Dr. S. Hedrick (University of California, San Diego, CA). The furin expression plasmid pMT-PACE has been described (17, 18).

The CHO and FD11 class II IE^k transfectants (CHIE; FDIE) were generated using 10 μg each of the IEα and IEβ expression plasmids, and 15 μl Lipofectamine (Life Technologies, Grand Island, NY) for 4 h in serum-free medium, followed by incubation in complete medium overnight. The cells were plated at 1×10^4 cells/well in 24-well culture plates for 24 h before the addition of G418 (1 mg/ml; Life Technologies). Viable cells were analyzed and cloned by limiting dilution. The vSAg7 transfectants CHIE/S7 and FDIE/S7 were generated by secondary transfection of CHIE and FDIE with pSRαvSAg7, followed by selection with 25 μg/ml puromycin (Sigma). The transient transfections were performed in an identical manner except that the cells were analyzed within 24 h.

Antibodies. The anti-vSAg antibodies VS7 and VS1, and the anti-IE antibody 14-4-4, have been described (2, 19).

Flow Cytometry. Flow cytometry was performed using a FACScan® flow cytometer (Becton Dickinson & Co., Mountain View, CA). Before analysis, cells were treated with 0.25% trypsin/1 mM EDTA in HBSS, washed, and incubated in Complete Tumor Medium for 4 h at 37°C to allow surface re-expression of vSAg7.

Cell Lines. All cells were cultured in Complete Tumor Medium, composed of Eagle's MEM supplemented with 10 μM 2-mercaptoethanol, 10% fetal calf serum, 10% Mishell-Dutton Nutrient cocktail (20), 100 μg/ml gentamycin, 100 U/ml penicillin G, and 200 μg/ml streptomycin sulfate (Sigma). CHO cells and the furin-negative cell line FD11 have been described (15). The T cell hybridomas Kmls13.11, K25-59.10, 5KC-73.8/51.6, and the vSAg7 expressing B cell lymphoma CH12/S7 have been described (2, 21). Other T cell hybridomas used in this study were generated from BALB/c T cells after incubation for four days with the vSAg7 expressing cell line CHIE/S7, followed by 4 d incubation with murine IL-2 (625 U/ml). The T cell blasts were fused to the thymoma $BW\alpha^-\beta^-$ (22) using standard methods, and cloned by limiting dilution. $BW\alpha^-\beta^-$, X63-IL-2, and HT-2 cells were generously provided by Drs. J. Kappler and P. Marrack (Howard Hughes Medical Institute, Denver, CO).

T Cell Stimulation Assays. Assays were performed using 1×10^5 cells each of a hybridoma and an APC. After 16–24 h supernatants were assayed for IL-2 using the indicator cell line HT-2 (23). 24 h after the addition of the HT-2 cells [^{3}H]thymidine was added (100 μCi/ml; Amersham Corp., Arlington Heights, IL), the cultures were incubated for an additional 8–16 h, and thymidine incorporation was measured using a Betaplate Liquid Scintillation Counter (Wallac Instruments, Gaithersburg, MD). IL-2 units were measured by comparison with murine IL-2 obtained from supernatants of the cell line X63-IL-2. Data were analyzed using the computer program MKSET (Dr. J. Kappler, Howard Hughes Medical Institute, Denver, CO).

Protein Chemistry. Surface radiolabeling, lysate preparation, and immunoprecipitations have been described previously (2). Antigen was eluted from the Sepharose beads using 1 ml of 50 mM diethylamine (pH 11.6), lyophilized, and electrophoresed in 8–20% gradient polyacrylamide gels. Gels were dried and autoradiographed for 5 d at −70°C using Hyperfilm ECL (Amersham) and an intensifying screen.

Results

vSAg7 Presentation by Furin-negative CHO Cells. To study the presentation of vSAg7 in the presence and absence of furin, CHO (furin-positive) and FD11 (furin-negative) cells were transfected with plasmids encoding the class II IE^k α and β chains. IE^k-positive transfectants underwent a second transfection with the vSAg7 expression plasmid pSRαvSAg7, and puromycin-resistant cell lines were recovered. The doubly-transfected lines were tested for their ability to stimulate the vSAg7-reactive hybridoma Kmls13.11 (Vβ6). 60% of the furin-positive transfectants (CHIE/S7; $n = 20$), but none of the furin-negative transfectants (FDIE/S7; $n = 13$), presented vSAg7 (data not shown). Several cell lines were cloned by limiting dilution before further analysis. Surface expression of IE^k and vSAg7 on the transfectants was confirmed using flow cytometry (Fig. 1). The FDIE/S7 transfectants exhibited slightly reduced surface expression of IE^k, and two- to fourfold lower levels of vSAg7, relative to CHIE/S7 (Fig. 1 and data not shown).

Both the furin-positive and furin-negative transfectants were capable APC, as shown by their ability to efficiently stimulate IL-2 production from a moth cytochrome c (mcc)-reactive T cell hybridoma upon addition of mcc (88-103) peptide, and their ability to present SEA to a Vβ3-bearing T cell hybridoma (Fig. 2 *a*). However, in contrast to the results described above, the data generated using the cloned APCs indicated that weak presentation of vSAg7 did occur in the absence of furin (Fig. 2 *b*). In general, responses to vSAg7

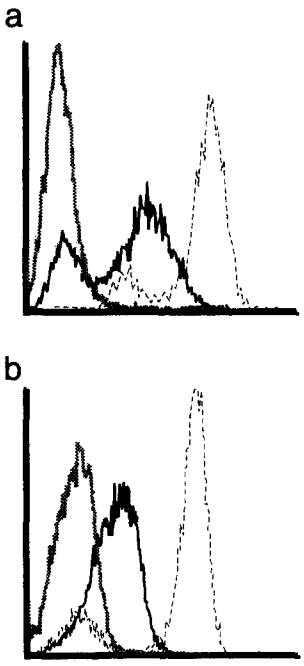

Figure 1. Surface expression of vSAg7 and IE^k. Adherent cells were isolated after treatment with trypsin/EDTA, allowed to incubate in medium at 37°C for 4 h to allow re-expression of the vSAg, and stained with biotinylated VS7 (vSAg7-specific; *solid lines*), or biotinylated 14-4-4 (IE-specific; *dashed line*) followed by PE-coupled streptavidin, or with PE-coupled streptavidin alone (*dotted lines*), and analyzed by flow cytometry. (*a*) The furin-positive transfectant CHIE/S7-16.4. The mean peak fluorescent intensities (MPFI) were 2.8 (PE control), 60.4 (VS7 positive cells), and 542 (14-4-4 positive cells). (*b*) The furin-negative transfectant FDIE/S7-2.1. MPFIs were 6.5 (PE control), 24.7 (VS7 positive cells), and 307 (14-4-4 positive cells). The ordinate represents the relative fluorescence (log scale) and the abscissa the relative cell number.

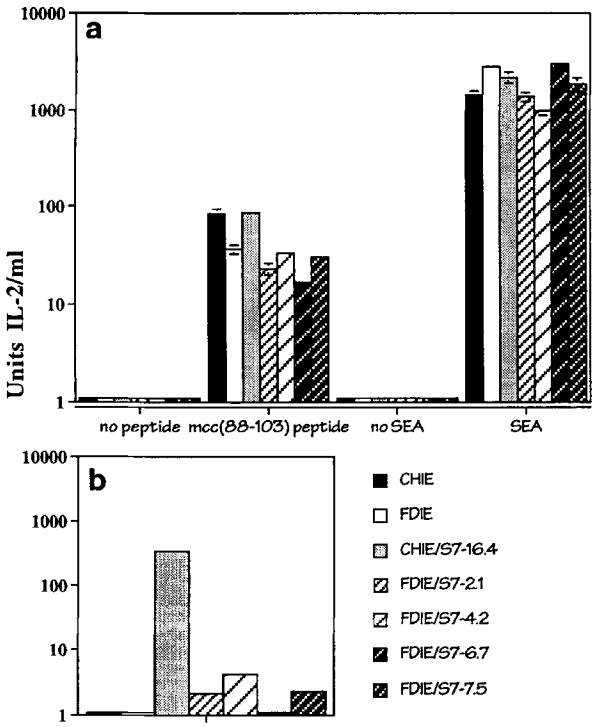

Figure 2. Presentation of antigen and superantigen by furin-negative cell lines. (*a*) T cell hybridomas were incubated with furin-positive (CHIE, CHIE/S7) and furin-negative (FDIE, FDIE/S7) APCs (1×10^5 each), with or without the addition of moth cytochrome c (88-103) peptide (mcc; 10 μM), or SEA (10 μg/ml). The T cell hybridomas were 5KC-73.8/51.6 (Vβ3; moth cytochrome-specific) and K25-59.10 (Vβ3; SEA-reactive). CHIE and FDIE express IE^k in the absence of vSAg7. Assay was for IL-2 production, as measured by assay for [^{3}H]thymidine incorporation by the IL-2-dependent cell line HT-2. Each histogram represents the average determination from triplicate cultures. Error bars indicate the standard deviation of the mean, where greater than 10%. (*b*) The T cell hybridoma Kmls13.11 (Vβ6) was incubated with the indicated APC, and IL-2 production was measured, as in *a*.

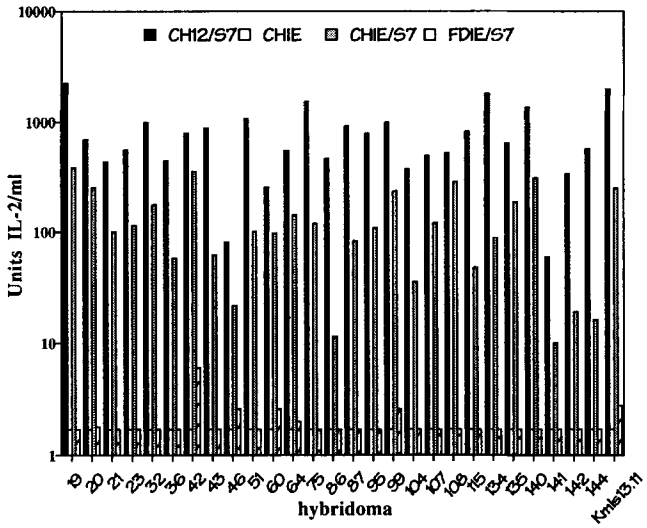

Figure 3. T cell hybridomas are deficient in their ability to recognize vSAg7 in the absence of furin. BALB/c T cells were cultured for 4 d with CHIE/S7, transferred to media containing IL-2 for 4 d, and fused to the thymoma BWα$^-$β$^-$. The T cell hybridomas (Omls, numbered as shown) were tested for reactivity against the indicated APC. CH12/S7 is a B cell lymphoma vSAg7 transfectant ($H\text{-}2^k$).

presented by the furin-negative cell lines were 5- to 10-fold lower than responses elicited by the furin-positive APC.

Several other T cell hybridomas were tested for responses to vSAg7 presented by the CHO cells. Most available T cell hybridomas, however, failed to detect vSAg7 on CHIE/S7. To circumvent this problem, hybridomas were generated that responded to vSAg7 presented by CHIE/S7. IL-2 production from a representative panel of hybridomas is shown in Fig. 3. Although responses to CH12/S7 and to CHIE/S7 were readily detected, responses to FDIE/S7 were either undetectable or very low (less than 6 U IL-2/ml). Of the 27 new hybridomas, only four generated detectable levels of IL-2. Thus, the inefficiency in the presentation of vSAg7 by the furin-negative cells was readily generalized upon examination of additional hybridomas.

Reintroduction of the Furin Gene Restored vSAg7 Presentation. To determine if the defect in the FDIE/S7 was indeed due to a lack of furin activity, the *furin* gene was reintroduced into FDIE/S7 by transient transfection. Transient furin expression restored significant vSAg7 activity to FDIE/S7, as detected by the response of five different hybridomas (Fig. 4). Surface expression of vSAg7 was not upregulated after reintroduction of furin (data not shown). The data indicated that the defect in presentation by the FDIE/S7 transfectants was due to a lack of furin activity, and suggested that proteolytic processing by furin played an important role in vSAg7 presentation. The low levels of vSAg7 activity observed in the furin-negative antigen presenting cells suggested either that vSAg7 retained some activity in the absence of proteolytic processing, or that proteases other than furin also processed and activated vSAg7.

Leupeptin Inhibited the vSAg7 Activity in Furin-negative Cells. To determine if leupeptin-sensitive proteases were responsible for the activity detected in FDIE/S7, assays were performed in the presence or absence of the protease inhibitor leupeptin. Furin activity is unaffected by leupeptin (9). Leupeptin treatment eliminated the residual vSAg7 activity detected by Omls42.6, but had no effect on presentation by CHIE/S7 (Fig. 5 *a*), or on SEA presentation (data not shown). The effect of leupeptin was dose and time dependent (Fig. 5 *b*). The failure to detect any activity under conditions of optimal leupeptin treatment indicated that vSAg7 was not presented in the absence of proteolytic processing. The data strongly suggested that proteolytic processing was necessary for vSAg7 presentation to T cells. The identity of the leupeptin-sensitive protease(s) has not been determined.

Minimal Processing of vSAg7 in the Absence of Furin. To determine if the antigen presentation deficiency in FDIE/S7 was due to a failure to process vSAg7, a biochemical analysis was performed. CHIE/S7 and FDIE/S7 cells were surface radiolabeled with ^{125}Iodine, and vSAg7 was precipitated using the antibody VS1, which recognizes the amino

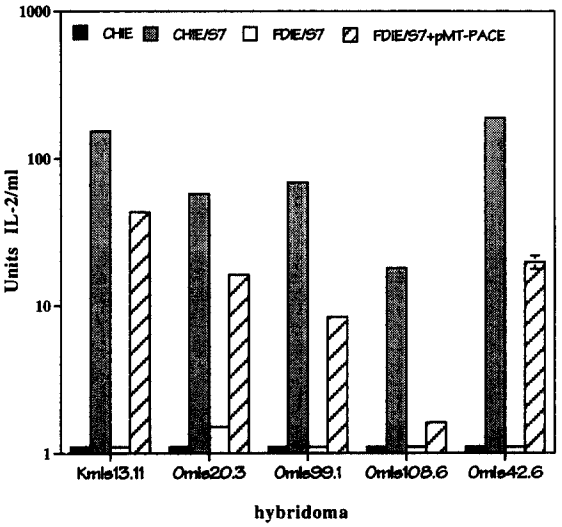

Figure 4. Presentation of vSAg7 is restored by reintroduction of the *furin* gene. FDIE/S7 was transfected with 10 μg of the furin expression plasmid pMT-PACE (*cross-hatched histograms*), or mock-transfected (*open histograms*) 24 h before addition of hybridoma cells. T cell hybridomas (numbered as in Fig. 3, followed by the clone designation) were tested for responses to the indicated APC. Each histogram represents the average determination from triplicate cultures. The standard deviation of the mean was less than 10% for all samples.

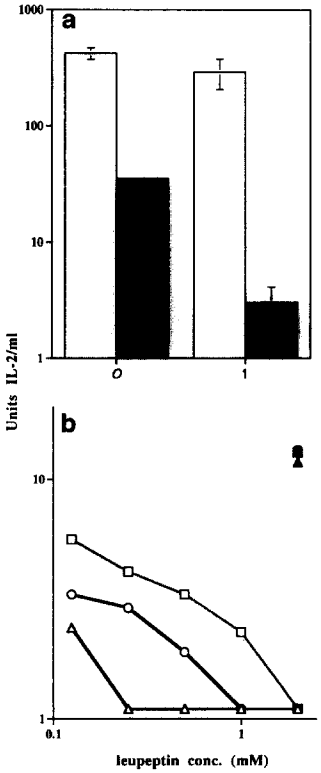

Figure 5. Treatment of furin-negative cells with leupeptin eliminated vSAg7 activity. (*a*) APC were untreated, or incubated with leupeptin for 8 h before and subsequent to the addition of the T cell hybridoma Omls42.6. Filled histograms: CHIE/S7; open histograms: FDIE/S7. The data represent the mean and standard deviation of three determinations. (*b*) Dose response. Antigen presenting cells were treated without (*closed symbols*), or with leupeptin (*open symbols*) at the indicated concentrations, for 24 h (*triangles*), 8 h (*circles*), and 4 h (*squares*) before and after the addition of the T cell hybridoma Omls 42.6. Each data point represents a single determination. The transfectant cell lines were routinely passaged with trypsin/EDTA. However, the vSAg7 activity detected with FDIE/S7 was not an artefact of trypsinization, because the SAg activity could be detected when the cells were disrupted using only EDTA, or when disruption was performed mechanically (data not shown).

terminus of all vSAgs. In previous experiments, the radiolabeled carboxy-terminal processing product was bound noncovalently to the amino terminus (3) and was precipitated by VS1. The previously characterized 18.5-kD carboxy-terminal processing product was readily precipitated from lysates of CHIE/S7, but was nearly undetectable in lysates of FDIE/S7 (Fig. 6). The majority of the vSAg7 detected in FDIE/S7 migrated with a mobility in the range of 105–120 kD, and represented the unprocessed vSAg7. Other vSAg7 proteins detected on CHIE/S7 represent amino-terminal proteolytic cleavage products, or perhaps some unprocessed vSAg7 (Fig. 6).

Discussion

On the basis of the data presented, it can be concluded that proteolytic processing is necessary for activation of T cells by vSAg7. In the absence of furin most T cell hybridomas were unable to respond to vSAg7, and the low level responses detected with some hybridomas were completely inhibited by addition of leupeptin. Reintroduction of the *furin* gene into the furin-negative cells rescued vSAg7 presentation, indicating that the failure of the furin-negative cells to present vSAg7 was indeed due to a loss of furin activity. It has not been formally demonstrated that rescue of vSAg7 presentation was accompanied by restored proteolytic processing. However, furin is a well-characterized protein convertase, and has been demonstrated to process vSAg7 in vitro, so the most likely explanation is that reintroduction of the *furin* gene restored vSAg activity through proteolytic processing. Biochemical analyses revealed a lack of vSAg7 processing in the furin-negative cells, because nearly all of the detectable vSAg7 migrated with a high electrophoretic mobility (>105 kD). However, because a convertase recognition sequence is found in vSAg7 at position 68-71, vSAg7 processed only at this amino-terminal position would not have been detected after immunoprecipitation with VS1. The data are nevertheless consistent with the simplest explanation, that only very limited processing occurred in the absence of furin.

In a previous study it was also concluded that proteolytic processing was required for vSAg activity (13). Analysis of a mutant form of vSAg7, in which the convertase recognition sequence at position 168-171 was destroyed, revealed that the mutant protein was not transported to the cell surface in the B cell lymphoma CH12.1. In contrast, the surface expression of unprocessed vSAg7 observed in the furin-negative cells indicated that proteolytic processing was not required for surface expression, nor did reintroduction of furin upregulate vSAg7 surface expression. It is possible that the defect in intracellular transport observed in studies of the mutated vSAg7 was due to the amino acid substitutions, which may have interfered with surface expression. Alternatively, the requirements for surface expression of vSAg7 may differ between B cells and CHO cells.

Although the requirement for proteolytic processing has been demonstrated here for vSAg7, it is likely that this requirement exists for all viral superantigens. Most vSAg contain consensus convertase recognition sequences at positions 68-71, 168-171, and 190-194 (numbering as in vSAg7). However, it is not known at which positions processing must take place for vSAg activation to occur. Not all vSAg

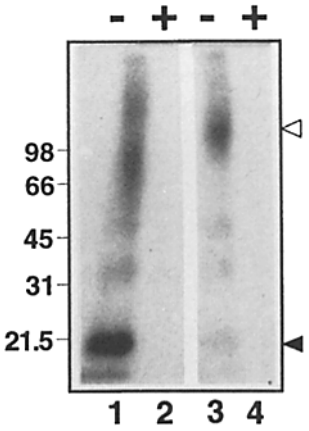

Figure 6. vSAg7 is largely unprocessed in furin-negative cells. Furin-positive (CHIE/S7, lanes *1* and *2*) and furin-negative (FDIE/S7, lanes *3* and *4*) cells (5×10^7) were surface radiolabeled with 0.5 mCi ^{125}Iodine, lysed in PBS containing 1% NP-40 and protease inhibitors, and vSAg7 was precipitated with the anti-vSAg antibody VS1. −/+ refers to the absence or presence of VS1-specific competitor peptide during the precipitation. Solid arrow indicates the carboxy-terminal proteolytic cleavage product (lane *1*); open arrow indicates the presumptive unprocessed vSAg7 (lane *4*).

proteins in an individual cell undergo processing at all possible recognition sites. Thus, processing may generate several forms of vSAg, and the differentially processed vSAg may function as superantigens, or perhaps perform yet other roles in viral pathogenesis (24).

The data also suggest a role in vivo for furin as a vSAg convertase. This does not suggest, however, that other convertases might not also process vSAg. For example, PACE4, and the newly discovered PC7 (25), are also expressed in cells that are known to express vSAg.

The biochemical data revealed that in the absence of proteolytic processing, vSAg7 migrated in SDS polyacrylamide gels with a mobility of 105–120 kD. The apparent size is even greater than that detected previously in B cells, where vSAg7 was observed to migrate at ~82 kD (3), and may be due to cell type differences. The size heterogeneity of vSAg7 has been shown to be due at least in part to the addition of N-linked carbohydrate. The core molecular weight of vSAg7 is 37 kD, so posttranslational modifications may contribute as much as 80 kD to the mature protein. The high levels of carbohydrate may be important for folding or intracellular trafficking, because mutant vSAgs that carried a reduced amount of N-linked oligosaccharide exhibited impaired surface expression (McMahon, C., L. Bogatzki, and A. Pullen, manuscript submitted for publication).

Why do vSAgs require processing for activation? Proteolytic processing is generally used by cells and by pathogens to regulate biological activity. For example, processing of influenza hemagglutinin reveals a peptide that binds to a cell surface receptor and facilitates virus fusion (26). Previous experiments have suggested that processed vSAg7 may have a higher affinity for class II proteins than the unprocessed vSAg (3), so proteolytic processing may in a similar manner reveal a binding site on vSAg for class II MHC proteins.

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