Superantigen Trafficks Independently of Major Histocompatibility Complex Class II Directly to the B-Cell Surface by the Exocytic Pathway

MICHAEL E. GRIGG,†‡ CHRISTOPHER W. MCMAHON,† STANISLAW MORKOWSKI,‡ ALEXANDER Y. RUDENSKY,†‡ AND ANN M. PULLEN†‡* 

Howard Hughes Medical Institute† and Department of Immunology,‡ University of Washington School of Medicine, Seattle, Washington 98195

Received 13 October 1997/Accepted 22 December 1997

Presentation of the Mtv-1 superantigen (vSag1) to specific Vβ-bearing T cells requires association with major histocompatibility complex class II molecules. The intracellular route by which vSag1 trafficks to the cell surface and the site of vSag1-class II complex assembly in antigen-presenting B lymphocytes have not been determined. Here, we show that vSag1 trafficks independently of class II to the plasma membrane by the exocytic secretory pathway. At the surface of B cells, vSag1 associates primarily with mature peptide-bound class II αβ dimers, which are stable in sodium dodecyl sulfate. vSag1 is unstable on the cell surface in the absence of class II, and reagents that alter the surface expression of vSag1 and the conformation of class II molecules affect vSag1 stimulation of superantigen reactive T cells.

T lymphocytes respond to peptide antigens presented by either major histocompatibility complex (MHC) class I or class II molecules. Many viruses have evolved sophisticated strategies that interfere with antigen presentation by infected cells in order to escape recognition by T lymphocytes. Most strategies studied rely on disrupting MHC class I presentation, either by affecting components of the processing machinery that generate and transport viral peptides into the endoplasmic reticulum (ER) or by retarding transport or targeting class I molecules into the degradation pathway (for a review, see reference 73). In contrast, mouse mammary tumor virus (MMTV) utilizes T-cell stimulation to promote its life cycle. MMTVs encode into the degradation pathway (for a review, see reference 73). It is thought that an 18-kDa C-terminal fragment binds MHC class II products (75). It has also been suggested that vSags associate weakly with class II in the ER and that proteolytic processing is required for the efficient assembly of vSag-class II complexes for presentation to T cells (46, 49, 75). As yet, the intracellular route that vSags take to the cell surface, the compartment in which they bind class II, and whether they associate with peptide-loaded class II dimers have been enigmatic.

Newly synthesized MHC class II αβ heterodimers assemble with invariant chain (Ii), a type II integral membrane protein, to form an oligomeric complex in the ER (37). Ii prevents class II heterodimers from binding peptides in the ER and Golgi complex (55), and signals in its cytoplasmic tail sort the complex into the endocytic pathway (4, 42). In this acidic, protease-rich compartment, Ii is degraded and class II binds antigenic peptides. After the formation of peptide-class II dimers, the complexes are exported to the plasma membrane (8, 48). In the absence of Ii, class II αβ heterodimers exhibit defective post-ER transport, and their conversion into functionally mature, sodium dodecyl sulfate (SDS)-stable compact dimers by peptide antigens is affected (7, 16, 22, 70).

A specialized endosomal compartment where class II peptide loading occurs, termed the MHC class II-enriched compartment (MIIC or CIIV), has been found recently in antigen-presenting cells (2, 50, 53, 58, 68, 71). Whether nascent Ii-class II complexes traffic directly to the MIIC from the trans-Golgi network (TGN) or transit first to early endosomes, either directly or via the cell surface, before entering late endocytic vesicles and MIIC is still under debate (26, 56, 57). Transport by all these routes most probably occurs to ensure the capture and loading of antigenic peptides throughout the endocytic pathway (12). MIIC vesicles are enriched for lysosome-associated membrane proteins (LAMPS) and cathepsin D and are under debate (18, 32, 59), proteins that facilitate the catalytic exchange of class II-associated invariant peptide chain (CLIP) for antigenic peptides (19, 61, 62). The
ultrastructural colocalization of DM with intracellular peptide-class II complexes suggests that the MIIC is a main site where class II dimers bind exogenous and endogenous peptide antigens (47, 58).

Determining the route by which Vsg protein(s) trafficks to the cell surface and the cellular location where VsgI processing and assembly with class II molecules occurs is central to understanding the mechanism whereby Vsg activate T cells to maintain the viral life cycle. It has been unclear whether Vsgs traffic independently by the constitutive exocytic pathway or with class II and MHC to the MIIC before reaching the cell surface. Reagents that alter class II expression have been shown to affect Vsg presentation (43, 46). Furthermore, mice lacking Ig show reduced intrathymic Vp-specific T-cell deletion (70), suggesting that Ig play a role, either by ensuring proper degradation of class II dimers or by targeting Vsg-class II complexes to the MIIC, in promoting efficient Vsg-induced immune responses.

To investigate these issues, we used immunochemical detection of VsgI protein in combination with subfractional separation and surface reexpression assays. We show that class II is required for stable VsgI surface expression. VsgI trafficks directly to the cell surface independently of class II, and reagents that alter the conversion of newly synthesized class II into peptide-loaded SDS-stable dimers affect functional VsgI surface expression.

**MATERIALS AND METHODS**

**Cell lines.** The mouse B-cell lymphoma CH27 cell line (H-2K d) transfected with Mtv-1 Sag was described previously (45). Transfection of the M12.C3 cell line (23) with the Mtv-1 Sag under neomycin selection and the E k gene under bygromycin selection was carried out as described previously (45). The TI-Ia a cell line was obtained from P. Cresswell, Yale University. The T-cell hybridomas reporting lysozyme by assaying for the following markers: plasma membrane by labelling cells at 4°C with antibodies. The following antibodies were used: monoclonal antibody (MAB) IN-1 recognizing mouse Ig (35); MAB VS1 recognizing the N terminus of all Vsgs (75); rabbit anti-VsgI serum P2 recognizing a C-terminal peptide unique to VsgI (45); rabbit anti-VsgI serum (goat) raised against gel-purified baculovirus-expressed VsgI (9), a kind gift from J. Butel; MAB ABL-93 specific for mouse LAMP-2, obtained from the Developmental Studies Hybridoma Bank; rabbit anti-rab7 serum recognizing the C terminus of canine rab7 (53), a kind gift from A. Wadinger-Ness; rabbit polyclonal sera specific for the cytoplasmic tail of mouse MHC class I (RS0, (51)); and rabbit anti-I E5 specific for K553 recognizing mouse H-2M (20), a kind gift from L. Karlsson; MAB TR-310 recognizing mouse V 7 (HB 219); MAB H57-597 recognizing mouse pan-TCR a (HB 218); and MAB 145-2C11 recognizing mouse CD3 (59). The MHC class I- and II-specific antibodies used were anti-K a AF3-12.13 (HB 160), anti-I A 3 10-2.16 (TIB 93), anti-I A 3 H116/32.R5 (27), anti-I E5 14-4-4S (HB 32), and anti-I E5 17-3-3S (HB 6). All MABs were purified from culture supernatants by protein G-Sepharose chromatography, and some were biotinylated for use in flow cytometry. Western blot, or enzyme-linked immunosorbent assay (ELISA) analyses. The following MABs were purchased from Pharmigen as biotin or fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (IgG) Fab′ (Boehringer Mannheim). All MABs used for staining the surface of cells were coupled with either goat anti-FITC, Phycocyanin-conjugated streptavidin (Tago, Inc.) was used to detect biotinylated MABs.

**T-cell stimulation assays.** Stimulation assays were carried out as described previously (45). Some assays were performed in the presence of purified anti-class II antibodies at a concentration of 5 mg/ml. Cells were cultured over a time course of 5 h at 37°C to allow for surface reexpression, some in the presence of the following inhibitors: 1 mg/ml leupeptin, 50 mg/mL chloroquine (Sigma), 2 mg of brefeldin A (BFA; ICN) per ml, or 10 mg of cytochalasin D (Sigma) per ml. After culture, the cells were centrifuged, resuspended in ice-cold fluorescence-activated cell sorter (FACS) buffer, and processed for cytofluorimetric analysis as above. The percent recovery was calculated by counting the mean channel fluorescence at each time point, subtracting the mean channel fluorescence of the irrelevant isotype- and species-matched antibody or plus-peptide control, and dividing the resulting value by the mean fluorescence intensity of the same antibody combination from the mock-transfected cells.

**Immunoprecipitation and Western analysis.** CH27, TI-Ia a or Mtv-1 Sag transfected using chloroquine-resistant Mycobacterium bovis (Bacillus Calmette-Gue rin) (BCG) attenuated H37Rv rotor at 27,000 rpm. Then 10 4 to 10 6 cell equivalents of clarified lysates were immunoprecipitated at 4°C for 3 h with 25 to 100 ml of a 50% solution of either VSI, H57-597, or 145-2C11 covalently coupled to CNBr-activated Sepharose (Pharmacia) at 2 mg of antipeptide/ml of Sepharose. Immunoprecipitates were washed at 4°C in PBS-1% NP-40-0.4 M NaCl (pH 7.0) followed by 1 mM phosphate buffer (pH 7.0). Bound material was eluted either in Tris-buffered 2% SDS-1% mercaptoethanol-10% glycerol at room temperature for 20 min or in 50 mM dithylmaleate (pH 11) for lysis buffer. The resulting supernatant was resuspended in either 0.1 M sodium phosphate buffer (pH 7.0) or 0.1 M sodium citrate buffer (pH 5.5) containing 0.5% SDS-1% mercaptoethanol, heated to 100°C for 10 min, and treated with 0.5 mg of n-glycanase (PNGase F) or endo H (New England Biolabs), respectively, for 3 h at 37°C. Immunoprecipitates were separated in 8 to 20% gradient gels by SDS-polyacrylamide gel electrophoresis at 20 mA. The separated proteins were transferred to Hybond-C nitrocellulose (Amersham Corp.). Western analyses with biotinylated affinity-purified polyclonal or MABs were detected with avidin D-horseradish peroxidase (HRP; Vector Labs). Rabbit polyclonal antiserum were detected with a goat anti-rabbit IgG-HRP secondary antibody (Amersham Corp.). Antibody binding was detected by enhanced chemiluminescence (Pierce Chemical Co.).

**Subcellular fractionation.** Subcellular fractionation and analysis of organelle-spanning markers were performed as described previously (47). Briefly, Mtv-1 Sag transfected (1.4 10 10 4 cells/ml) were washed in ice-cold PBS (pH 7.0). The cells were resuspended at 2 10 9 cells/ml in homogenization buffer (HB) (10 mM imidazole, 0.25 M sucrose, 2 mM EDTA, 0.2 mM TMCL, 1 mM PMF (pH 7.0)), and successive 1.0-ml suspensions were homogenized in a Dounce homogenizer (Wheaton). Centrifugation of homogenate at 900 g for 2 h at 4°C followed by a 2,000 g centrifugation over a 100-mL Supernatant was layered onto 10 mL of 27% (vol/vol) Percoll in HB and centrifuged for 2 h at 9000 3 gA. A total of 24 fractions were collected from the bottom of the tube. Equivalent fractions from each gradient were pooled, diluted to 10 mL with HB, and centrifuged overnight at 100,000 g to pellet membranes. Consecutive membrane fractions were pooled and solubilized in 1% NP-40 lysis buffer. After centrifugation of solubilized membranes at 100,000 3 g for 30 min, each fraction was immunoprecipitated with VSI-Sepharose as above.

**Organelle distribution in the Percoll gradient fractions was determined by assaying for the following markers: plasma membrane by labelling cells at 4°C with 111-I transferrin, early endosomes by internalization of 125-I transferrin, lysosomes by assaying for β-hexosaminidase activity and Western blotting for LAMP-2 with ABL-93, late endosomes by immunoblotting with the rabbit anti-rabbit serum against rab7, ER by immunoblotting with 10C3 specific for BiP, and TGN by assaying for β-1,4-galactosyltransferase activity. The MBC was defined by several criteria: by a sandwich ELISA to quantitate H-2M and class II levels, and by a presentation assay.**

**Surface reexpression assay.** CH27/SagI B-cell transfectants were washed twice with ice-cold Hanks balanced salt solution (HBSS) and resuspended at 5 10 6 cells/ml in prewarmed (37°C) HBSS (mock) or HBSS containing 2 mg of pronase (Calbiochem) per ml. The cells were incubated for 10 min at 37°C before pronase activity was quenched with 10 mL of ice-cold HBSS supplemented with 5% fetal calf serum. After three washes, the cells were incubated for 5 min in the presence of 0.5 mM PMF and 0.1 mM TMCL to inactivate any remaining pronase activity. Pronase- and mock-treated cells were resuspended at 5 10 9 cells/ml in ice-cold complete tumour medium (CTM) containing 10% fetal calf serum and were more than 98% viable by trypan blue staining. The cells were either left on ice or cultured over a time course of 5 h at 37°C to allow for surface reexpression. Of the following inhibitors: 1 mM leupeptin, 50 mg/mL chloroquine (Sigma), 2 mg of brefeldin A (BFA; ICN) per ml, or 10 mg of cytochalasin D (Sigma) per ml. After culture, the cells were centrifuged, resuspended in ice-cold fluorescence-activated cell sorter (FACS) buffer, and pro- cessed for cytofluorimetric analysis as above. The percent recovery was calculated by recording the mean channel fluorescence at each time point, subtracting the mean channel fluorescence of the irrelevant isotype- and species-matched antibody or plus-peptide control, and dividing the resulting value by the mean fluorescence intensity of the same antibody combination from the mock-transfected cells.

2578 GRIGG ET AL. J. VIROL. Downloaded on November 7, 2017 by guest from http://jvi.asm.org/
RESULTS

vSag1 must associate with class II for stable cell surface expression in B cells. To determine formally whether association with class II is required for stable vSag1 surface expression in B cells, the lymphoma variant M12.C3 was sequentially transfected with vSag1 and Eβk. This cell line lacks class II surface expression due to the absence of Abd mRNA and a structural mutation in Eβd that results in the intracellular accumulation of EaEkd (11, 25).

M12.C3 cells transfected with vSag1 alone and stained with the P2 antiserum specific for a C-terminal peptide unique to vSag1 did not express detectable levels of Sag protein on their cell surface (Fig. 1). Intracellular staining with VS1, a MAb specific for the N-terminal 13 amino acids common to all vSags, confirmed that vSag1 protein was present in the M12.C3vSag1 transfectant despite its lack of surface expression. After transfection of Eβk and restoration of class II EaEkd surface expression, vSag1 protein was readily detected on the B-cell surface. These results show that vSag1 is not present at detectable levels on the surface of B cells in the absence of class II. Furthermore, vSag1 was not detected on the surface of the class II negative Ltk- cell line after vSag1 transfection (data not shown), arguing that vSag1 was not being retained inside the M12.C3vSag1 transfectant associated with intracellular EαEβd. vSag1 therefore appears to require class II for surface stability or alternatively for trafficking to the cell surface.

Leupeptin inhibits vSag1 surface expression and presentation to Vβ3+ T-cell hybrids. To evaluate the importance of a replenishing pool of mature class II heterodimers on both cell surface expression and vSag1-mediated T-cell activation, the functional expression of vSag1 was investigated in the presence of leupeptin, a serine and thiol protease inhibitor. Leupeptin blocks the conversion of newly synthesized class II molecules into compact, peptide-loaded SDS-stable dimers by limiting Ii as well as nominal antigen proteolysis, resulting in the retention of Ii-class II complexes in endocytic vesicles (48).

Western blot analysis of leupeptin-treated CH27vSag1 B-cell transfectants by using IN-1 MAb specific for the cytoplasmic tail of Ii revealed a dose-dependent accumulation of LIP and SLIP (8), products of incomplete Ii chain degradation that remain associated with class II (Fig. 2A). As expected, leupeptin-treated cells showed diminished IEk-restricted presentation of the exogenous protein antigen, pigeon cytochrome c (PCC) (Fig. 2B). In contrast, presentation of moth cytochrome c peptide 88 to 104 (MCC 88–104) was significantly increased during leupeptin treatment. This may reflect an escape of nascent class II complexed with loosely bound peptide, which can be easily displaced at the cell surface with exogenous peptide antigens.

Leupeptin treatment had no effect on the surface expression of MHC class I proteins that traffic by the default secretory pathway (Fig. 2C and D). However, there was a slight dose-dependent decrease in class II surface staining, presumably because less de novo synthesized class II was trafficking to the cell surface. More strikingly, there was a significant and reproducible decrease in the level of vSag1 surface staining despite only a small decline in class II surface expression. Furthermore, three of the six Sag-reactive T-cell hybrids tested were...
FIG. 2. Leupeptin treatment of CH27\(v\)Sag1 transfectants. (A) Cells were incubated with increasing concentrations of leupeptin for 24 hours. Cell lysates solubilized with 1% NP-40 were separated by gradient SDS-PAGE (8 to 20% polyacrylamide), transferred to nitrocellulose, and immunoblotted with IN-1 to detect the \(\alpha\) chain. (B) Cells incubated in the absence or presence of 500 \(\mu\)M leupeptin were assessed in a stimulation assay for their ability to present PCC or MCC\(88-104\) to the IE\(k\)-restricted T-cell hybrid HOD6.8.26. (C) Cells were stained with MAb H116/32, 14-4-4S or AF3 or the P2 antiserum to detect class II \(\alpha\)\(\kappa\) and IE\(k\), class I \(\kappa\), and \(v\)Sag1, respectively, after incubation in the absence (thin line) or presence (thick line) of 500 \(\mu\)M leupeptin for 24 h. Competitive peptide (25 \(\mu\)M; P2) and an isotype-matched antibody (dotted line) served as negative staining controls. (D) Cells were incubated in the absence or presence of graded concentrations of leupeptin for 24 h and stained for surface markers as in panel C. The data represent the mean and standard deviation from three independent experiments. (E) \(\nu\)Sag1 T-cell hybrids were cocultured with untreated or leupeptin-treated CH27\(v\)Sag1 transfectants, or exposed to immobilized anti-CD3 antibody, for 18 h in a stimulation assay performed in the continuous presence of leupeptin. IL-2 was assayed as in panel B.
inefficiently stimulated by the leupeptin-treated CH27vSag1 transfectants (Fig. 2E). The effect was specific because leupeptin did not affect the release of interleukin-2 (IL-2) by these V_{δ}^{3+} T-cell hybrids upon CD3 cross-linking with antibody. Moreover, all hybrids tested were inhibited equally upon titration of the P2 antiserum used to limit vSag1 presentation, suggesting that the differences in reactivity patterns are not merely explained by the affinity of the TCR-vSag interaction (data not shown).

The inclusion of leupeptin significantly alters the proportion of surface-expressed class II dimers containing loosely bound peptide (Fig. 2B). The nonresponsiveness exhibited by several of the Sag-reactive T-cell hybrids might therefore indicate that the structural conformation adopted by class II associated with loosely bound peptides may influence the Sag reactivity of individual T-cell hybrids.

Biochemical detection of vSag1 proteins by Western blot analysis. To investigate whether vSag1 assembles with class II and Ii upon synthesis in the ER or whether vSag1 and class II traffic independently to the cell surface before associating, the different molecular forms of vSag1 protein were purified and their glycosylation status and the biochemical form of the class II molecules associated with purified vSag1 were then determined.

The characterization of vSag molecular forms has proven extremely difficult because of their relatively low abundance in cells and the paucity of high-affinity antibodies. These technical limitations necessitate transfection to achieve vSag protein levels sufficient for biochemical analysis. To date, only vSag7 has been fully characterized biochemically. However, sequence data predict that different vSags encoded by both infectious viruses and proviral integrants will be highly conserved structurally with only slight variations due to differences in their number of potential glycosylation motifs, proteolytic cleavage sites, and their sequence polymorphism at the C terminus (76).

Immunoprecipitation of vSag1 with VS1 followed by Western blot analysis revealed that vSag1, like vSag7, exists predominantly as a 45-kDa ER-resident glycoprotein with high mannose-type carbohydrate additions as determined by its sensitivity to the glycosidase endo H (Fig. 3A). The heterogeneous array of higher-molecular-weight forms identified were endo H resistant, indicating that these forms had been modified further by the addition of complex-type glycans upon transit through the Golgi stacks. vSag1 was specifically detected in the CH27vSag1 transfectant but not in the control lysates, indicating that the low levels of endogenous vSags 8, 9, 17, and 30 present in CH27 are below the level of detection. Moreover, the irrelevant control antibody H57-597 did not precipitate vSag1. Digestion of purified vSag1 with PNGase F (N-glycanase) to remove N-linked oligosaccharides resolved the gp45 species to the predicted 37-kDa deglycosylated core and revealed two bands at 27 and 44 kDa, presumably derived from the higher-molecular-weight vSag1 forms. The p27 has been described previously and represents the amino-terminal proteolytic cleavage product (75), indicating that vSags 1 and 7 are similarly processed. The 44-kDa form has not been described previously and may represent an unprocessed Golgi intermediate. Both forms are approximately 8 kDa larger than the predicted N-terminal cleavage product (19 kDa) and deglycosylated core (37 kDa) and most probably are the result of incomplete digestion with PNGase F or alternatively are modified in some other way.

Immunoprecipitation with the polyclonal rabbit antiserum α-gp45, followed by Western blotting with VS1, identified the same molecular forms in the CH27vSag1 transfectant and not in the CH27 control, confirming that the proteins identified were vSag1 (Fig. 3B). Furthermore, the vSag1-specific P2 antiserum, although unable to detect vSag1 in Western analyses, immunoprecipitated the same molecular species, verifying that the amino- and carboxy-terminal proteolytic cleavage fragments of vSag1 remain noncovalently associated (Fig. 3C).

vSag1 associates with both isotypes of class II. To confirm that vSag1 is presented by both class II isotypes expressed on the surface of CH27vSag1 transfectants, MAbs specific for each class II molecule were used to block vSag1 presentation to T cells. Table 1 illustrates that both IA^{b} and IE^{b} present vSag1 to T cells. Interestingly, all additional IA^{b}-specific antibodies tested were more effective at inhibiting the T-cell response than were IE^{b}-specific antibodies, suggesting that IA^{b} presents vSag1 to these V_{δ}^{3+} T-cell hybrids more efficiently (data not shown). This is in agreement with vSag7 presentation by the CH12 B-cell lymphoma (75) and may suggest that IA^{b} presents vSag more efficiently than does IE^{b}, an intriguing
observation given that it is generally believed from in vivo studies that I-E is better at presenting viral Sags to T cells (44). Association with both isotypes is not merely the result of overexpression of vSag1 with a strong promoter, because transfection of I\(^a\) or E\(^b\) separately into M12.C3 similarly showed that both class II molecules present the endogenous M12vSag to a panel of V\(_{b^7}\) T cell responders (data not shown).

To show that vSag1 forms a stable complex with MHC class II molecules, CH27vSag1 cell lysates were immunoprecipitated with VS1 or the control antibody, 145-2C11. Of the several chain-specific MAbs that bind only the A\(^a\) or E\(^b\) heterodimers (21), this is highly unlikely, since numerous chain-specific MAbs that bind only the A\(_{\alpha}\) or E\(_{\beta}\) subunit in the A\(_{\alpha}\)E\(_{\beta}\) mixed isotype heterodimers (54), served as controls to rule out nonspecific binding of class II molecules during the immunoprecipitation procedure with VS1 antibody. Although Western analysis cannot formally rule out that vSags associate exclusively with A\(_{\alpha}\)E\(_{\beta}\) mixed isotype heterodimers (21), this is highly unlikely, since numerous chain-specific MAbs that bind only the A\(_{\alpha}\) or E\(_{\beta}\) subunit in the A\(_{\alpha}\)E\(_{\beta}\) heterodimer completely blocked presentation by each class II isotype, ruling out the presence of significant association of vSag1 with A\(_{\alpha}\)E\(_{\beta}\) dimers (data not shown).

To investigate whether II is present in vSag1-class II complexes, the same experimental approach was adopted. vSag1 was isolated by VS1 immunoprecipitation, and the eluate was probed for II and a similarly abundant protein, LAMP-2, which served as a control for the immunoprecipitation procedure. Although both II and LAMP-2 were identified by Western analysis in the control lysate, II was not found associated with vSag1 (Fig. 4B). The blot was stripped and reprobed for class II and vSag1 to confirm the presence of both proteins (data not shown). The data suggest that the majority of II stably associated with vSag1 is not complexed with II.

**SDS-stable, endo II-resistant class II associates with vSag1.** To identify the maturation status of class II molecules associated with vSag1, a series of experiments was performed to investigate the glycosylation and SDS stability of these class II molecules. Immunoprecipitation with VS1 antibody followed by deglycosylation with endo H or PNGase F identified only endo H-resistant class II A\(_{\alpha}\) molecules associated with vSag1 (Fig. 5A). Endo H treatment was clearly efficient, as evidenced by the reduction of vSag1 gp45 to its core 37-kDa form (Fig. 5A), and by its ability to deglycosylate class II molecules detected from whole cell lysates (data not shown). The class II molecules were modified with complex-type glycans because the A\(_{\beta}\) chain detected was sensitive to PNGase F digestion. Similar results were obtained for IE\(^b\) (data not shown), suggesting that assembly of vSag1-class II complexes does not occur in the ER. Furthermore, a significant proportion of the class II molecules bound to vSag1 were compact dimers resistant to dissociation in 2% SDS (Fig. 5B). These results suggest that vSag1 most probably binds class II molecules after the exchange of CLIP for antigenic peptide either in the MIIC or at the cell surface.

It could be argued that vSag-class II complex formation occurs in the ER (75) and that this association does not preclude later peptide loading and conversion of class II molecules to SDS-stable heterodimers. To address this possibility, CH27vSag1 transfectants were incubated for 5 h in the presence of BFA, an inhibitor of protein transport between the ER and Golgi complex (41), to enrich for ER-resident vSag1. In the absence of BFA, VS1 immunoprecipitated all the vSag1 proteins and the class II associated was endo H-resistant (Fig. 5C). Strikingly, after 5 h of BFA treatment, no higher-molecular-weight vSag1 protein was detected, nor was any class II protein bound. These results illustrate that class II does not associate with ER-resident gp45 vSag1 and that the mature vSag1 protein is relatively short-lived, demonstrated by the absence of higher-molecular-mass vSag1 proteins which normally resolve at 27 and 44 kDa after PNGase F digestion (Fig. 5C).

*Fig. 4.* Both isotypes of class II associate with vSag1. (A) CH27, CH27vSag1, and T1-IA\(^a\) cells were solubilized in 1% NP-40 lysis buffer and immunoprecipitated with either VS1 or the isotype- and species-matched control antibody, 145-2C11. Immunoprecipitated proteins were eluted in 50 mM diethylamine (pH 11) and lyophilized. The eluate was resuspended in SDS-PAGE loading buffer, separated by gradient SDS-PAGE, and immunoblotted. Class II E\(_{\beta}\) and A\(_{\alpha}\) chains were identified with the cytoplasmic tail-specific rabbit antiserum R4226 and R5015, respectively. (B) As in panel A, except that II was detected with MAAb IN-1 and LAMP-2 was detected with MAAb ABL-93.

**TABLE 1.** Both class II isotypes present vSag1 to T cells.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>IL-2 released (U/ml) by T-cell hybrid:</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>K25-49.16, K25-59.6, BR-146.11</td>
</tr>
<tr>
<td>10-2.16</td>
<td>IA(<em>{\alpha})(</em>{\alpha})</td>
<td>650, 1,400, 190 (V(<em>{b^3})^+), (V(</em>{b^1})^+), (V(_{b^3})^+)</td>
</tr>
<tr>
<td>14-4-4S</td>
<td>IE(<em>{\alpha})(</em>{\alpha})</td>
<td>24, 290, 10 (V(<em>{b^3})^+), (V(</em>{b^1})^+), (V(_{b^3})^+)</td>
</tr>
<tr>
<td>Both</td>
<td>IA(<em>{\alpha})(</em>{\alpha})+IE(<em>{\alpha})(</em>{\alpha})</td>
<td>–, –, –</td>
</tr>
<tr>
<td>GK1.5</td>
<td>CD4</td>
<td>–, –, –</td>
</tr>
</tbody>
</table>

\(^a\) Antibodies were added to a final concentration of 10 μg/ml.  
\(^b\) c <10 U of IL-2 per ml.
VOL. 72, 1998

SUPERANTIGENS TRAFFIC INDEPENDENTLY OF MHC CLASS II

β-hexosaminidase activity to identify lysosomes and β-1,4-galactosyltransferase activity to mark the TGN. The binding of 125I-transferrin to its surface receptor at 4°C identified the plasma membrane, and internalization of 125I-transferrin at 37°C for 10 min marked the early endosomes (data not shown). Identification of class II IAβ by sandwich ELISA demonstrated that the first two fractions in the dense portion of the gradient were enriched for class II αβ heterodimers. An ELISA for H-2M revealed that H-2M was similarly distributed in these gradients (Fig. 6). Moreover, endocytic vesicles isolated from the first two fractions contained MHC class II dimers capable of binding peptide antigen and stimulating T cells in a presentation assay, confirming the presence of functional class II in this portion of the gradient (Fig. 6). Western blotting for grp78 (BiP) and rab7 identified the ER and late endosomes, respectively (Fig. 7A). The presence of the lysosomal antigen LAMP-2 and class II α chains, and the relative absence of intact Ii, further suggested that fractions 1 and 2 contained the peak of lysosomes and MIIC (Fig. 7A). A second peak of LAMP-2 and β-hexosaminidase activity (fractions 5 to 8) overlapped with fractions containing the majority of the other cellular membranes including the ER, plasma membrane, TGN, and early and late endosomes. This peak

FIG. 5. Endo H-resistant, SDS-stable class II associates with vSag1. (A) CH27vSag1 cells (2 × 10⁸ cells per lane) were lysed in 1% NP-40 buffer and immunoprecipitated with either VS1 or 145-2C11 followed by deglycosylation and detection of vSag1 by Western analysis essentially as described in the legend to Fig. 3. Class II αβ chains were detected as described in the legend to Fig. 4A. (B) 10-2.16 (α-IAβ), VS1, or 145-2C11 immunoprecipitates from CH27vSag1 1% NP-40 lysates (2 × 10⁶ cells per lane) were incubated in 2% SDS–1% 2-mercaptoethanol for 20 min and then either heated to >95°C for 5 min (lanes B) or loaded directly into SDS-PAGE gradient gels without heating (lanes NB). Class II αβ chains were detected as described in the legend to Fig. 4A. (C) CH27vSag1 cells (2 × 10⁶ cells per lane) were incubated in the absence or presence of BFA at 2 μg/ml for 5 h prior to solubilization with 1% NP-40 lysis buffer. VS1 immunoprecipitates were deglycosylated, and vSag1 and class II αβ were detected as described above.

FIG. 6. Distribution of CH27vSag1 membrane organelles in 27% Percoll density gradients. Organelle distribution was determined by assaying for the following: plasma membrane by 125I-transferrin (cpm), lysosomes by β-hexosaminidase activity (fluorescence units), TGN by β-1,4 galactosyltransferase activity (gal-tf; cpm), dense MIIC (fractions 1 and 2) and plasma membrane (fractions 6 to 8) by sandwich ELISA for H-2M and class II (optical density units at 405 nm), and the T-cell stimulation assay for MCC88–104 presented by IEk (units of IL-2 per milliliter).

FIG. 7. Distribution of CH27vSag1 membrane organelles in 27% Percoll density gradients. Organelle distribution was determined by assaying for the following: plasma membrane by 125I-transferrin (cpm), lysosomes by β-hexosaminidase activity (fluorescence units), TGN by β-1,4 galactosyltransferase activity (gal-tf; cpm), dense MIIC (fractions 1 and 2) and plasma membrane (fractions 6 to 8) by sandwich ELISA for H-2M and class II (optical density units at 405 nm), and the T-cell stimulation assay for MCC88–104 presented by IEk (units of IL-2 per milliliter).
probably reflects the presence of late endosomes and early MIICs (47), because these fractions were also enriched for H-2M (Fig. 6 and 7).

To characterize the steady-state distribution of vSag1 molecules in Percoll gradients, membranes from the different subcellular fractions were solubilized in NP-40 and vSag1 was immunoprecipitated with VS1-Sepharose. vSag1 molecular forms were identified in the gradient fractions by VS1 Western blotting (Fig. 7B). Treatment with PNGase F demonstrated that the vSag1 higher-molecular-weight forms present in fractions 1 and 2 were composed exclusively of mature proteolytically processed vSag1, because only the p27 amino-terminal cleavage product was detected. Endo H-sensitive, gp45 ER-resident forms that migrate at 37 kDa after deglycosylation were detected only in fractions 7 and 8, containing the ER and the bulk of the other organelles. Moreover, the unprocessed 44-kDa form, revealed after PNGase F treatment, was identified only in fractions 6 to 9, further suggesting that only mature, processed forms of vSag1 are present in vesicles cosedimenting with MIIC and lysosomes (fractions 1 and 2).

To investigate whether vSag1-class II complexes are present in the MIIC, vSag1 was isolated by VS1 immunoprecipitation from Percoll gradient fractions and the eluate was probed for class II molecules. Although endo H-resistant class II molecules were identified in fractions 6 to 8 in three separate experiments, we were unable to detect vSag1-class II complexes in dense fractions containing the MIIC (data not shown), suggesting that the vSag1 identified in fractions 1 and 2 reflects mature protein internalized from the plasma membrane into the lysosomal pathway for degradation. Pulse-chase analysis to monitor the intracellular trafficking of vSag1 proved impractical due to insufficient sensitivity (25a).

vSag1 traffics directly to the plasma membrane prior to association with class II molecules. To assess the kinetics of vSag1 surface expression, CH27vSag1 cells were incubated in the presence of pronase to remove proteins from the cell surface. The protease-treated cells were then placed at 37°C and allowed to recover surface expression in order to monitor the kinetics of nascent vSag1 trafficking. BFA was used to distinguish de novo surface expression of newly synthesized protein from surface reexpression from a recycling pool of preexisting protein.

Incubation of CH27vSag1 cells in the presence of pronase for 10 min selectively removed a number of B-cell surface proteins, including vSag1, HSA, CD19, and CD69, while having relatively little effect on the expression of IgM and class I and II molecules, as assessed by flow cytometry (Fig. 8A; data not shown). The treated cells were then incubated at 37°C over a time course of up to 5 h to monitor the kinetics of surface reexpression in the absence or presence of the lysosomotropic agent chloroquine or leupeptin. The latter inhibitor was included to specifically block nascent class II molecules from progressing through the endocytic pathway in order to elucidate whether vSag1 associates with class II molecules in the MIIC before surface expression.

Pronase treatment did not affect the ability of the transfected to present peptide fragments and protein antigens to reactive T-cell hybrids (Fig. 8B). Both chloroquine and leupeptin effectively blocked presentation of the protein antigen PCC, while chloroquine had little effect on the loading and presentation of exogenously supplied peptide. Pronase-treated cells incubated in the presence of leupeptin exhibited very efficient presentation of exogenously provided MCC88–104 peptide, as observed previously in Fig. 2B.

As expected, chloroquine and leupeptin treatment slightly altered or had little effect on the surface reexpression of HSA or CD19, proteins that traffic by default secretion (Fig. 8C). In fact, both markers reappeared at the plasma membrane within 30 to 60 min, kinetics expected of proteins that travel directly to the cell surface upon synthesis (34). Importantly, vSag1 displayed kinetics of reexpression similar to HSA and CD19 (30 to 60 min), even in the presence of chloroquine or leupeptin (Fig. 8C). The reappearance of vSag1, HSA, and CD19 on the cell surface was effectively blocked by the addition of BFA, indicating that there were few, if any, recycling molecules in

FIG. 7. Steady-state intracellular distribution of vSag1 after Percoll density centrifugation. (A) Dense MIIC and plasma membrane by Western blotting for class II Aβ; ER by intact Ii and BiP; lysosomes (fractions 1 and 2), early MIIC and plasma membrane (fractions 5 to 8) by LAMP-2; late endosomes by rab7. (B) Intracellular distribution of vSag1 in Percoll gradient fractions by VS1 immunoprecipitation. Methods of detection and deglycosylation were as described in the legend to Fig. 3A.
FIG. 8. vSag1 trafficks by the exocytic pathway directly to the B-cell surface. (A) CH27vSag1 cells were incubated in the absence (mock) or presence of pronase for 10 min at 37°C and stained with the P2 antiserum, M1/69 (specific for HSA), 1D3 (CD19), or H116/32 (IAk) to assess their surface expression after protease treatment. (B) Pronase-treated cells were tested in a stimulation assay for their ability to present PCC or MCC 88–104 to HOD6.8.26 as described in the legend to Fig. 2B. Presentation of these antigens was assessed in the absence or presence of leupeptin (500 μM) or chloroquine (50 μM). (C) Pronase-treated cells were cultured at 37°C over a time course of 5 h to monitor the recovery of stripped surface markers. The cells were cultured in medium alone or medium containing 2 μg of BFA per ml, 50 μM chloroquine, or 500 μM leupeptin. The recovery of vSag1, HSA, and CD19 surface expression was assessed by flow cytometry. Percent recovery was determined as described in Materials and Methods.
the pronase-treated cells. It therefore seems likely that vSag1 trafficks by the default secretory pathway and becomes stabilized by the class II molecules present at the cell surface, because the addition of chloroquine and leupeptin would have been expected to block transport and alter the kinetics of surface reexpression if vSag1-class II complexes formed in the MIIC prior to surface expression.

**DISCUSSION**

The data presented indicate that class II molecules are required for the stable and functional surface expression of vSag1. Only mature vSag1 protein bound class II, associating exclusively with endo H-sensitive vSag forms, the great majority of which were SDS stable and loaded with high-affinity antigenic peptides. De novo-synthesized vSag1 did not associate with class II or Ii in the ER but, rather, trafficked directly to the plasma membrane by the exocytotic secretory pathway, assembling with class II molecules on the B cell surface. Leupeptin treatment altered class II conformation and expression, significantly decreasing the amount of vSag1 protein at the plasma membrane. Several Sag-reactive T cells were very sensitive to leupeptin-induced decreases in vSag1 expression, which may suggest that some TCRs are greatly affected by subtle changes in the conformation of the vSag-peptide-class II complex.

**Class II is required for stable vSag1 surface expression in B cells.** Mice lacking class II molecules do not delete vSag-reactive T cells, and viral spread during MMTV infection is severely reduced, establishing the importance of class II molecules in the efficacious presentation of vSags during the MMTV life cycle (6). In general, most class II molecules present vSags to T cells, although the efficiency of presentation depends on the class II allele present (31). vSags associate poorly with IAα, and B cells bearing this class II allele do not express vSags on their cell surface, suggesting that, at least in B cells, association with class II molecules facilitates surface expression (74). However, transfection experiments in class II deficient fibroblasts have implied that class II is not critical for the surface expression of viral superantigens (74, 75). While low level surface expression of vSag7 is possible in some class II-deficient cell lines (45a, 74), we have not been able to demonstrate appreciable surface expression of vSag1 in the absence of class II molecules in B cells (Fig. 1) or fibroblasts (data not shown). The sequential transfection experiments presented in Fig. 1 clearly show that class II expression is required for vSag1 surface detection. Furthermore, transfection of class II into M12.C3vSag7 significantly increases vSag7 surface levels, underlining the importance of class II in the surface expression of viral superantigens (data not shown).

Although these experiments do not demonstrate whether class II molecules are required for vSag1 trafficking to the cell surface, the data illustrate that they are necessary for the efficient display of vSags on the B-cell surface and are consistent with the results of Lund et al. (43), who showed that conditions which increase nascent class II expression facilitate the functional surface expression of vSags. In the absence of class II, vSags are shed into the external medium when in culture (17), perhaps explaining their relative lack of expression on the surface of class II-negative cells.

**Intracellular transport of vSag1 and assembly with class II molecules.** It has been reported previously that the gp45 endo H-sensitive vSag form associates intracellularly with class II molecules in the ER (75). It has also been suggested that vSags, like αi, assemble with class II heterodimers in the ER, facilitated by a CLIP-like motif that stabilizes the complex and promotes its egress to the surface (3). Although our data do not exclude the possibility that a minor population of viral superantigen molecules traffic by this pathway, our data are not consistent with this model, since we have been unable to isolate endo H-sensitive class II molecules associated with vSag1 by V51 immunoprecipitation in any of four detergents used for solubilization (Fig. 5). Instead, the class II associated with purified vSag1 is endo H resistant, and most of it is stable in the presence of SDS, implying that complex assembly occurs in a post-Golgi environment, after Ii removal and the generation of peptide-loaded class II dimers. Experiments in which BFA was used to block transport from the ER illustrate that class II molecules do not associate intracellularly with ER-resident vSag1, and the rapid surface reexpression kinetics imply that vSag1 trafficks by default secretion, presumably binding with mature class II dimers on the surface of B cells. It is formally possible that the NP-40 solubilization and washing conditions we used did not preserve a complex between ER-resident vSag1 and class II or class II-Ii complexes; however, using the same experimental conditions as those of Winslow et al. (75), we identified the gp45 vSag1 form in not only our anti-class II IAα (10-2.16) and IEd (17-3-3S), but also our irrelevant control anti-class I Kk (AF3, Y3) eluates, raising the possibility that this association is an experimental artifact and probably does not occur in vivo (25a).

Upon egress from the ER, vSags become proteolytically processed en route to the cell surface, and there is evidence that endoprotease-mediated cleavage by furin in the Golgi improves vSag presentation (46, 49). Whether proteolytic processing of vSags evolved to improve vSag binding with mature class II molecules on the cell surface or allow for their intracellular transfer (17) is still unclear. Given the rapid surface reexpression of vSag1 and the absence of detectable vSag1-class II complexes in lysosomes and MIIC, our data suggest that vSags do not require trafficking to low-pH, proteolytically active endosomes for processing and efficient association with class II molecules. Moreover, vSags possess no obvious endosomal localization motif, and cytoplasmic tail truncations do not appear to affect their functional expression (14). Our observation that vSag1 and class II molecules do not associate in the ER raises the question of what prevents their association. Ii is the most likely candidate to exclude vSag1 from interacting stably with class II heterodimers in the ER and Golgi complex, raising the intriguing possibility that Ii, by ensuring the proper maturation of class II molecules loaded with antigenic peptides, facilitates the formation of effective vSag-peptide-class II complexes that are capable of stimulating T cells. In this regard, mice lacking Ii show reduced Vα8-specific T-cell deletion (70).

Considerable information now exists about the importance of antigenic peptide binding on the class II structure and how proper assembly of the peptide-MHC class II trimolecular complex influences the presentation to T cells (10, 16, 64). Numerous studies have shown that class II molecules exist in several distinct forms based on their stability in SDS. Our data imply that vSag1 associates with functionally mature, SDS-stable class II dimers, arguing against the hypothesis that vSags bind with only the minor fraction of “empty” (containing loosely bound peptide) class II present on the surface of B cells (43). Given the paucity of vSag relative to class II on the B-cell surface, it seems likely that vSags can associate only with a restricted set of class II molecules. In this regard, binding of the bacterial superantigen toxic shock syndrome toxin 1 to class II is highly dependent on the particular peptide bound by the class II molecule (33, 66). It seems plausible then that vSag-class II association and presentation may be similarly influ-
ence by the peptide bound in the polymorphic groove of class II dimers. Sequencing of peptides eluted from class II molecules complexed with vSags would address this question.

It is not immediately apparent why there were marked differences in the vSag-reactive T-cell response after leupeptin treatment of the CH2v/Sag1 transfectants. The results could not be explained by differential sensitivity among hybrids to the decline in vSag1 surface levels, since titrating the P2 antisera in a blocking experiment to limit vSag1 presentation affected all V\(\alpha\)1.3 T cell hybrids equally (25a). Several intriguing possibilities therefore exist; leupeptin affects either the proteolytic activation of vSags or alters the conformation of class II, or, alternatively, a combination of the two effects may result in ineffective presentation of vSag1, affecting the extent and efficiency of vSag1 recognition by some but not other Sag-reactive T cells. It is well established that the V\(_\gamma\) element of the TCR influences the reactivity to vSags (63, 69) and that not all TCRs bearing reactive V\(_\gamma\) chains are stimulated by vSags in vitro (51); therefore, conformational differences in class II structure may influence the mode of vSag presentation and affect T-cell recognition. In this regard, alloresponsive T-cell clones have been shown to be very sensitive to the conformation of class II molecules (15).

The leupeptin-induced block in class II trafficking exhibited by other class II haplotypes (8, 48) might not be complete in H-2g-bearing cells because both class II IA\(^b\) and IE\(^k\) exhibit very low affinities for Ii fragments containing CLIP (60), and so it is conceivable that some class II molecules loaded with loosely bound peptide fragments escape to the cell surface during leupeptin treatment. In fact, the leupeptin-induced increase in IE\(^k\)-restricted presentation of MCC\(_{88-104}\) peptide may reflect an increased amount of conformationally altered class II molecules on the surface of treated B cells. Taken together, the stimulation data may suggest that some Mtv-1 Sag-reactive T-cell receptors are sensitive to subtle changes in the conformation of the vSag-peptide-class II complex.

We have purified the Mtv-1 superantigen and investigated its intracellular route and site of assembly with MHC class II molecules to understand better the mechanism whereby MMTV encoded superantigens, functioning as potent virulence factors, bypass the exquisite specificity of the class II–T-cell receptor interaction and deftly activate T cells to maintain the viral life cycle. It is becoming increasingly evident that a number of B-cell-tropic viruses target the class II molecule to establish potent infections (38, 40, 65, 67). These examples illustrate how several different viruses have converged on a potent strategy for B-cell-tropic viruses target the class II molecule to establish patent infections (38, 40, 65, 67). These examples illustrate how several different viruses have converged on a potent strategy for


