Processing of a viral glycoprotein in the endoplasmic reticulum for class II presentation

Endogenous processing of viral glycoproteins for presentation to CD4+ T cells is a poorly investigated aspect of antigen processing and presentation. This pathway may involve not only pathogens, but also self proteins, and may thus be involved in self-tolerance. We have characterized the processing of the endoplasmic reticulum-restricted glycoprotein (G) of vesicular stomatitis virus, termed poison tail (Gpt), biochemically and enzymatically, and by T cell recognition assays. Expressed with a vaccinia vector, Gpt remains endoglycosidase H-sensitive and does not mature to endoglycosidase D sensitivity. The protein is degraded in the ER with a T1/2 of 4 h. Gpt peptides are not secreted since Gpt-infected cells are unable to sensitize uninfected antigen-presenting cells in an innocent bystander assay. Using flow cytometry, Gpt is undetectable on the plasma membrane; in contrast, wild-type G is readily found on the surface or secreted into the milieu as soluble G following infection of A20 cells with a vaccinia recombinant expressing G. The degradation of Gpt is sensitive to the thiol reagent diamide and occurs optimally at physiological pH. A series of proteolytic inhibitors were tested: 3,4-dichloroisocoumarin and 1-chloro-3-tosylamido-7-amino-2-heptanone inhibited degradation, which suggests the involvement of a serine protease. The degradation does not require transport to the Golgi complex, and is not sensitive to a variety of lysosomotropic agents. We show that the degradation products include the immunogenic epitopes recognized by a panel of T cell clones and hybridomas.

1 Introduction

The main function of the immune system is the natural defense against infections. Detailed research over recent years has illuminated the cellular and molecular nature of these immune reactions. The T cell receptor (TCR) does not recognize native antigens, but binds peptides derived from partially degraded proteins [1, 2] bound in MHC molecule [3]. The precise site(s) of antigen processing and subsequent MHC loading with antigen are not completely defined. A complex picture has emerged, indicating the existence of multiple pathways of Ag/MHC assembly [4, 5]. Most commonly, a distinction between two principal pathways, utilized for the loading of Class I and II molecules has been recognized [6–8]. The access of the antigen to specific intracellular compartments is thought to be critical in determining whether it is presented in association with MHC class I or II molecules. Newly synthesized proteins [9], or intact protein molecules artificially introduced to the cytosol [10], are processed and efficiently presented in association with class I MHC molecules [11, 12]. Proteins entering the endosomal compartment are processed and preferentially charge class II MHC molecules [9].

Although these generalizations may provide a framework for understanding MHC-restricted Ag recognition, more recent investigations have shown that class II-restricted T cells also recognize endogenous Ag [13–27]. A number of examples of endogenous protein processing involved cell surface molecules or secreted proteins. This may reflect the internalization of cell surface membrane Ag (recycling), allowing them to become accessible to endosomal processing, and subsequent presentation by class II molecules. Class II-restricted presentation of endogenous cytoplasmic Ag [17–19] has also been described. Thus, it may be possible for certain Ag to translocate directly from the cytoplasm into the vacuolar compartment, or alternatively, these Ag may be transferred to endosomes by autophagy [17, 18, 20, 27]. In other systems, some endogenously synthesized Ag retained within the endoplasmic reticulum (ER) are processed for presentation by class II molecules, suggesting that the loading of peptide Ag may take place within the ER for certain antigenic determinants [13].

Previous studies of the immune response to vesicular stomatitis virus (VSV) led to the observation that immunization of BALB/c and BALB/c-H-2dm2 (dm2) mice with VSV elicited VSV glycoprotein (G)-specific MHC-class II-restricted CTL that were specific for the immunizing serotype [28]. Further observations have shown that recombinant vaccinia virus encoding an ER-retained form of VSV glycoprotein (vacc-Gpt) can be presented to T cells [23].
Further studies showed that the presentation of vacc-Gpt by infected B cells is inhibited when the targets are incubated with chloroquine, methylamine or ammonium chloride, emetine, or brefeldin-A (BFA) [22, 23]. The vacc-Gpt system was chosen because no G is secreted; that is, no soluble G (Gs) or plasma membrane G is made and therefore cannot reenter cells by endocytosis to be processed by the APC as an exogenous Ag. Also, vaccinia infection, unlike VSV infection, is not affected by drugs altering the pH of endosomes and lysosomes [30].

The relative contribution of the endogenous pathway of class II-restricted presentation is likely to depend upon the degree to which individual determinants are present in the cytoplasm. ER/salvage compartment and endosomal compartments; the abundance of the determinant; the capacity of the Ag determinant to compete with other peptides for MHC occupancy; and theoretically the ability of the antigenic peptide to displace invariant chain (II) from class II molecules. The last possibility must be considered in the case of class II MHC/peptide loading within the ER. This might happen with ER-retained proteins that are malformed or possess a retention signal, and would therefore be subject to proteolytic degradation within the ER. Recent data, though, has suggested that Ii association with class II molecules inhibits peptide binding [31].

Our understanding of the different proteolytic events an Ag undergoes in the cell is far from complete. The intracellular compartments where Ag processing occurs for endogenous proteins is not well defined, and may differ among APC of distinct lineages. In this study using the vacc-Gpt model, we investigated the mechanisms involved in determining the site(s) where the Ag is processed into peptides and partially characterize the proteases that may be involved.

2 Materials and methods

2.1 Cell lines

A20.2, an H-2d, Ia- B cell lymphoma cell line, CTLL 20, an IL-2-dependent T cell line [32, 33], CV-1 monkey kidney cells, and Chinese hamster ovary cells (CHO) were maintained in vitro as described [34].

2.2 T cell clones and T-T hybridomas

CTL clones 9F-1 and BALB/c #4 derived from VSV-immunized BALB/c ir2 mice and BALB/c mouse strains, respectively, by limiting dilution culture, have been shown to be I-E\(^d\)-restricted and to recognize distinct determinants of the ectodomain of the VSV Indiana serotype G [28, 34, 35]. The murine T cell hybridomas 3DV155.14 and 3DV315.11 are restricted by class II MHC I-A\(^d\) and I-E\(^d\), respectively, and are both G-specific [36].

A20, CHO, CV-1, and T cell hybridomas were grown in RPMI (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco), 2 mM t-glutamine, 15 mM Hepes, 100 μg/ml penicillin, 100 μg/ml streptomycin and 2 μg/ml gentamycin. CTL clones and CTLL 20 cells were cultured in medium supplemented with 1% conditioned medium from Con A-stimulated rat splenocytes as a source of IL-2 [37, 38]. CTL clones were maintained by stimulating bi-weekly with irradiated (1500 rad) syngeneic splenocytes sensitized with a temperature-sensitive mutant of VSV (tsG41) as described [34]. CTL were then used in cytolytic assays 4–6 days post-stimulation.

2.3 Viruses

VSV, San Juan isolate, Indiana serotype, was grown in CHO cells and twice purified on sucrose gradients as described [28]. VSV was isolated by affinity chromatography from culture supernatants as described [33]. The engineering of the vaccinia recombinants vacc-G and vaccpt12G/vacc-Gpt, which express the full-length VSV G or longer poison tail construct, respectively, and their characterization, have been described [23]. In vacc-Gpt, the 29 amino acid cytoplasmic tail of the wild-type G has been replaced by the 12-amino acid tail PSRDRSRHDKIH [29].

The propagation, titering, and purification of the vaccinia recombinants were carried out as described [39]. Briefly, vaccinia recombinants were propagated on confluent CV-1 monolayers. After 48 h, cells were trypsinized and pelleted by centrifugation. The cells were resuspended in MEM and subjected to three cycles of freezing and thawing to release the virus. Virus was purified from the crude lysates by two rounds of sucrose density gradient ultracentrifugation to remove contaminating proteins. This was especially important for presentation studies, where it is necessary to exclude exogenous G. Western blot analysis confirmed that no residual G protein remained (data not shown). The amount of virus was determined by plaque assay on CV-1 monolayers. Titers were expressed as plaque forming units (pfu)/ml.

2.4 Antibodies

A polyclonal rabbit anti-VSV hyperimmune serum was provided by Dr. Alice Huang (New York University, NY). Hybridomas used in the production of anti-VSV G monoclonal antibodies were provided by Dr. Leo Lefrancois (University of Connecticut). 30.5.7, 28.14.8s, 34.5.8s, 34.2.12S, 31.3.4S, and 10.2.16 were from the American Type Culture Collection (Rockville, MD).

2.5 Immunologic assays

2.5.1 Cytolytic assay

Cytolytic activity was measured by standard 4-h 51Cr-release assays as described [23].

2.5.2 Proliferation assay

A20 cells were pretreated with the indicated inhibitor for 30 min, virus [multiplicity of infection (moi) of 10] or Gs (1 μg/10⁶ cells) was added in the presence of the inhibitor, and cells were incubated for 6 h at 37°C. APC were washed and fixed in 1 ml of 0.5% paraformaldehyde for
20 min at room temperature, then washed and adjusted to 10^6 cells/ml. Cells (10^5 in 100 μl) were added to wells containing medium only or 10^7 T hybridoma cells, and the plates were incubated for 24 h at 37°C, then frozen until containing 200 pCi/ml of [35S] methionine (DuPont-NEN, Boston, MA) for metabolic labeling of newly synthesized proteins. Following the labeling period, the cells were resuspended in complete medium. When used, drugs and inhibitors were included in the chase medium, except where pretreatment is indicated. At appropriate time intervals, the chase was terminated by placing the cells on ice and washed twice with cold HBSS. The cells were lysed for 15 min in ice-cold lysis buffer containing 1% Triton X-100 in PBS and the protease inhibitors 100 μM PMSF, 1 μM leupeptin, and 1 μM pepstatin. Triton X-100-insoluble material was removed by sedimentation at top speed in a microcentrifuge (Model Z 230 M, Vanguard Int.) for 2 min. SN were precleared twice with 20 μl of protein G-agarose (Boehringer Mannheim, Indianapolis, IN). An aliquot from a pool of anti-G mAb was added to the SN for immunoprecipitation at 4°C overnight. Immunoprecipitates were washed once in 0.1% Triton X-100-PBS to remove nonspecifically adsorbed proteins and three times in PBS. Bound antigen was eluted from the beads by boiling in 15 μl 1% SDS sample buffer containing 3% β-mercaptoethanol. The sample was divided into three parts: one was mixed with 20 μl of 0.1 M citrate pH 5.5, and another with 20 μl of 0.02 M sodium phosphate pH 6.5 for Endo H or Endo D digestion, respectively, and boiled for 2 min. The third sample was mock-treated, and to the other two samples either 5 μU of Endo D or 5 μU of Endo H (both from Boehringer Mannheim) was added. Digestion was carried out at 37°C for 20–24 h. Samples were re-immunoprecipitated with anti-G mAb after 10-fold dilution with lysis buffer. Samples were then mixed with 2 × SDS sample buffer containing 2-ME. The samples were analyzed by SDS-PAGE on 10% gels (5% stacking gel), using the buffer system of Laemmli [40]. Gels were then fixed, impregnated with 1 M sodium salicylate, dried, and exposed to X-ray film at −80°C. To quantify radioactivity incorporated into protein, band intensities were determined by exposing the dried gel to a Model GS-250 Imaging Screen Cassette-HS (Bio-Rad, Hercules, CA) which has a high sensitivity screen for low-energy isotopes like 35S. The resulting bands were analyzed on a Phosphor Imager (Bio-Rad) using volume analysis.

2.7 Flow cytometry

A20 cells were infected as described above and incubated at 37°C for up to 24 h before staining. Cells were plated at 10^5 cells in 96-well plates and maintained at 4°C. Culture supernatant (100 μl) containing the indicated mAb was then added to the cells. Cells were incubated for 60 min at 4°C and then washed three times. Cells were resuspended in secondary antibody (FITC-goat anti-mouse; Tago, Burlingame, CA) diluted 1:100 in RPMI and incubated for a further 60 min, washed and resuspended in 1% paraformaldehyde in PBS. Stained, infected cells were analyzed on a FACSort using Consort 30 and Lysys software (Becton Dickinson, Mountain View, CA).

2.6 Metabolic labeling, immunoprecipitation, and electrophoresis

Approximately 10^7 cells per time point were starved for 30 min in methionine-free medium. The cells were then incubated for 15 min in methionine-free culture medium containing 200 μCi/ml of [35S]methionine (DuPont-NEN, Boston, MA) for metabolic labeling of newly synthesized proteins. Following the labeling period, the cells were resuspended in complete medium. When used, drugs and inhibitors were included in the chase medium, except where pretreatment is indicated. At appropriate time intervals, the chase was terminated by placing the cells on ice and washed twice with cold HBSS. The cells were lysed for 15 min in ice-cold lysis buffer containing 1% Triton X-100 in PBS and the protease inhibitors 100 μM PMSF, 1 μM leupeptin, and 1 μM pepstatin. Triton X-100-insoluble material was removed by sedimentation at top speed in a microcentrifuge (Model Z 230 M, Vanguard Int.) for 2 min. SN were precleared twice with 20 μl of protein G-agarose (Boehringer Mannheim, Indianapolis, IN). An aliquot from a pool of anti-G mAb was added to the SN for immunoprecipitation at 4°C overnight. Immuno-precipitates were washed once in 0.1% Triton X-100-PBS to remove nonspecifically adsorbed proteins and three times in PBS. Bound antigen was eluted from the beads by boiling in 15 μl 1% SDS sample buffer containing 3% β-mercaptoethanol. The sample was divided into three parts: one was mixed with 20 μl of 0.1 M citrate pH 5.5, and another with 20 μl of 0.02 M sodium phosphate pH 6.5 for Endo H or Endo D digestion, respectively, and boiled for 2 min. The third sample was mock-treated, and to the other two samples either 5 μU of Endo D or 5 μU of Endo H (both from Boehringer Mannheim) was added. Digestion was carried out at 37°C for 20–24 h. Samples were re-immunoprecipitated with anti-G mAb after 10-fold dilution with lysis buffer. Samples were then mixed with 2 × SDS sample buffer containing 2-ME. The samples were analyzed by SDS-PAGE on 10% gels (5% stacking gel), using the buffer system of Laemmli [40]. Gels were then fixed, impregnated with 1 M sodium salicylate, dried, and exposed to X-ray film at −80°C. To quantify radioactivity incorporated into protein, band intensities were determined by exposing the dried gel to a Model GS-250 Imaging Screen Cassette-HS (Bio-Rad, Hercules, CA) which has a high sensitivity screen for low-energy isotopes like 35S. The resulting bands were analyzed on a Phosphor Imager (Bio-Rad) using volume analysis.

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2.8 Reagents

Chemicals were obtained from Sigma (St Louis, MO), unless stated otherwise. BFA was purchased from Epicenter Technologies. Iliimaquinone (IQ) was generously provided by Dr. V. Malhotra (USCD).

3 Results

3.1 Poison tail VSV G is completely ER associated in A20 cells

An engineered form of VSV G, Gpt, developed by Rose and Bergmann [29] does not become Endo H resistant in transfected COS-1 cells even after 3–4 h [29]. We obtained similar results with murine A20 B lymphomas, in which the half-life of Gpt in vacc-Gpt-infected A20 cells was approximately 4 h (data not shown). To determine the significance of the ER in Gpt processing in vacc-Gpt-infected A20 cells, it was necessary to exclude the possibility that Gpt could leave the ER, either to be secreted or expressed on the cell surface. This was achieved by examining the glycosylation patterns of Gpt through sensitivity of the protein to Endo H and Endo D. Endo H recognizes and cleaves high-mannose side chains of glycoproteins present in the ER or cis-Golgi compartments. Trimming of oligosaccharides from the high-mannose form of the glycoprotein found in the ER is accomplished by α-mannosidase I, presumably acting in the cis- or medial-Golgi compartments, where the enzyme resides [41]. Endo D recognizes and cleaves the more-processed oligosaccharides of the structure (Man)_3(GlcNAc)_n [42]. Sensitivity of the protein to either enzyme will result in a band whose mobility would be similar to that of the unglycosylated form of the protein. Both enzymes cleave at the same site, the β-1,4
linkage of the N-acetylgalcosamine residues [42]. Thus, Endo H resistance and Endo D sensitivity is a measure of progress through the Golgi.

To determine in which compartments Gpt could be found, cells were pulsed with \[^{[35}S\] methionine and chased in unlabeled medium for up to 8 h. Gpt was immunoprecipitated and then treated with Endo H or Endo D. Fig. 1 shows that labeled Gpt remains Endo H-sensitive through 8 h of chase, as indicated by the faster-migrating band. Gpt is not susceptible to Endo D, indicating that degradation of the protein to peptides occurs prior to entry into the cis-Golgi.

### 3.2 Presentation of endogenous Gpt to CD4\(^+\) T cells involves neither surface expression nor secretion of the protein

A20 cells infected with vacc-Gpt are readily recognized by class II-specific CTL [23]. To determine whether the engineered protein, like its wild-type counterpart, was expressed on the cell surface of the APC, surface staining and flow cytometry was performed. Cell surface expression of Gpt during infection would allow the protein to be internalized by endocytosis and processed as an exogenous antigen. Fig. 2 shows the comparative analysis of the cell surface of A20 cells either uninfected or infected with wild-type VSV or vacc-Gpt at 24 h post-infection. All infected cells showed high levels of expression of class I molecules on the surface (anti-H-2\(^d\); left column) and were not reactive with the isotype control (anti-I-A\(^d\); middle column). As can be seen in the right column, cells infected with wild-type VSV show high levels of surface expression of G, while cells infected with vacc-Gpt do not express detectable levels of G on the surface. Vacc-G infected cells were similar to wild-type VSV-infected A20 cells (not shown). Comparable results were seen at 5 h and 12 h post-infection (not shown).

Gpt is retained in the ER and appears to undergo proteolytic cleavage in this compartment. Although Gs is not formed or secreted by CV-1 cells infected with vacc-Gpt [23], it is still possible that immunogenic peptides formed in the ER of A20 cells could be secreted into the medium, and subsequently taken up as exogenous antigen. To test this possibility, SN were obtained from A20 cells infected with wild-type VSV 5 h post-infection, or vacc-G or vacc-Gpt at 12 h post-infection by centrifuging the cultures at 259,000 \(x\) g for 30 min, which pellets viral particles but not protein. The virus-free SN were then used to sensitize \(^{51}\)Cr-pulsed A20 cells as targets for CTL assays. As seen in Fig. 3, SN obtained from wild-type VSV- and vacc-G-infected cells were readily able to sensitize uninfected A20 cells for targeted lysis by the VSV G-specific CD4\(^+\) CTL clone 9F-1. SN obtained from vacc-Gpt-infected cells did not sensitize uninfected A20 cells. Gs was included as a positive control for exogenous sensitization.

In parallel experiments, an innocent bystander assay was performed with vacc-Gpt-infected CHO cells which were incubated with \(^{51}\)Cr-pulsed uninfected A20 cells. Uninfected A20 cells were not lysed by G-specific CD4\(^+\) T cells, while SN from vacc-G-infected CHO cells were able to sensitize \(^{51}\)Cr-A20 (not shown). We interpret these results to show that immunogenic peptides are not secreted by vacc-Gpt-infected A20 cells. Thus, the sensitization of the cells for class II-restricted recognition is unlikely to be due to the exogenous pathway.

These functional studies, therefore, are not only more sensitive but also corroborate the biochemical analysis of the protein. These experiments indicate that processing of endogenous G for class II-restricted recognition takes place independently of G release and re-uptake.
3.3 Degradation of Gpt does not require transport through the Golgi complex and is nonlysosomal

To determine whether intracellular degradation of the protein required passage through the Golgi complex, we examined the effect of inhibitors of intracellular transport on degradation of Gpt. One possible mechanism for Gpt degradation is direct transport from the ER to lysosomes via autophagic vesicles during stress, possibly caused by the viral infection [43]. Since it had been observed in our laboratory that NH₄Cl, a lysosomotropic agent, blocked endogenous presentation of G protein in vacc-Gpt-infected A20 cells [22, 23], it was imperative to determine whether lysosomes were involved in the degradation of Gpt. To address this possibility, we tested the effects of drugs that inhibit lysosomal activity (Fig. 4). Degradation of Gpt in A20 cells was marginally affected by treatment with NH₄Cl, a weak base that raises intravesicular pH, thus inactivating low pH-dependent lysosomal enzymes [44]. Leupeptin, an inhibitor of endosomal and lysosomal proteases such as cathepsin B [45], and the ionophore monensin, which blocks ER-to-Golgi transport, had no effect on the rate of Gpt degradation. These results argue against lysosomal and autophagic involvement in the degradation of Gpt, and indicate a pre-Golgi, most probably the ER compartment as the site of Gpt degradation.

No inhibition of Gpt degradation was observed after treatments with BFA or IQ (data not shown), two agents which block transport from the ER to the Golgi by disrupting the integrity of the Golgi [46–50]. Thus, transport to or through the Golgi complex is not required for Gpt proteolysis.

3.4 Effect of pH on pre-Golgi degradation

As discussed in the preceding section, lysosomotropic agents elevate the pH of acidic intracellular compartments, thereby inactivating acid-dependent protease activities. The failure of these agents to inhibit pre-Golgi degradation of Gpt in A20 cells raises the question of the role, if any, of pH on this nonlysosomal proteolytic pathway.

We used the ionophore carbonyl cyanide m-chlorophenylhydrazone (CCCP), to pH-clamp the internal compartments of vacc-Gpt-infected cells to explore the role of pH on the degradation of Gpt by the pre-Golgi pathway. When cells are incubated with 0.5 μM CCCP, an approximate pH equilibrium with the external medium across the plasma membrane is established [51]. When the initial pH of vacc-Gpt-infected A20 cells was clamped at pH 8 using CCCP, a dramatic inhibition of the degradation rate was observed (Fig. 5) resulting in 80% of the protein remaining intact after 8 h. This effect was not observed with infected cells incubated with CCCP at pH 7.0, where the rate of degradation resembled that of control cells (Fig. 5). As the external pH was reduced below neutral, Gpt degradation was somewhat retarded, though not significantly (Fig. 5). These results indicate that intracellular pH has a major effect on the dynamics of degradation by the non-lysosomal pathway. Proteolysis of Gpt proceeds optimally at pH 7.0, which is characteristic of the ER environment. This further evidence suggests that the ER is the site of Gpt degradation.

3.5 Involvement of free sulfhydryl groups in pre-Golgi Gpt degradation

One drug which inhibited significantly the degradation of Gpt was diamide. Treatment of vacc-Gpt-infected A20 cells with this agent, which oxidizes glutathione [52], the
were chased in medium (control), in the presence of 1 mM diamide, or after washout and resuspension in medium containing 5 mM 2-ME (Fig. 6). Diamide for 8 h, or in the presence of 1 mM diamide for 4 h and washed and resuspended in medium containing 5 mM 2-ME for a further 4 h. Proteins were solubilized and immunoprecipitated with mAb to VSV G and analyzed by SDSPAGE. The turnover rate of Gpt during each treatment was quantitated by volume analysis on a phosphorimager, 0 h (■), 4 h (□), and 8 h (■).

**Figure 6.** Requirement of a reducing environment for efficient degradation of Gpt. Vacc-Gpt-infected A20 cells were metabolically labeled for 15 min at 37°C with [35S]methionine. The cells were chased in medium (control), in the presence of 1 mM diamide for 8 h, or in the presence of 1 mM diamide for 4 h and washed and resuspended in medium containing 5 mM 2-ME for a further 4 h. Proteins were solubilized and immunoprecipitated with mAb to VSV G and analyzed by SDS-PAGE. The turnover rate of Gpt during each treatment was quantitated by volume analysis on a phosphorimager, 0 h (■), 4 h (□), and 8 h (■).

chymotrypsin-like serine proteases, had no significant inhibitory effect. The cysteine protease inhibitor E-64 did not inhibit degradation (data not shown), and as shown previously, nor did leupeptin (Fig. 4). The spectrum of inhibitors suggests that a serine-type protease is involved in the degradation of Gpt.

major redox buffer in the secretory pathway [53], inhibited degradation (Fig. 6). Analysis of the resulting bands by phosphor-imager (Fig. 6) showed that in the presence of 1 mM diamide, only 30% of the labeled protein was degraded in 4 h, in marked contrast to untreated control cells, which was around 73%. Inhibition of degradation was reversed when cells were washed and resuspended in fresh medium containing 5 mM 2-ME (Fig. 6). Diamide has also been shown to inhibit pre-Golgi degradation of several sensitive substrates such as chimeric CD4-TCRα [54] and CH12x [55]. The data suggest that reduced sulfhydryl groups are required for rapid degradation. Details of this process remain to be established, although one possibility may be the involvement of enzymes such as the cysteine or serine proteases [56, 57].

**3.6 Gpt degradation is inhibited by serine protease inhibitors**

To determine the specificity of the degradation, several types of membrane-permeable protease inhibitors were tested. The most effective of them was the mechanism-based serine protease inhibitor 3,4-dichloroisocoumarin (DCI) [58]. A time course of Gpt degradation in the presence of DCI (Fig. 7) showed that 94% of newly synthesized Gpt was protected from degradation after 2 h, and that 77% remained intact even after 8 h. 1-Chloro-3-tosylamido-7-amino-2-heptanone (TLCK), an inhibitor of trypsin-like serine proteases, also inhibited degradation, but was less effective than DCI (Fig. 7). L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) which blocks

Table 1 summarizes data gathered from experiments designed to test the ability of A20 cells to process and present different forms of G to T cell hybridomas in the presence of different inhibitors. With the use of BFA, presentation of peptides derived from both exogenous as well as endogenous forms of VSV G is impeded, while sensitization of targets occurs in the presence of another Golgi-disrupting drug, IQ. The data suggest that BFA, unlike IQ, may affect other cellular processes involved in Ag processing and presentation, in addition to retrograde transport of Golgi membranes to the ER, while IQ appears to be more specific in its effect. The mechanisms by which BFA and IQ affect ER- to Golgi transport are known to be distinct [47-49]. The data also indicate the possibility that peptides or peptide-MHC class II complexes may travel into an early compartment and be targeted to the cell surface or to the endosome/lysosome. The effect of IQ and BFA on the disruption of the Golgi in A20 cells has been verified by immunofluorescence studies (manuscript in preparation).

Upon incubation of vacc-Gpt-infected A20 cells in 3,4-DCI, which has been shown to affect the T1/2 of the protein (Fig. 7), there was virtually no presentation of G epitopes, implicating serine proteases in the production of epitopes from the ER-retained Gpt. The protease inhibitor did not appear to prevent class II molecules from acquiring peptides, since A20 cells sensitized with Gs (exogenous pathway), as well as vacc-G-infected A20 cells (exogenous and endogenous), were able to present G epitopes efficiently.

**3.7 Dissection of the pathway involved in the processing and presentation of Gpt**
This study was undertaken to define the pathway(s) by which processing of the viral glycoprotein is readily detected [23].

VSV elicited class I1-restricted G-specific CTL and T cell hybridomas [28]. We also observed that endogenous processing of the viral glycoprotein is readily detected [23]. This study was undertaken to define the pathway(s) by which endogenous VSV glycoprotein is processed and presented with class II molecules. For these studies, the ER-retained form of the glycoprotein expressed in a vaccinia vector, vacc-Gpt, was used. Additionally, possible exogenous glycoprotein would be processed and presented by the APC.

Although the Endo H sensitivity of the mutant protein has been established by Rose in transfected COS-1 cells [29], it was important to determine that no other forms of the protein were found in post-ER organelles, especially in the cis-Golgi, of A20 cells. Similar studies done by Kitleston et al. [59] using intracellularly expressed influenza hemagglutinin showed that the protein form that was efficiently expressed on APC was processed in the cis compartment of the Golgi, and was then transported by vesicles to the compartment of peptide loading (CPL). To investigate whether this was the case in our system, Gpt was tested for sensitivity to Endo D, which cleaves complex sugars present on trimmed oligosaccharides in the cis-/medial-Golgi [63]. Gpt remained Endo H sensitive and Endo D resistant while it was being degraded (Fig. 1), indicating that proteolysis was restricted to the ER.

It only takes a few molecules undetected by conventional biochemical analysis to elicit an immune response; in the case of Gs, fewer than $10^3$ molecules/cell is sufficient [35]. To verify that no immunogenic material was secreted into the medium or present on the cell surface available to reenter the APC by endocytosis or recycling, more sensitive functional immunological assays were carried out. No surface glycoprotein was present on vacc-Gpt-infected A20 cells as determined by flow cytometry (Fig. 2). In parallel experiments, innocent bystander assays, as well as exposure of uninfected A20 cells to supernatants from virus-infected cells, showed no target sensitization of APC for G-specific class I1-restricted CTL (Fig. 3). These results eliminate the possibility that an exogenous form of the antigen is responsible for the presentation of Gpt by class II molecules.

Earlier studies showed that the sensitivity of targets infected by vacc-Gpt for G-specific, class I1-restricted CTL was inhibited by late NH$_4$Cl treatment, implicating the involvement of a low-pH compartment [22]. Autophagosomes are thought to arise from the ER and have been implicated in the turnover of the ER proteins, glucosidase II and cytochrome P-450 [43, 60]. In fact, there is evidence that enhanced protein degradation, observed when cells are starved for serum or nutrients, proceeds by autophagic vesicle formation and fusion of these vesicles to lysosomes [61, 62] which contain acid hydrolases. Plasma membrane, Golgi and endosomal membrane antigens were not found in these vacuoles, in contrast to rough ER luminal proteins, which were detected in these autophagous vesicles [43]. In a sense, a cell may be considered to be starved upon viral infection due to the production of viral proteins rather than cellular proteins. Therefore, we tested whether the degradation of the mutant glycoprotein occurred in the ER, or whether fusion with lysosomes, essential for autophagy, was necessary. Ammonium chloride, which inhibits low-pH dependent lysosomal/endosomal enzymes [44], leupeptin, a serine- and thiol-protease inhibitor which inhibits lysosomal protease activities without altering intravesicular pH [45], monensin, and BFA or IQ, agents that disrupt the integrity of the Golgi [47–49], were added to cultures of vacc-Gpt-infected A20 cells in the presence of a radioactively labeled amino acid. No significant retardation in degradation occurred in the presence of these drugs (Fig. 4), consistent with the interpretation that the ER is the site of Gpt degradation. These results also indicate that autophagy is unlikely to be involved. Further evidence that the ER is the site of Gpt degradation is provided by the results of our determination of the pH optimum; degradation of Gpt occurs maximally in a neutral environment (Fig. 6).

That a reducing environment is required for degradation of Gpt, demonstrated by the reversible inhibition with diamide (Fig. 5), can be explained in several ways. The lumen of the ER is oxidizing, and this redox status is likely to be maintained by a glutathione-based redox buffer [53]. Oxidizing conditions promote protein folding by allowing newly synthesized membrane proteins to form disulfide bonds. Addition of reducing agents to cells in vivo inhibits disulfide bond formation within the ER [63, 64]. The improperly folded proteins could expose protease-sensitive sites that are otherwise conformationally masked under normal oxidizing conditions. Indeed, several groups have shown that the conformation of the ectodomain of hemagglutinin [63] of the mouse hepatitis coronavirus glycoprotein [64, 65], as well as that of VSV G [66] is highly dependent on disulfide bond formation.

A second interpretation of the results could be that free sulfhydryl groups are critical for the activity of ER protea-
The proteinase responsible for Gpt degradation in the ER are the inhibitor studies narrow the possible site of Gpt processing to the ER. Some of these inhibitors have also been used to examine the delivery of nascent MHC class II-Li complexes to lysosomal compartments for subsequent loading of peptide upon removal of Ii. Ammonium chloride and leupeptin have been shown to inhibit not only Ii breakdown, but also the conversion of class II molecules to an SDS-resistant complex [72, 73], which is indicative of class II molecules being occupied by peptide [74]. Morphological and immunocytochemical studies of B cells have implicated a post-Golgi organelle, either early endosomes [75] or a prelysosomal structure designated CPL [76], as the entry point of class II-Li complexes to the endocytic pathway. To determine whether ER-Golgi transport is important in the presentation of the resulting peptides from Gpt by class II molecules, the antigen-presenting capacity of infected A20 cells was assayed. In Table 1, BFA and Iq, known to prevent ER-to-Golgi transport, it is shown that treatment with Iq did not interfere significantly with the presentation of peptides derived from Gpt; in contrast, BFA blocked recognition by the hybridomas. It is likely that in the case of BFA, the integrity or transport of class II molecules not only from ER to Golgi, but also from Golgi to the CPL and further to the plasma membrane, may be compromised.

The mechanisms by which BFA and Iq impede transport from the ER to the Golgi are distinct. BFA causes fusion of the Golgi with the ER, resulting in retrograde transport of Golgi proteins, as well as inhibition of the association of transport vesicle coat components β-COP and ARF [47-49]. BFA has also been shown to affect traffic between the endosome and lysosome [77]. Upon treatment of NRK cells with Iq, Golgi membranes become highly fragmented and are subsequently dispersed throughout the cytoplasm; thus, proteins synthesized on rough ER and targeted to the cell surface exit the ER, but fail to reach the mediol Golgi membranes [50]. IQ has the same effect on A20 cells (Barto, Stein, and Reiss; manuscript in preparation).

DCI inhibited recognition of class II-G complexes on fixed vacc-Gpt A20 cells by T cell hybridomas, while recognition of these complexes was readily detected on A20 cells treated with exogenous Gs (Table 1). This result suggests the involvement of a serine proteinase in the processing of Gpt.

The findings reported here indicate that nascent proteins which fail to meet the structural criteria for transport to the Golgi can be rapidly and efficiently degraded by the cell, and that the site of degradation is either part of or closely related to the ER. Peptides resulting from proteolysis in the ER, along with those generated in the cytosol and endosome, may contribute to the array of antigenic fragments displayed by MHC class II molecules on the cell surface. Where does the charging of ER-generated Gpt peptides on class II molecules occur? It is possible that Gpt peptides may charge class II molecules in the ER, as proposed by Weiss and Bogen [78] for peptides derived from ER proteins. We have recently detected the presentation of epitopes from vacc-Gpt-infected splenic cells derived from II−/− mice using either I-Ak or I-Ek restricted T cell hybridomas (manuscript in preparation). In the absence of invariant chain, A0.1, and AI0.1 molecules assemble efficiently and are conformationally similar to mature wild-type heterodimers [79]. Alternatively, peptides may traffic with the class II molecules into an early Golgi compartment and charge the class II molecule there, and then be directly targeted to the surface. Finally, peptides may travel with class II to a late endosome/lysosome for charging on a recycling class II molecule. Work is in progress to distinguish among these possibilities.

One significant aspect of this phenomenon might be the development of T cell tolerance to self proteins, since during development of the T lymphocyte repertoire, peptides derived from intracellular proteins may be generated, leading to negative selection of self-reactive T lymphocytes. Peptides found in association with murine class II Aα have been mostly found to be derived from secretory or integral membrane proteins [80]. The importance of the contribution of the ER compared to the cytosol or endosome in the generation of processed peptides for self- and non-self-discrimination remains to be determined.

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