Cytoplasmic Processing Is a Prerequisite for Presentation of an Endogenous Antigen by Major Histocompatibility Complex Class II Proteins

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Abstract

Biochemical and functional studies have demonstrated major histocompatibility complex (MHC) class II–restricted presentation of select epitopes derived from cytoplasmic antigens, with few insights into the processing reactions necessary for this alternate pathway. Efficient presentation of an immunodominant epitope derived from glutamate decarboxylase (GAD) was observed regardless of whether this antigen was delivered exogenously or via a cytoplasmic route into human histocompatibility leukocyte antigen class II–DR4+ antigen-presenting cells. Presentation of exogenous as well as cytoplasmic GAD required the intersection of GAD peptides and newly synthesized class II proteins. By contrast, proteolytic processing of this antigen was highly dependent upon the route of antigen delivery. Exogenous GAD followed the classical pathway for antigen processing, with an absolute requirement for endosomal/lysosomal acidification as well as cysteine and aspartyl proteases resident within these organelles. Presentation of endogenous GAD was dependent upon the action of cytoplasmic proteases, including the proteasome and calpain. Thus, translocation of processed antigen from the cytoplasm into membrane organelles is necessary for class II–restricted presentation via this alternate pathway. Further trimming of these peptides after translocation was mediated by acidic proteases within endosomes/lysosomes, possibly after or before class II antigen binding. These studies suggest that processing of exogenous and cytoplasmic proteins occurs through divergent but overlapping pathways. Furthermore, two cytoplasmic proteases, the proteasome and calpain, appear to play important roles in MHC class II–restricted antigen presentation.

Key words: proteasome • calpain • cathepsin • cytoplasm • protease

Introduction

MHC class II molecules selectively present peptides derived from self- and foreign antigens to CD4+ T cells, providing a physiological survey of host integrity. Biochemical studies to define the source of antigenic peptides associated with class II molecules have revealed a predominance of peptides derived from exogenous antigens, as well as epitopes from the plasma membrane and endosomal proteins (1, 2). Such ligands would predictably intersect class II protein in transit to the cell surface via the endosomal pathway (3, 4). By contrast, the observation that peptides derived from cytoplasmic antigens are also presented by class II proteins appears more difficult to reconcile. Epitopes from both soluble and membrane-bound proteins localized within the cytoplasm have been observed to be efficiently presented by class II complexes using human and murine APCs (5, 6). Furthermore, studies suggest that some viral epitopes derived from cytoplasmic antigens gain access to class II proteins for presentation (7, 8). Cytoplasmic antigens have long been established as the primary source of peptides for MHC class I molecules, although studies suggest that in specialized APCs, class I molecules may access proteins within endosomal/phagosomal compartments (9, 10). Likewise, class II proteins may have evolved mechanisms to sample antigenic peptides within the cytoplasm, potentiating helper T cell responses to intracellular pathogens and the maintenance of self-tolerance (6).
Peptides derived from extracellular or exogenous antigens are generated by processing reactions localized within a complex network of endosomal and lysosomal vesicles (11). Antigen denaturation by the acid pH within these organelles coupled with the action of resident endopeptidases and carboxypeptidases appears to be a key step in the generation of ligands for MHC class II complexes. Chief among these proteases are the cathepsins with acidic pH optima and a broad range of substrate specificity (12). These include the cysteine proteases cathepsins B, H, S, and L and the aspartic hydrolases cathepsins D and E. Cathepsins have been implicated in the processing of a variety of exogenous antigens for class II–restricted presentation (13, 14). By contrast, much less is known concerning the enzymes required for endogenous antigen processing. Nascent class II proteins are associated with an intracellular chaperone protein, the invariant chain (Ii), which must be proteolytically processed before peptide binding by these MHC antigens. Ii serves a dual purpose in protecting the ligand-binding groove of class II complexes, as well as guiding class II molecules to endocytic compartments rich in antigenic peptides. Studies of Ii processing may provide some insights into the processing environment encountered by endogenous antigens within endosomes and lysosomes, as both cysteine and aspartic cathepsins have been implicated in Ii degradation (14–16). Studies using mice genetically targeted to inactivate cathepsin S suggest this protease functions at a late stage of Ii processing within professional APCs, whereas cathepsin L appears important in catalyzing this terminal reaction within thymic cells (17). Thus, endogenous antigens trafficking through endosomes may likely encounter similar proteases to those functioning in the processing of exogenous proteins. Remarkably, endogenous cytoplasmic antigens also access the class II presentation pathway, as shown by class II loading with epitopes derived from resident cytoplasmic proteins (18). It remains unclear whether acidic cathepsins or cytoplasmic proteases, optimally active at neutral pH, are required for processing cytoplasmic antigens for MHC class II–restricted presentation.

This study addresses the requirements for processing and MHC class II–restricted presentation of the autoantigen glutamate decarboxylase (GAD) derived exogenously, as well as from its natural cytoplasmic environment. GAD has been localized to the cytoplasmic face of membrane organelles in neuroendocrine cells, and this enzyme represents a key target autoantigen in several autoimmune disorders, including insulin-dependent diabetes mellitus (IDDM) (19). Our data demonstrate divergent antigen processing pathways for exogenous and cytoplasmic GAD. Although both exogenous and endogenously derived GAD access endosomal/lysosomal proteases, a distinct cytoplasmic processing pathway was identified for GAD originating in the cytosol. Thus, presentation of cytoplasmic-derived GAD required the function of a cytoplasmic Ca\(^{2+}\)-dependent protease, calpain, and the multicatalytic proteasome. These results demonstrate that processing reactions within the cytoplasm govern the class II–restricted presentation of a subset of endogenous antigens. These studies also revealed that after translocation from the cytoplasm into membrane organelles, cytoplasmic antigens are further processed by endosomal/lysosomal proteases before presentation by class II complexes.

### Materials and Methods

#### Cell Lines

The B lymphoblastoid cell line (B-LCL) Priess homozygous for the expression of HLA-DR4 (DRA*10101, DRB*0401) was maintained in Iscove’s medium (GIBCO BRL) supplemented with 10% heat-inactivated calf serum. Retroviral transduction of the parental cell line Priess with the gene encoding the 65-kD form of human GAD resulted in a cell line constitutively expressing this endogenous antigen, termed PriessGAD. Endogenous GAD expression levels were equivalent using retroviral gene transduction or standard transfection methods with GAD expressed in the pMCFRpacGAD vector (20). Conditioned medium was generated from PriessGAD cells by culturing overnight, followed by passage of this spent medium through a 0.2-µm filter. The T cell hybridoma 33.1 is specific for the peptide GAD273–285 presented by HLA-DR4 (provided by Linda Wicker, Merck Research Laboratories, Rahway, NJ). This cell line was maintained in RPMI (GIBCO BRL) supplemented with 10% FCS, 0.1% β-mercaptoethanol, and 100 U/ml penicillin and 100 µg/ml streptomycin. Control studies with the class II–negative cell line, T2, confirmed the DR4 restriction of the 33.1 T cell. T2 is a B–T cell hybrid with a large deletion of the MHC class II locus and was maintained in the same tissue culture medium as the Priess cell lines.

#### Inhibitors, Purified Antigens, and Synthetic Peptides

Chloroquine and leupeptin were purchased from Sigma Chemical Co. and were solubilized in PBS. Cathepsin B inhibitor II, calpeptin, PD 150606, and lactacystin were purchased from Calbiochem. These inhibitors, in addition to pepstatin A and brefeldin A (BFA; Sigma Chemical Co.), were solubilized in DM SO. The recombinant 65-kD form of human GAD was produced in bacteria and purified to homogeneity using affinity chromatography, as published previously (21). Studies indicated that both a truncated form of recombinant GAD lacking the NH\(_2\)-terminal domain and full-length GAD were presented equivalently by APCs GAD273–285 (IAFTSEHSHFSLK) was synthesized using an Applied Biosystems peptide synthesizer and FMOC technology. Peptide purity and structure was confirmed by reverse phase HPLC and mass spectroscopy.

#### Electroporation of Antibody into APCs

PriessGAD cells were washed twice in ice-cold PBS (GIBCO BRL) and resuspended at 2 x 10\(^6\) cells/ml in ice-cold PBS. 50 µl of this cell solution was placed into 0.4-cm electroporation cuvettes (Bio-Rad) and placed on ice. 2 µl of mAb raised against calpain I or II (Calbiochem), or an IgG1 isotype control (Sigma Chemical Co.) was added (final antibody concentration of 0.16 µg/ml, 0.4 µg/ml, and 40 µg/ml, respectively). Each cuvette was gently mixed and placed on ice for 10 min, then pulsed at 0.270 kV, 0.125 µF (400 ohms resistance). Immediately afterwards, 1 ml of warm media was added, and the cells were transferred to a 48-well tissue culture plate. The cells were incubated at 37°C for 20 h and fixed in 1% paraformaldehyde, and GAD was presentation assessed.
Subcellular Fractionation and Immunoblotting. To localize intracellular GAD, cytosolic and membrane organelle fractions were isolated from PriessGAD as described previously (22). In brief, cells were lysed using a Balch homogenizer, then differential centrifugation was performed to isolate a) soluble cytoplasmic, and membrane organelle fractions. Membrane organelles were washed in a high salt buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 10 mM benzamidine, 0.1 mM p-chloromercuri phenylsulfonic acid [p-CMPS]) to disrupt loosely bound proteins. The isolated particulate and soluble fractions were assayed by Western blot analysis. For each fraction, equal amounts of protein were boiled in reducing sample buffer and separated by 12% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane (Micron Separations, Inc.) and probed with the GAD-specific mAb GAD6 (23). Bound GAD6 was visualized using goat anti-mouse horseradish peroxidase (Jackson Laboratories) followed by epichrominunescence (Amersham Pharmacia Biotech).

Elution of Peptides from Surface MHC Class II. To remove acid-labile peptides from cell surface class II complexes, PriessGAD cells were washed twice in ice-cold 160 mM NaCl, then resuspended in 160 mM NaCl/citric acid, pH 4.0 and incubated for 30 min on ice. These cell preparations were neutralized with 160 mM NaCl, 10 mM benzamidine, 0.1 mM p-chloromercuri phenylsulfonic acid [p-CMPS] to disrupt loosely bound proteins. The isolated particulate and soluble fractions were assayed by Western blot analysis. For each fraction, equal amounts of protein were boiled in reducing sample buffer and separated by 12% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane (Micron Separations, Inc.) and probed with the GAD-specific mAb GAD6 (23). Bound GAD6 was visualized using goat anti-mouse horseradish peroxidase (Jackson Laboratories) followed by epichrominunescence (Amersham Pharmacia Biotech).

Endogenously expressed GAD associates with the cytoplasmic face of synaptic vesicles as demonstrated by microscopy and subcellular fractionation (19). Sequences within the N-formyl peptide for an additional 2 h. Peptide controls were used to ensure that inhibitors affect only antigen processing and not class II maturation or cell surface expression. To monitor endogenous GAD processing and presentation, PriessGAD cells were incubated with or without inhibitors for 18 h with no visible loss of viability. For studies with the calpain inhibitor PD 150606, APCs were cultured in Ca²⁺-free medium. B-LCLs typically display a high Ca²⁺ influx because of the expression of the EBV-encoded latent membrane protein L (24). In some experiments, PriessGAD cells were acid washed before incubation with inhibitors. In all cases, APCs were subsequently fixed in 1% paraformaldehyde for 10 min at room temperature and washed extensively in cold tissue culture medium. After fixation, 2 × 10⁴ APCs were cocultured with 10⁴ GAD-specific T hybridoma cells for 24 h at 37°C. IL-2 production was measured using the IL-2-dependent cell line, HT-2. HT-2 cells were incubated for 8–16 h with aliquots of T cell culture supernatants, followed by the addition of [³²P]thymidine. After an additional 8–16 h of incubation at 37°C, HT-2 cells were harvested using a 96-well plate cell harvester (Skatron). [³²P]thymidine incorporation was measured using a Wallac microplate reader. The viability of APCs was monitored in all studies with inhibitors, and the concentrations of drugs were determined to be nontoxic. The concentrations of proteases inhibitors tested did not disturb peptide processing or general class II protein function, as was demonstrated in control studies monitoring II peptide complexes using PAGE (13). All T cell assays were performed in triplicate with the mean and SD calculated for each data point. The percentage of T cell activation of inhibitor-treated APCs was calculated relative to untreated samples. All figures are representative of a minimum of three independent experiments.

Results

G A D Localizes to the Cytoplasmic Face of Membrane Vesicles in the B-LCL PriessGAD. In GABA-secreting neurons, GAD associates with the cytoplasmic face of synaptic vesicles as demonstrated by microscopy and subcellular fractionation (19). Sequences within the N-formyl peptide for an additional 2 h. Peptide controls were used to ensure that inhibitors affect only antigen processing and not class II maturation or cell surface expression. To monitor endogenous GAD processing and presentation, PriessGAD cells were incubated with or without inhibitors for 18 h with no visible loss of viability. For studies with the calpain inhibitor PD 150606, APCs were cultured in Ca²⁺-free medium. B-LCLs typically display a high Ca²⁺ influx because of the expression of the EBV-encoded latent membrane protein L (24). In some experiments, PriessGAD cells were acid washed before incubation with inhibitors. In all cases, APCs were subsequently fixed in 1% paraformaldehyde for 10 min at room temperature and washed extensively in cold tissue culture medium. After fixation, 2 × 10⁴ APCs were cocultured with 10⁴ GAD-specific T hybridoma cells for 24 h at 37°C. IL-2 production was measured using the IL-2-dependent cell line, HT-2. HT-2 cells were incubated for 8–16 h with aliquots of T cell culture supernatants, followed by the addition of [³²P]thymidine. After an additional 8–16 h of incubation at 37°C, HT-2 cells were harvested using a 96-well plate cell harvester (Skatron). [³²P]thymidine incorporation was measured using a Wallac microplate reader. The viability of APCs was monitored in all studies with inhibitors, and the concentrations of drugs were determined to be nontoxic. The concentrations of proteases inhibitors tested did not disturb peptide processing or general class II protein function, as was demonstrated in control studies monitoring II peptide complexes using PAGE (13). All T cell assays were performed in triplicate with the mean and SD calculated for each data point. The percentage of T cell activation of inhibitor-treated APCs was calculated relative to untreated samples. All figures are representative of a minimum of three independent experiments.

Figure 1. Endogenously expressed GAD associates with the cytoplasmic face of membranes in the B-LCL PriessGAD. Parental Priess (lanes 1, 3, 5, and 7) and PriessGAD (lanes 2, 4, 6, and 8) cells were lysed using an ice-cold Balch homogenizer. After the removal of nuclei, particulate (M) and soluble (S1) fractions were separated by centrifugation at 100,000 g. The membrane pellet was washed to elute loosely associated membrane proteins, and was centrifuged as before to yield the washed membrane (W M) fraction and the corresponding supernatant (S2; reference 22). All fractions were then solubilized in 2% Triton X-100. Equal amounts of total protein from each fraction were separated by gel electrophoresis and probed with the mAb GAD6.
previously identified using exogenous GAD in DR4+ human APCs and transgenic mice (20). Immunoblot analysis failed to detect secreted GAD in conditioned medium from these cells, suggesting cytoplasmic GAD was processed through an endogenous mechanism for class II presentation (data not shown). To confirm that minute quantities of GAD or GAD-derived peptides were not released by PriessGAD and presented after endocytosis, functional studies were conducted to screen for GAD secretion by these APCs (Fig. 2). Conditioned medium was prepared from PriessGAD cells, and upon incubation with the parental DR4+ cell Priess, this medium failed to stimulate GAD-specific T cells. Incubation of conditioned medium from PriessGAD cells with viable PriessGAD cells also failed to significantly enhance or inhibit the inherent presentation of this endogenous antigen. These results indicate exogenous GAD or GAD fragments were not released by transduced cells and could not contribute to the class II-restricted presentation of this antigen. Thus, efficient presentation of the cytoplasmic self-antigen GAD was not dependent upon secretion and endocytosis of the antigen via the classical MHC class II processing pathway. Rather, this cytoplasmic antigen gains direct access to class II proteins through an endogenous processing mechanism. Newly Synthesized MHC Class II Molecules Are Required for Presentation of GAD

Derived from both Exogenous and Cytoplasmic GAD. Studies with exogenous antigens have established that MHC class II proteins access antigenic peptides via two distinct pathways (3, 26). Newly synthesized class II α/β heterodimers en route to the cell surface intersect a wide variety of peptide ligands within late endosomal- and lysosomal-like compartments such as MHC class II compartment (11). By contrast, a pool of mature cell surface class II proteins transit through recycling early endosomal vesicles acquiring distinct antigenic epitopes via the process of peptide editing. The fungal product BFA has been widely used to differentiate these pathways (27, 28). This inhibitor of anterograde movement from the endoplasmic reticulum to the Golgi complex blocks presentation of epitopes by newly synthesized class II proteins without perturbing the display of peptides by recycling class II molecules. As expected, this inhibitor blocked exogenous GAD presentation by HLA-DR4 (Fig. 3 A). Similarly, incubation of PriessGAD cells in the presence of BFA resulted in a significant reduction in cytoplasmic GAD presentation (Fig. 3 B). Presentation of the synthetic GAD peptide was not altered by BFA, as was expected based on the binding of the peptide to preexisting cell surface class II complexes. Together, these data indicate that presentation of both cytoplasmic and exogenous GAD was dependent upon the binding of GAD epitopes to newly synthesized MHC class II α/β heterodimers on route to the cell surface.

Cytoplasmic GAD is constitutively expressed in the PriessGAD cell line, such that abundant levels of preformed GAD peptide-class II complexes are always present on the surface of these APCs. Exposing these cells to mild acid pH or buffers with high ionic strength was found to release GAD epitopes from class II DR4 molecules on the cell surface, and permitted analysis of newly formed intracellular peptide-class II complexes in transit to the cell surface (29). These treatments did not alter surface class II protein peptide binding, nor was the export of newly generated GAD peptide-class II complexes to the cell surface perturbed. Disruptions in GAD epitope presentation were observed in cells using drugs such as BFA regardless of acid stripping; however, this inhibition was more complete when the acid stripping protocol was employed.

MHC Class II Presents Cytoplasmic GAD Independent of Endosomal/Lysosomal Acidification. Once resident within the endocytic pathway, newly synthesized MHC class II

Figure 2. PriessGAD secretes neither GAD nor GAD fragments for reuptake via the classical MHC class II pathway. Priess (white bars) or PriessGAD (gray bars) cells were incubated for 18 h in fresh media or PriessGAD-conditioned media (CM). These cells were then incubated with the T cell 33.1 to assess GAD epitope presentation. T cell activation was not enhanced by incubation of APCs with conditioned media. The addition of 10 μM GAD273-285 to control APCs resulted in equivalent stimulation of the T cell 33.1, indicating nearly identical levels of functional HLA-DR 4 in both APCs.

Figure 3. Presentation of both exogenous and cytoplasmic forms of GAD required newly synthesized MHC class II molecules. (A) Priess cells were preincubated with or without BFA (50 ng/ml), then exogenous GAD was added, and the cells were incubated at 37°C for 18 h (white bars). (B) Acid-stripped PriessGAD cells (gray bars) were incubated for 18 h in the presence or absence of 50 ng/ml BFA. Control samples were pulsed with 8 μM GAD273-285 (A and B, black bars) 2 h before fixation. The cells were then paraformaldehyde fixed, and antigen presentation was assessed using the 33.1 T cell hybridoma as described in Materials and Methods. Maximal [%H] thymidine incorporation was 14,439 cpm and 32,097 cpm for exogenous and cytoplasmic GAD presentation, respectively. Maximal cpm for corresponding peptide-pulsed samples were 416,378 cpm and 340,856 cpm, respectively. Background (T cells alone) was 3,602 cpm.
molecules encounter an acidic environment favoring antigen denaturation and hydrolysis (11). The low pH in late endosomes and lysosomes may also facilitate conformational alterations in MHC class II dimers, enhancing interactions with HLA-DM and peptide binding (30). However, some class II–restricted antigenic epitopes are presented independent of pH, and there is evidence that peptides may also be generated in early endosomes with only mildly acidic pH (3, 31).

To determine whether HLA-DR4–restricted presentation of GAD epitopes was dependent upon the acidification of vesicular organelles, APCs were incubated with the lysomotropic agent chloroquine. This drug has been shown to block the acidification of late endosomes and lysosomes, thereby inhibiting endosomal processing by resident cysteine proteases. Presentation of the peptide GAD273-285 derived from exogenous antigen was completely abrogated at low concentrations of chloroquine (Fig. 4 A). However, only minimal inhibition in presentation of the same epitope derived from cytoplasmic GAD was observed, with a significant portion of this endogenous processing pathway proving resistant to the action of chloroquine (Fig. 4 B). These results demonstrate that processing and presentation of endogenous GAD, in contrast with exogenous forms of the antigen, does not display absolute dependence upon endosome/lysosomal acidification. The measurable endogenous GAD epitope presentation in the presence of chloroquine further suggests that, at these concentrations, this lysomotropic agent does not abolish intracellular ligand binding to class II molecules or the transport of class II–peptide complexes to the cell surface, steps common to both endogenous and exogenous antigen presentation. Studies of exogenous peptide presentation by class II proteins were unaltered by chloroquine (Fig. 4), in agreement with control experiments (data not shown) demonstrating no change in II fragmentation or the formation of stable class II–peptide complexes using low drug concentrations. Thus, class II antigen function and surface expression were not grossly perturbed by the concentrations of chloroquine tested. In total, these findings suggest that presentation of exogenous GAD is absolutely dependent upon the acidic environment found within late endosomes and lysosomes. In contrast, this critical component of the classical MHC class II presentation pathway plays a less essential role in processing GAD originating in the cytoplasm of APCs.

Exogenous and cytoplasmic GAD are processed through divergent proteolytic pathways. Endocytosed antigens are cleaved by proteases active within the acidic environment of endosomal and lysosomal vesicles. By contrast, cytoplasmic proteases function to degrade self- and foreign proteins at neutral pH. To dissect the relative contributions of these two distinct types of proteases in exogenous and cytoplasmic GAD processing and presentation, functional studies were conducted using class-specific proteolytic inhibitors. Leupeptin, which inactivates serine and thiol proteases including cathepsins B, S, and L, completely blocked the

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Figure 4. HLA-DR4–mediated presentation of exogenous but not cytoplasmic GAD requires acidification of endosomal and lysosomal vesicles. (A) Priess cells pulsed with exogenous GAD were incubated in the presence or absence of increasing concentrations of chloroquine for 18 h (O). (B) PriessGAD cells (□) were incubated with increasing concentrations of chloroquine. Control APCs (A [●] and B [■]) were pulsed with 10 \( \mu \text{M} \) GAD273-285 3 h before fixation and subsequent assessment of GAD presentation, as described in Materials and Methods. Additionally, Priess cells (C) were exposed to the cathepsin B inhibitor (CBI II; 10 \( \mu \text{M} \)), followed by the addition of exogenous GAD (white bars). (D) Acid-stripped PriessGAD cells (gray bars) were treated with the cathepsin B inhibitor (CBI II; 10 \( \mu \text{M} \)). Control APCs (C and D, black bars) with or without cathepsin B inhibitor treatment were pulsed with 8 \( \mu \text{M} \) GAD273-285 2 h before fixation in 1% paraformaldehyde. GAD–specific presentation was assessed as described in Materials and Methods.

Figure 5. Class II–restricted presentation of exogenous and cytoplasmic GAD displays differential requirements for endosomal cysteine proteases. (A) Priess cells pretreated with leupeptin were incubated with exogenous GAD (O). (B) PriessGAD (□) were treated with increasing concentrations of leupeptin. After exposure to leupeptin, control APCs were pulsed with 10 \( \mu \text{M} \) GAD273-285 (A [●] and B [■]) 3 h before fixation and subsequent assessment of GAD presentation, as described in Materials and Methods. Additionally, Priess cells (C) were exposed to the cathepsin B inhibitor (CBI II; 10 \( \mu \text{M} \)), followed by the addition of exogenous GAD (white bars). (D) Acid-stripped PriessGAD cells (gray bars) were treated with the cathepsin B inhibitor (CBI II; 10 \( \mu \text{M} \)). Control APCs (C and D, black bars) with or without cathepsin B inhibitor treatment were pulsed with 8 \( \mu \text{M} \) GAD273-285 2 h before fixation in 1% paraformaldehyde. GAD–specific presentation was assessed as described in Materials and Methods. M animal [H] thymidine incorporation for untreated samples was 56,384.7 cpm and 28,241.2 cpm for exogenous and cytoplasmic GAD presentation, respectively. M animal [H] thymidine incorporation for corresponding peptide-pulsed samples was 65,503.3 cpm and 58,802.9 cpm, respectively. Background (T cells alone) was 1,563.7 cpm.
presentation of exogenous GAD (Fig. 5 A). This result is consistent with the transport of GAD through endosomal or lysosomal compartments for presentation by class II antigens. By contrast, endogenous GAD processing was only partially reduced upon incubation of PriessGAD cells with this peptidyl aldehyde (Fig. 5 B). The concentrations of leupeptin tested did not perturb overall class II antigen maturation or surface expression, as demonstrated using synthetic peptides (Fig. 5) and experiments to monitor the formation of SDS-stable α/β dimers and Ii proteolysis (data not shown).

To further define the role of acidic cysteine proteases in cytoplasmic antigen processing, GAD presentation was examined in cells treated with cathepsin B inhibitor II, a leupeptin analogue that has been shown to preferentially block the action of cathepsin B (32). A partial reduction in both exogenous and cytoplasmic GAD presentation was observed with inhibition of this protease as measured by antigen-specific T cell activation (Fig. 5, C and D). Cathepsin B has amino- and carboxypeptidase specificity, and within endosomes this enzyme may serve in the final stages of trimming GAD epitopes before or after binding to class II complexes. This is supported by the observation that cathepsin B has an occluding loop within its active center, favoring exopeptidase activity while limiting its endopeptidase activity (33). Overall, these results demonstrate that cytoplasmic GAD or fragments of this antigen gain access to endosomal/lysosomal proteases necessary to promote efficient interactions with class II complexes and T cell recognition. Equally important, the observation of leupeptin-insensitive GAD presentation suggests that alternate processing steps are required for this cytoplasmic antigen.

Pepstatin A is a peptide inhibitor that blocks the activity of endosomal and lysosomal aspartyl proteases and degradation of endocytosed antigens in macrophages and B cells (14). To define a role for these endosomal hydrolases in the processing of exogenous and cytoplasmic GAD, APCs were incubated with pepstatin A. This inhibitor of cathepsins D and E efficiently blocked presentation of GAD\(^{273–285}\) derived from the exogenous form of this antigen (Fig. 6). By contrast, presentation of cytoplasmic-derived GAD was not affected by exposure of cells to pepstatin A. Control studies confirmed that the expression and function of surface class II proteins were not altered by pepstatin A treatment of cells. These data clearly suggest that MHC class II presentation of GAD epitopes derived from both exogenous and cytoplasmic antigens follows divergent but overlapping processing pathways.

Extra-lysosomal Processing Mechanisms Are Involved in MHC Class II–restricted Presentation of Cytoplasmic GAD. Cytoplasmic proteases function in supplying peptides for class I–restricted presentation. However, the role of these enzymes in generating epitopes for class II molecules remains unclear. The proteasome, a large multicatalytic complex found in the nucleus and cytoplasm, is well established as playing a critical role in class I–mediated immune responses (34). To examine the role of the proteasome in cytoplasmic antigen presentation via MHC class II molecules, B cells were treated with lactacystin. This proteasome-specific inhibitor has been shown to block MHC class I presentation of cytoplasmic antigens (35). As expected, lactacystin treatment of APCs did not inhibit MHC class II–restricted presentation of exogenous GAD (Fig. 7 A). In fact, a small but reproducible enhancement of exogenous GAD presentation via lactacystin treatment of cells was observed, suggesting that downmodulation of the proteasome may also indirectly enhance antigen processing via the endosomal/lysosomal pathway. Yet when the GAD-expressing APC, PriessGAD, was incubated with this proteasome inhibitor, a marked reduction in GAD epitope presentation

![Figure 6](image-url)

**Figure 6.** A aspartyl protease activity is required for exogenous but not cytoplasmic GAD processing. Priess cells exposed to variable concentrations of pepstatin A were then incubated for 18 h with exogenous GAD (○). PriessGAD cells (□) were treated with increasing concentrations of pepstatin A for an identical time. Control APCs (● and ■) with or without pepstatin A treatment were pulsed with 10 μM GAD\(^{273–285}\) 3 h before fixation in 1% paraformaldehyde. GAD-specific antigen presentation was assessed as described in Materials and Methods.

![Figure 7](image-url)

**Figure 7.** Proteasome inhibition selectively blocks endogenous GAD processing and MHC class II–restricted presentation. (A) Priess cells were preincubated with or without 250 nM lactacystin, a specific inhibitor of the proteasome, followed by 18-h exposure to exogenous GAD (white bars). PriessGAD cells (gray bars) were incubated for 18 h with or without the proteasome-specific inhibitor lactacystin (250 nM). Control APCs (A and B, black bars) were pulsed with 8 μM GAD\(^{273–285}\) 2 h before fixation in 1% paraformaldehyde. Antigen presentation was assessed as described in Materials and Methods. \(^{3}H\) thymidine incorporation for untreated samples was 62,397.6 cpm and 27,784.1 cpm for exogenous and cytoplasmic GAD presentation, respectively. \(^{3}H\) thymidine incorporation for corresponding peptide pulsed samples was 81,696.3 cpm and 95,691.4 cpm, respectively. Background (T cells alone) was 1,059 cpm.
was detected (Fig. 7 B). Given the reported specificity of lactacystin for the proteasome, these results clearly provide evidence that blocking proteasome activity can influence the MHC class II presentation pathway. These studies were conducted using concentrations of lactacystin demonstrated not to perturb cell viability or trigger apoptosis, even after 24 h of treatment (data not shown). Lactacystin has been used to inactivate the proteasome in APCs and blocks the generation of endogenous peptides required for assembly and stable surface expression of MHC class I (36). Surface class I antigen expression was reduced at least 30% in cells exposed to low concentration of this drug overnight, confirming proteasome inactivation (data not shown).

Complete inhibition of endogenous GAD presentation in lactacystin treated cells was not obtained, suggesting multiple enzymes may function in processing this cytoplasmic antigen for the class II pathway. Moreover, studies using lactacystin have established that certain class I epitopes are not dependent upon proteasome activity, thus alluding to the presence of additional cytoplasmic proteases involved in presentation of endogenous antigens (36, 37). In the cytoplasm, calcium-dependent cysteine proteases have been shown to modulate signal transduction pathways and transcription mechanisms through their endoproteolytic processing function (38). Calpains are found in the cytoplasm bound to intracellular membranes, as well as partitioning in the soluble fraction, prompting us to examine the role of this protease in processing endogenous GAD (39). Calpeptin is a membrane permeable inhibitor with a demonstrated specificity for cytoplasmic calpains (40, 41).

As expected, treatment of APCs with low concentrations of calpeptin did not significantly alter class II presentation of exogenously derived GAD (Fig. 8 A). In contrast, incubation of PriessGAD with calpeptin completely inhibited the presentation of cytoplasmic-derived GAD (Fig. 8 B). Studies with control APCs pulsed with synthetic GAD273-285 indicated that exposing cells to calpeptin did not effect overall class II antigen expression and function, nor was the formation of peptide-class II complex formation perturbed, as assessed by SDS-stable dimer stability assays and analysis of Ii proteolysis (data not shown). At higher concentrations of calpeptin (>10 μM), inhibition of exogenous GAD presentation could be detected. However, this was accompanied by a concomitant decrease in presentation of synthetic peptide, suggesting perturbation in overall class II maturation. Indeed, at high dosages of calpeptin, Ii cleavage fragments indicative of disturbances in endosomal protease function were detected (data not shown). Nonetheless, at the calpeptin concentrations used in this functional study, only the presentation of cytoplasmic-derived GAD was significantly altered in APCs (Fig. 8).

To further explore the novel prospect that calpains were involved in MHC class II-restricted presentation of cytoplasmic GAD, we employed the nonpeptidyl inhibitor PD150606. This mercaptoacrylic acid derivative has been shown to selectively block calpain activity by reversibly binding to the Ca2+-binding domain of the protease (42). Thus, unlike peptidyl inhibitors, there is no requirement for competitive binding within the active site of the hydrolyase, preventing any cross-reactivity of this inhibitor with calcium-independent proteases such as cathepsins B, L, and S. Treatment of APCs with PD150606 caused a marked reduction in cytoplasmic GAD presentation (Fig. 8 C).

Figure 8. Inhibition of the neutral cytoplasmic protease calpain decreases presentation of cytoplasmic GAD. (A) Priess cells were pretreated with 5 μM calpeptin, then incubated for 18 h in the presence of exogenous GAD (white bars). (B) Acid-washed PriessGAD cells (gray bars) were treated with or without the calpain-selective inhibitor calpeptin (5 μM). These samples were run in parallel to those described in Fig. 7, and percentage T cell activation was calculated relative to untreated samples shown in Fig. 7. (C) Acid-washed PriessGAD cells (gray bars) were resuspended in Ca2+-free DMEM/10% heat-inactivated calf serum and incubated in the presence of the calpain-selective inhibitor PD150606 for 20 h. Maximal 3H-thymidine incorporation was 158,388 cpm for untreated APCs and 192,482 cpm for the corresponding peptide control. In both experiments (A-C), control APCs (black bars) were treated plus or minus inhibitor and incubated for an identical period, then pulsed with 8 μM GAD273-285 2 h before fixation. GAD-specific presentation was assessed as described in Materials and Methods.

An alternate technique to specifically interrupt the activity of intracellular enzymes is through the use of mAbs. Lukas et al. (43) have shown that mAbs specific for cyclin D1 introduced into viable cells through electroporation can arrest cell cycle progression. Two calpain isozymes have been identified in B cells: calpain I (requiring micromolar Ca2+ for activation) and calpain II (requiring millimolar Ca2+; reference 44). Remarkably, introduction of an mAb specific for calpain I into electrically permeabilized PriessGAD cells reduced cytoplasmic GAD presentation (Fig. 9). An antibody specific for calpain II displayed significantly less ability to inhibit GAD presentation. Parallel control studies with an irrelevant isotype control mAb failed to produce significant inhibition of class II-restricted presentation of this cytoplasmic antigen.

Taken together, these results demonstrate a key role for calcium-dependent calpains in the processing pathway of cytoplasmic antigens for presentation by MHC class II molecules. Epitopes derived from both soluble and membrane-
II molecules. Across membranes into organelles containing receptive class gens and possibly favoring the transport of nascent peptides, sensitizing its contact with both classes of cytoplasmic antigens bound to MHC class II proteins, suggesting that select intracellular antigens gain access to class II proteins. Further, in vivo studies have also demonstrated a role for cytoplasmic antigens in inducing central tolerance via class II-restricted presentation in the thymus. To begin to unravel the mechanisms underlying these critical immunologic functions, an in vitro model system was designed to directly compare the antigen processing mechanisms of exogenous and cytoplasmic forms of GAD.

Presentation of cytoplasmic antigens by class II proteins may occur through several potential mechanisms. During infection of APCs, pathogens may enter endosomes or phagosomes before delivery into the cytoplasm, leading to processing of pathogen-derived proteins by compartmentalized acidic proteases, and presentation by class II proteins within these organelles (47). Alternatively, APCs may endocytose pathogenic antigens released during lysis of infected host cells through the process of bystander presentation (48). Finally, as demonstrated in this study, cytoplasmic antigens may be processed by proteases within the cytoplasm of the APCs, then translocated into membrane organelles containing MHC class II proteins. Evidence that peptides derived from a cytoplasmic antigen, GAD, gain direct access to newly synthesized class II proteins was obtained in EBV-transformed B-LCLs. Presentation of endogenous GAD epitopes was only marginally dependent upon key features of the classical pathway for exogenous antigen processing and display, including acidification of endosomal/lysosomal compartments and activation of cysteine proteases resident within these organelles. Rather, an absolute requirement for cytoplasmic antigen processing was observed for presentation of endogenous GAD epitopes. Thus, proteases responsible for generating peptide ligands for MHC class I molecules appear to also function in supplying epitopes for presentation by class II complexes. Evidence was obtained suggesting that both the proteasome and a calcium-activated protease calpain participate in processing antigens for class II-restricted presentation. These studies suggest a sequential mechanism whereby cytoplasmic antigens are cleaved by neutral proteases before translocation into organelles containing class II molecules. The final stages of antigen processing or trimming of these epitopes may be mediated within endosomes by proteases such as cathepsin B, before or after binding to class II complexes.

The antigen selected for this study, GAD, has been identified as a key target in several autoimmune disorders involving the neuroendocrine system (19). In insulin-dependent diabetes, autoantibodies to GAD are among the earliest hallmarks of progression to disease, with measurable T cell responses to this antigen noted in both humans and mice (19). In addition, a strong genetic link between susceptibility to IDDM and specific MHC class II alleles has been found in both humans and rodents (49), with B cells playing an essential role in progression to disease (50). The development of diabetes can be ablated in NOD mice immunized with tolerogenic levels of GAD (51). Thus, studies to examine the processing of exogenous GAD may have relevance to both the induction and prevention of this autoimmune disorder. Presentation of an immunodominant epitope derived from exogenous GAD by APCs was dependent upon acidification of endosomes and lysosomes, suggesting that processing or binding of this epitope occurs in these acidic organelles. Consistent with this result, processing of exogenous GAD by both cysteine and aspartyl proteases was a prerequisite to efficient class II-restricted presentation of this autoantigen via B cells. Disruptions in autoantigen processing can block the development of autoimmune disease, as was recently demonstrated with cathepsin S knockout mice and collagen-induced arthritis (52). Although cysteine proteases such as cathepsin S play a role in processing a variety of antigens (53, 54), the role of aspartyl proteases in antigen presentation appears more restricted (12, 14, 15). Thus, inactivation of the aspartyl protease required for exogenous GAD processing may hold promise as a preventative treatment for IDDM.

Unexpectedly, endogenous GAD molecules produced within the cytoplasm of cells also gain access to the class II...
pathway for presentation (20). The processing of endogenous GAD was clearly distinct from the exogenous antigen, as determined by susceptibility to protease inhibitors. Thus, aspartyl proteases are not required for endogenous GAD presentation. Further, inhibitors of acidic cysteine proteases only partially block GAD epitope formation, suggesting that these enzymes may play a role in the final stages of epitope trimming. By contrast, at least two distinct cytoplasmic proteases, the proteasome and calpain, function in processing endogenous GAD for class II–restricted presentation.

A role for the multicatalytic proteasome in processing cytoplasmic GAD for class II–restricted presentation was demonstrated in this study using the specific inhibitor lactacystin. The proteasome rapidly catalyzes the turnover of short-lived and abnormally folded proteins within the cytoplasm (34). Yet, prior studies have shown that both long- and short-lived cytoplasmic antigens are presented by class II molecules (55). Ubiquination and processing by the proteasome also favors proteins with basic NH₂-terminal residues as defined by the N-end rule pathway (56). However, generation of cytosolic antigens with basic NH₂-terminal residues did not always favor class II–mediated presentation, and proteasome cleavage of substrates via the N-end rule appears to vary with cell type (8). These findings, along with the demonstration that acidification of endosomal/lysosomal compartments was sometimes important for presentation of many cytoplasmic antigens via class II molecules (7, 55), raised questions as to the requirement for the proteasome in the class II pathway. The requirement for endosomal/lysosomal acidification is not at odds with a role for the proteasome, as proteases in these organelles may play a role in the final trimming of peptides for class II presentation. Alternatively, the acid pH within these organelles may favor peptide binding to class II molecules (30). As the proteasome can generate peptides of variable length (57), it is reasonable to hypothesize that this complex may supply ligands not only for MHC class I–restricted but also for class II–restricted presentation.

Previous studies designed to implicate the proteasome in antigen processing for class I have alluded to the potential role of other cytoplasmic proteases. In fact, it has been shown that nonproteasomal hydrolytic activity within the cytoplasm can influence class I–restricted presentation (37, 58). Lymphoid and myeloid cells express at least two calpain isozymes, calpain I and calpain II, with the activation of these enzymes linked to cytoplasmic calcium levels (44). In this study, MHC class II–restricted presentation of an endogenous cytoplasmic antigen was ablated after inhibition of the calcium-dependent protease calpain with the peptidyl inhibitor calpeptin. Additionally, presentation of cytoplasmic GAD was diminished in the presence of PD150606, an inhibitor of calcium-dependent calpains as well as mAbs to calpains. Remarkably, both calpain and GAD share a similar subcellular distribution within the cytoplasm, existing in both membrane and soluble forms. GAD has been localized to the cytoplasmic face of granules within neuroendocrine cells (19, 22), whereas calpains have been observed to bind to the cytoplasmic face of the plasma membrane, coated vesicles, and the endoplasmic reticulum (39). Whether the membrane localization of GAD and calpain play a role in favoring the translocation of GAD epitopes into organelles rich in class II proteins is currently under investigation. In contrast with cathepsins and the proteasome, calpains exhibit greater substrate selectivity and have been implicated in cleavage of lL+3, the common γ chain receptor, and ZAP70 in immune cells (59, 60). Thus, engineering antigens for calpain cleavage and membrane translocation may hold potential as a novel methodology for vaccine delivery to the class II pathway. Calpains have been linked with several pathological conditions, including the autoimmune disorders arthritis, multiple sclerosis, and experimental allergic encephalomyelitis (44, 61). The importance of calpains in processing antigens for class I presentation remains unclear. Interestingly, however, several peptidyl aldehydes used to inactivate the proteasome and demonstrated to block class I presentation were originally isolated as calpain inhibitors.

MHC class II molecules bind and present peptides derived from antigens originating within the cytoplasm of the APC. Unfortunately, the mechanisms underlying this nonclassical mode of antigen presentation remain largely unknown. This study demonstrates for the first time that cytoplasmic resident proteases are involved in MHC class II presentation of endogenous antigens. The requirement for cytoplasmic antigen processing provides a key insight into this alternate pathway for antigen presentation. However, the method of translocation of cytoplasmic antigen into class II–containing vesicles remains a mystery. Delivery of intact cytoplasmic antigens into class II–rich compartments by autophagy had been speculated to be one means of facilitating the intersection of antigenic epitopes and class II molecules. Yet, the current study demonstrates a clear requirement for processing before antigen delivery into membrane organelles. Thus, a specific mechanism for peptide translocation and delivery to class II proteins appears to be necessary. Whether the transport of these epitopes is linked to transporter associated with antigen processing (7, 62), an endosomal/lysosomal heat shock cognate protein (63), or an alternate translocation machinery requires further investigation.

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