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Lamp-2a Facilitates MHC Class II Presentation of Cytoplasmic Antigens

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Summary

Extracellular antigens are internalized and processed before binding MHC class II molecules within endosomal and lysosomal compartments of professional antigen presenting cells (APC) for subsequent presentation to T cells. Yet select cytoplasmic peptides derived from autoantigens also intersect and bind class II molecules via an unknown mechanism. In human B lymphoblasts, inhibition of the peptide transporter associated with antigen processing (TAP) failed to alter class II-restricted cytoplasmic epitope presentation. By contrast, decreased display of cytoplasmic epitopes via class II molecules was observed in cells with diminished expression of the lysosome-associated membrane protein-2 (Lamp-2). Overexpression of Lamp-2 isoform A (Lamp-2a), an established component of chaperone-mediated autophagy, enhanced cytoplasmic autoantigen presentation. Manipulating APC expression of heat shock cognate protein 70 (hsc70), a cofactor for Lamp-2a, also altered cytoplasmic class II peptide presentation. These results demonstrate a novel role for the lysosomal Lamp-2a-hsc70 complex in promoting immunological recognition and antigen presentation.

Introduction

CD4 T cells recognize peptide-MHC class II complexes displayed on APC, leading to the activation or anergy of host cellular immune responses. Whereas class II molecules function in protective immunity against bacterial and protein antigens exogenously delivered into APC, autoantigen presentation by these MHC molecules is also critical for the induction of self tolerance. Class II molecules transit endosomal compartments en route to the cell surface, thus favoring their acquisition of both foreign- and self-peptide ligands generated within acidic endosomes and lysosomes. Yet, there is also evidence that class II molecules potentiate CD4 T cell responses to endogenous antigens in tumor and virally infected cells (Jacobson et al., 1989; Jaraquemada et al., 1990; Wang et al., 1999). Biochemical studies have revealed peptide ligands derived from cytoplasmic and nuclear antigens bound to MHC class II molecules (Chicz et al., 1993; Rudensky et al., 1991). Cytoplasmic processing by proteases is required for efficient presentation of these autoantigens to CD4 T cells, although the mechanisms responsible for delivering peptides to vesicular compartments rich in MHC class II molecules remain poorly defined (Lich et al., 2000).

TAP delivers cytosol-derived peptides into the endoplasmic reticulum (ER) for binding to MHC class I molecules. Studies with viral antigens and B lymphoblasts suggest that TAP is not involved in the transport of cytoplasmic peptides to MHC class II molecules (Malnati et al., 1993, 1992). A small protein derived from herpes simplex virus, ICP47, has been shown to bind to TAP and block peptide transport into the ER (Fruh et al., 1995; Hill et al., 1995). To elucidate the role of TAP in cytoplasmic autoantigen presentation via class II proteins, ICP47 was expressed in APC. T cell activation assays with ICP47-expressing B cells confirmed no alteration in MHC class II-restricted presentation of two cytoplasmic autoantigens.

A system, chaperone-mediated autophagy, that mediates the transport of cytoplasmic peptides and proteins directly into endosomes and lysosomes has been identified in hepatocytes and fibroblasts under resting conditions and in response to metabolic stress (Chiang et al., 1989; Cuervo and Dice, 1996). Two essential components of this pathway are the Lamp-2a isoform and the heat shock cognate protein 70 (hsc70), formerly termed hsc73. Lamp family members are conserved transmembrane glycoproteins predominantly localized in prelysosomes and lysosomes. Due to alternative splicing, human Lamp-2 has at least two isoforms, Lamp-2a and Lamp-2b (Konecki et al., 1995). Studies in rodent cells suggest that Lamp-2a, but not Lamp-2b, functions in delivering proteins from the cytoplasm to lysosomes (Cuervo and Dice, 2000). A model has been proposed whereby an accessory chaperone, hsc70, intersects target proteins in the cytoplasm and facilitates their delivery to Lamp-2a, residing in the membrane of lysosomes. Studies in hepatocytes and fibroblasts indicate both Lamp-2a and hsc70 serve essential functions in protein translocation into lysosomes, yet the role of this transporter complex in immunological responses has not previously been investigated. To address this question, cellular levels of Lamp-2a and hsc70 in APC were altered. Functional assays with these cells revealed corresponding changes in the presentation of

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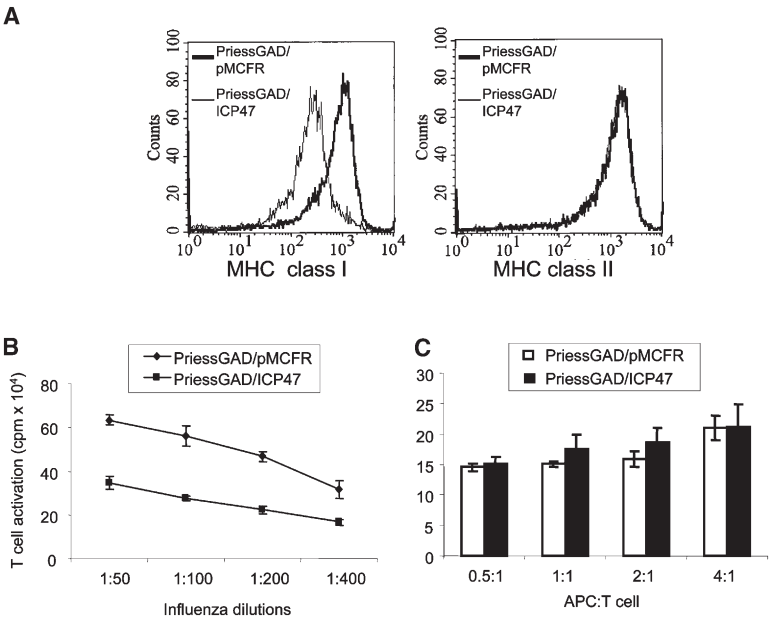

Figure 1. Inhibition of TAP Function Did Not Perturb Cytoplasmic GAD Presentation by MHC Class II

(A) Inhibition of MHC class I, but not class II, protein surface expression after B-LCL transfection with the TAP inhibitor ICP47. PriessGAD cells were transfected with a control vector (pMCFR) or a vector encoding ICP47. Cells were incubated with the anticlass I monoclonal antibody w6/32 or the anti-class II monoclonal antibody L243 followed by FITC-conjugated rabbit anti-mouse IgG. Cells were then analyzed by flow cytometry for surface MHC expression.

(B) Impaired class I antigen presentation in cells expressing ICP47. B-LCL cells with or without ICP47 expression were infected with serial dilutions of influenza A/PR/8/34 (1024 HA units/ml) followed by coculture with 4AV1 T cells recognizing $M1_{58–66}$. Error bars indicate the mean ± the standard deviation.

(C) Class II-restricted presentation of cytoplasmic GAD was unchanged with functional expression of ICP47 in B-LCL. PriessGAD B-LCL ± transfected ICP47 cDNA or the empty pMCFR vector was incubated with 33.1 T cells recognizing $GAD_{273–285}$. Variable APC:T cells ratios were used. Results are representative of three independent experiments. Error bars indicate the mean ± the standard deviation.

cytoplasmic antigens and peptides, suggesting that the Lamp-2a:hsc70 pathway promotes the intersection of cytoplasmic peptides with MHC class II molecules.

Results

Inhibition of TAP Function Did Not Affect Cytoplasmic Antigen Presentation by MHC Class II Molecules

The human B lymphoblastoid cell line (B-LCL) PriessGAD constitutively expressing the cytoplasmic antigen glutamate decarboxylase (GAD) was stably transfected with a plasmid encoding the TAP inhibitor ICP47. Expression of ICP47 in the transfected PriessGAD cells was confirmed by metabolic radiolabeling and immunoprecipitation (data not shown). Inhibition of TAP function was apparent by a significant decrease in cell surface MHC class I levels, as detected by flow cytometry with cells transfected with this viral protein compared to cells containing empty vector (Figure 1A). To further confirm the functional disruption of MHC class I antigen presentation in cells expressing the TAP inhibitor ICP47, CD8 T cell responses to viral antigens were tested with these B-LCL. Class I A2-restricted presentation of an influenza matrix protein-derived epitope was reduced at least 50% when comparing B-LCL with and without ICP47 expression (Figure 1B). Similarly, a 50%–75% reduction in human CTL responses to HLA-A2 and the BMRF1 antigen from Epstein Barr Virus (EBV) was detected when comparing these B cell lines with and without ICP47 (data not shown). Analysis of these B-LCL revealed that cell surface levels of MHC class II DR were not altered by ICP47 expression (Figure 1A). T cell activation assays with a T cell hybridoma specifically recognizing the $GAD_{273–285}$ epitope in the context of class II HLA-DR4 failed to reveal any change in cytoplasmic GAD presentation after inhibition of TAP function (Figure 1C). Similar studies were also carried out with another B cell line, Frev/SMA (FM), stably expressing cytoplasmic SMA, a mutated form of the autoantigen human immunoglobulin (Ig) κ chain, which is degraded in the cytosol by the proteasome (Dul et al., 2001; Lich et al., 2000). After inhibition of TAP function via expression of ICP47, presentation of two κ epitopes ($\kappa_{188–203}$ [κI] and $\kappa_{145–159}$ [κII]) from SMA was unaltered (data not shown). These results indicated that the transport function of TAP does not significantly contribute to MHC class II-restricted presentation of these cytoplasmically processed autoantigens in B cells.

Reduction in Total Cellular Lamp-2 Levels Decreased MHC Class II-Restricted Antigen Presentation

Lamp-2a has been suggested as the rate-limiting factor in chaperone-mediated autophagy (Cuervo and Dice, 1996). Here, in human B-LCL, the role of Lamp-2 isoforms in class II-restricted presentation of endogenous and exogenous antigens was examined. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis suggested that both the a and b isoforms of human Lamp-2 are expressed in primary monocytes, $CD19^+$ B cells, and B-LCL (data not shown). To test the role of Lamp-2 in class II pathways for antigen presentation, an expression plasmid encoding antisense Lamp-2 cDNA was constructed to decrease production of the a and b isoforms. PriessGAD cells were transfected with this cDNA or a control vector, and cell lines with overall decreased Lamp-2 protein levels (PriessGAD/AS-LAMP-2) were selected (Figure 2A). MHC class II levels

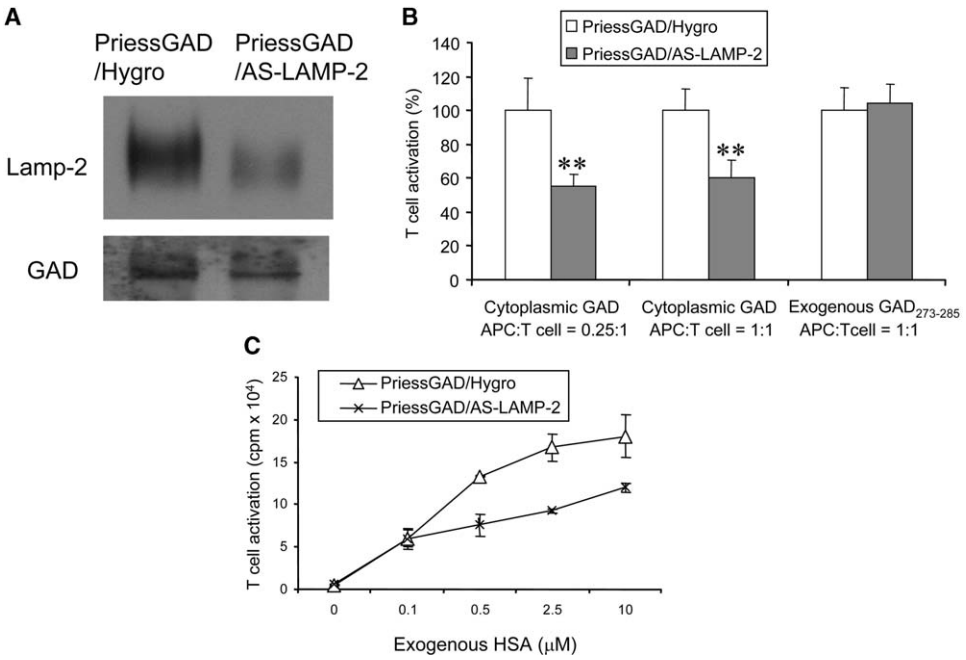

Figure 2. Decreased Lamp-2 Protein Levels Resulted in Decreased Cytoplasmic GAD Presentation

(A) Decreased Lamp-2 protein levels in antisense-transfected cells. Cell lysates were prepared from PriessGAD cells transfected with the control vector (PriessGAD/Hygro) or vector encoding Lamp-2 antisense (PriessGAD/AS-LAMP-2). Immunoblot analysis was performed with antibodies for Lamp-2 or the endogenous antigen GAD65. The Lamp-2 antibody equivalently detects a and b isoforms.

(B) Decreased class II presentation of cytoplasmic GAD by cells with low Lamp-2 levels. PriessGAD/Hygro and PriessGAD/AS-LAMP-2 cells were incubated with 33.1 T cells at variable APC:T cells ratios for 24 hr, and T cell activation was measured. As a control, APCs were preincubated with 10 μM $GAD_{237–285}$, and the presentation of exogenous GAD peptide was detected. Error bars indicate the mean ± the standard deviation. **$p < 0.01$, Student's t test.

(C) Decreased class II presentation of exogenous antigen HSA by Lamp-2-low cells. PriessGAD/Hygro and PriessGAD/AS-LAMP-2 cells were preincubated with increasing concentrations of HSA followed by incubation with 17.9 T cells specific for $HSA_{64–76}$. Results are representative of at least three independent studies. Error bars indicate the mean ± the standard deviation.

at the cell surface were unaltered in these transfected cells, as detected by flow cytometry (data not shown). The presentation of endogenous GAD in the context of HLA-DR4 was significantly decreased in these Lamp-2-low cells (Figure 2B). These functional studies were confirmed with multiple Lamp-2-low cell lines (data not shown). In contrast, the presentation of exogenous $GAD_{273–285}$ peptide was not affected (Figure 2B). The presentation of exogenous antigens, such as human serum albumin (HSA), was also decreased in cells with reduced Lamp-2a and Lamp-2b (Figure 2C). These results suggested that Lamp-2 isoforms were involved in antigen processing and presentation by MHC class II molecules.

Overexpression of Lamp-2a Increased Cytoplasmic, but Not Extracellular, Antigen Presentation via MHC Class II

Lamp-2a and Lamp-2b differ only in their transmembrane domains and short cytoplasmic tails, which are encoded by differential splicing of exon 9 (Konecki et al., 1995). To dissect the function of the individual isoforms and to further elucidate the role of Lamp-2 in antigen presentation, siRNA constructs specific for exon 9 were designed. However, by using these isoform-specific siRNA, decreases in Lamp-2 isoform levels were not attainable in B-LCL (data not shown). As an alternative, cellular overexpression of the individual isoforms was tested for effects on antigen presentation. Human B-LCL, PriessGAD, and FM were transfected with a plasmid encoding either Lamp-2a or Lamp-2b isoforms. Stable B cell lines overexpressing Lamp-2a were generated with enhanced levels of Lamp-2 protein as confirmed by Western blotting (Figures 3A and 3B). The total cellular content of cytoplasmic GAD65 antigen and MHC class II DR as well as cell surface MHC class II protein levels were unaltered in cells overexpressing Lamp-2a compared to cells transfected with empty vector alone (Figures 3A and 3B and data not shown). Studies of cytoplasmic antigen presentation revealed a significant increase in class II presentation of endogenous GAD only in cells overexpressing Lamp-2a (Figure 3C). As a control, the presentation of exogenous $GAD_{273–285}$ peptide was tested and found not to vary with changes in cellular Lamp-2a content (Figure 3C). The presentation of two Ig κ epitopes, κI and κII derived from the cytoplasmic antigen SMA, was also significantly increased by enhanced Lamp-2a expression in FM cells (Figure 3D). Interestingly, the presentation of exogenous antigens, including HSA, GAD, and tetanus toxoid (TT), was not affected by overexpressing Lamp-2a (Figure 3F and data not shown). PriessGAD cells

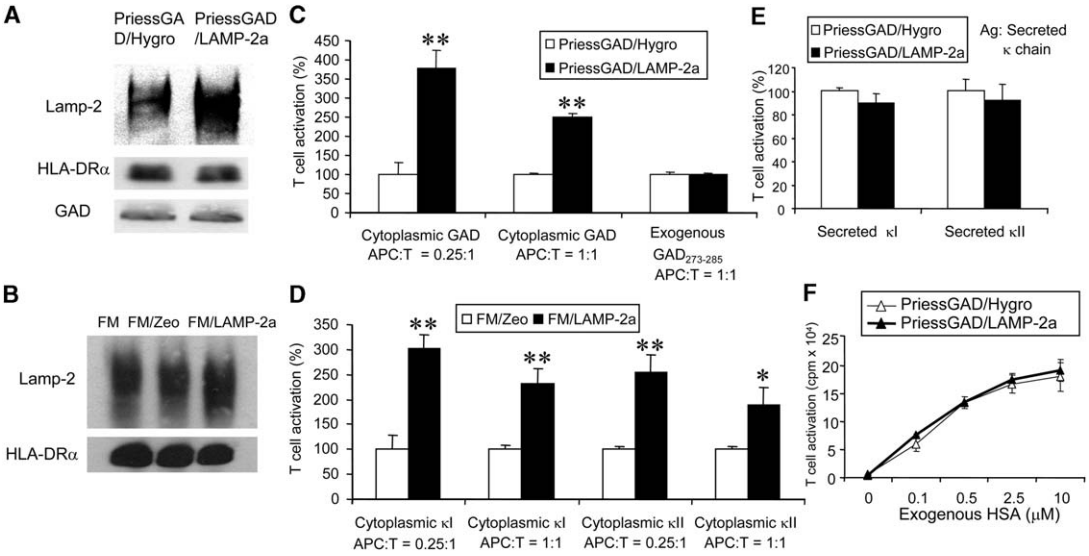

Figure 3. Overexpression of Lamp-2a in APC Increased Cytoplasmic, but Not Extracellular, Antigen Presentation via MHC Class II

(A) Overexpression of Lamp-2a in PriessGAD. Lysates were prepared from PriessGAD cells transfected with a control vector (PriessGAD/Hygro) or Lamp-2a vector (PriessGAD/LAMP-2a). Immunoblot analysis was performed by using antibodies for Lamp-2, GAD65, and HLA-DR α chain.

(B) Increased Lamp-2 protein levels were observed in FM cells transfected with Lamp-2a cDNA (FM/LAMP-2a) compared to cells transfected with a control vector (FM/Zeo).

(C) Increased class II presentation of cytoplasmic GAD by cells overexpressing Lamp-2a. PriessGAD/Hygro and PriessGAD/LAMP-2a cells were incubated with 33.1 T cells at variable APC:T cells ratios to monitor T cell activation. As a control, APCs were preincubated with 10 μM $GAD_{237–285}$, and the presentation of exogenous GAD peptide was detected. Error bars indicate the mean ± the standard deviation.

(D) Increased class II presentation of cytoplasmic SMA by cells overexpressing Lamp-2a. Presentation of SMA-derived κI and κII epitopes was tested with T cells 2.18 and 1.21. Error bars indicate the mean ± the standard deviation.

(E) Presentation of the secretory antigen Ig κ chain was not affected by cellular overexpression of Lamp-2a. PriessGAD/Hygro and PriessGAD/LAMP-2a cells were incubated with T cells 2.18 and 1.21 followed by analysis of T cell activation. Error bars indicate the mean ± the standard deviation.

(F) Presentation of exogenous antigen HSA was unaltered by overexpressing Lamp-2a in APC. PriessGAD/Hygro and PriessGAD/LAMP-2a cells were preincubated with human serum albumin (HSA). APCs were cultured with 17.9 T cells prior to analysis of T cell activation. Results are representative of three individual experiments. Error bars indicate the mean ± the standard deviation.

*$p < 0.05$; **$p < 0.01$, Student's t test.

constitutively produce secretory Ig κ, which serves as an endogenous intravesicular source of antigen. The presentation of κI and κII epitopes derived from this intravesicular Ig κ chain was not affected by changes in the cellular content of Lamp-2a (Figure 3E). These results were further confirmed with multiple Lamp-2a-high cell lines (data not shown). The role of Lamp-2b in cytoplasmic antigen presentation was also tested with B cell lines overexpressing this Lamp-2 isoform (Figure 4A). Remarkably, neither cytoplasmic nor exogenous antigen presentation by MHC class II molecules was affected by Lamp-2b overexpression (Figures 4B–4D). Together, these studies demonstrate that Lamp-2a, but not Lamp-2b, functions specifically in promoting cytoplasmic antigen presentation by MHC class II molecules.

Lamp-2 proteins are expressed at low levels on the surface of cells relative to their distribution in endosomes and lysosomes. Increased levels of Lamp-2 are found on the surface of metastatic tumors, possibly suggesting that Lamp-2 isoforms function as adhesion molecules (Saitoh et al., 1992). In Lamp-2a-overexpressing B cell lines, surface Lamp-2 levels were also increased slightly as detected by flow cytometry (data not shown). To test whether enhanced surface Lamp-2a promotes T cell and B cell interaction, thus affecting antigen presentation, the following experiment was performed. B cells were aldehyde fixed and incubated with increasing concentrations of $GAD_{273–285}$ peptide to directly load this ligand onto surface class II proteins in the absence of endocytosis. The ability of these fixed B cells to activate T cells was not altered regardless of enhanced B cell surface expression of Lamp-2a (Figure 5A). Studies with live B lymphoblasts also failed to reveal any change in the presentation of surface-loaded exogenous peptides with Lamp-2a overexpression (data not shown). In addition, blocking B cell surface Lamp-2 with specific antibodies had no effect on T cell activation in response to epitopes derived from either cytoplasmic or exogenous antigens (data not shown). Lamp-2a overexpression in APC did not induce any change in overall cell morphology as detected with electron microscopy (data not shown). Nor did overexpression of the Lamp-2a isoform alter the content of autophagic vacuoles in cells, as determined with the autophagosome marker monodansylcadaverine (MDC) (Figure 5B). Further analysis of Lamp-2a overexpressing cells also failed to reveal any measurable change in

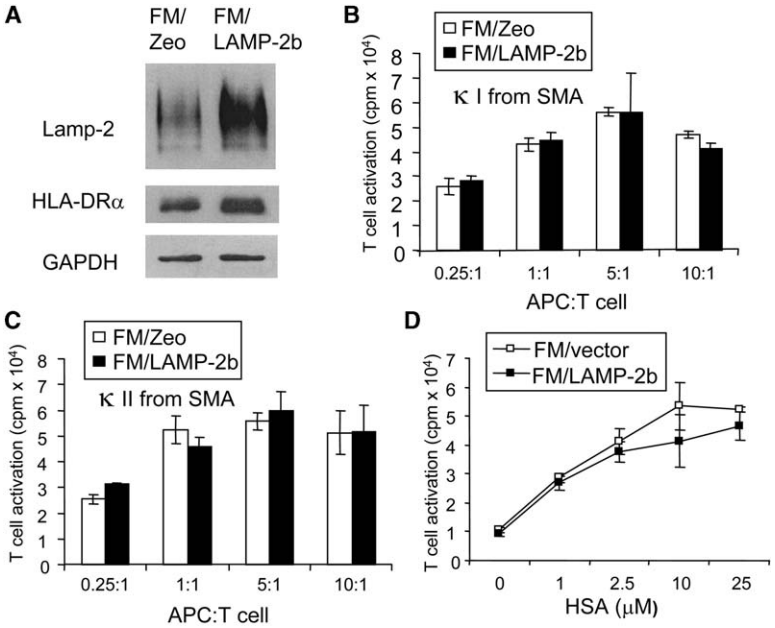

Figure 4. Overexpression of Lamp-2b in APC Had No Effect on MHC Class II-Restricted Antigen Presentation

(A) Enhanced Lamp-2 protein levels were detected in B-LCL after transfection with a plasmid encoding Lamp-2b. FM cells were transfected with pCDNA3.1/Zeo(−) encoding Lamp-2b cDNA (FM/LAMP-2b) or a control vector (FM/Zeo). Cell lysates were subjected to immunoblot analysis with antibodies specific for Lamp-2, HLA-DR α chain, and GAPDH.

(B and C) Cytoplasmic SMA presentation was not altered by overexpressing Lamp-2b. APCs (FM/Zeo and FM/LAMP-2b) were incubated with 2.18 and 1.21 T cells at the ratios indicated for 24 hr. T cell activation was detected as described previously. Error bars indicate the mean ± the standard deviation.

(D) Extracellular antigen (HSA) presentation was not altered by overexpressing Lamp-2b. FM/Zeo and FM/LAMP-2b cells were preincubated with increasing concentrations of HSA followed by culturing with 17.9 T cells. T cell activation was measured. Representative results from three studies are shown. Error bars indicate the mean ± the standard deviation.

lysosomal protease maturation or distribution (Figures 5C and 5D). Thus, consistent with the lack of change in class II exogenous antigen presentation, cathepsin D maturation and subcellular distribution were unchanged in APC overexpressing Lamp-2a.

Alteration of hsc70 Level Affected Class II Presentation of Cytoplasmic Antigens

The chaperone protein hsc70 has been demonstrated to cooperate with Lamp-2a in transporting proteins from the cytoplasm into lysosomes. To directly test the role of hsc70 in MHC class II-restricted cytoplasmic antigen presentation, PriessGAD cells were transfected with plasmids encoding human hsc70 sense or antisense DNA. Hsc70 is very abundant in cells, often representing 1%–2% of the total cellular protein (Newmyer and Schmid, 2001). The abundance of hsc70 in cells may limit overexpression of this molecule, yet in several independent B cell transfectants a slight increase in the total cell content of hsc70 could be detected (Figure 6A, insert). B cells transfected with antisense DNA for hsc70 were found to contain reduced levels of this heat-shock-like protein (Figure 6A, insert). Analysis of class II-restricted cytoplasmic antigen presentation revealed that cells transfected with hsc70 sense DNA displayed increased cytoplasmic GAD presentation, whereas cells transfected with hsc70 antisense DNA had slight decreases in GAD antigen presentation (Figure 6A). Altering cellular hsc70 levels had no significant effect on B cell presentation of exogenous antigens such as HSA (data not shown). These results suggest that hsc70 can modulate MHC class II-restricted cytoplasmic Ag presentation. Both the chaperone hsc70 and the lysosomal membrane protein Lamp-2a have been observed to bind to select cytoplasmic substrates, potentially to enhance transport and proteolytic processing. Here, in GAD-expressing APC, monoclonal antibodies were used to precipitate this self antigen, and Western blotting analyses were performed to detect the presence of bound hsc70 and Lamp-2. Results indicate that each of these molecules can be detected complexed with GAD autoantigen (Figure 6B).

Overexpression of Lamp-2a Increased Transport of Cytoplasmic GAD Peptide to Class II Complexes

To directly test the possible role of Lamp-2a in the translocation of antigenic peptides, the $GAD_{273–285}$ peptide was electroporated into the cytoplasm of FM cells with or without Lamp-2a overexpression. Electroporated cells were then monitored for peptide binding and presentation via class II proteins. Presentation of the peptide via class II HLA-DR4 was detected only in electroporated cells, suggesting that delivery of the epitope into the cytoplasm of cells was required prior to translocation and class II binding. T cell responses to APC electroporated with the $GAD_{273–285}$ peptide were enhanced nearly 2-fold in cells overexpressing Lamp-2 compared to control cells (Figure 7A). To detect the formation of MHC class II and GAD peptide complexes directly, C terminus biotin-labeled $GAD_{273–285}$ peptide was introduced into the cytosol of APC by electroporation followed by incubation for 16 hr. Stable peptide-class II complexes can be detected on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), migrating as molecular complexes with an apparent molecular mass of 60 kDa. Such complexes containing the biotinylated $GAD_{273–285}$ peptide were observed after peptide electroporation into the cytoplasm of cells (Figure 7B). In agreement with functional studies, the relative abundance of these GAD peptide-class II complexes was increased in cells overexpressing Lamp-2a. These results support the hypothesis that Lamp-2a overexpression in B cells enhances epitope delivery from the cytoplasm into endosomal and lysosomal

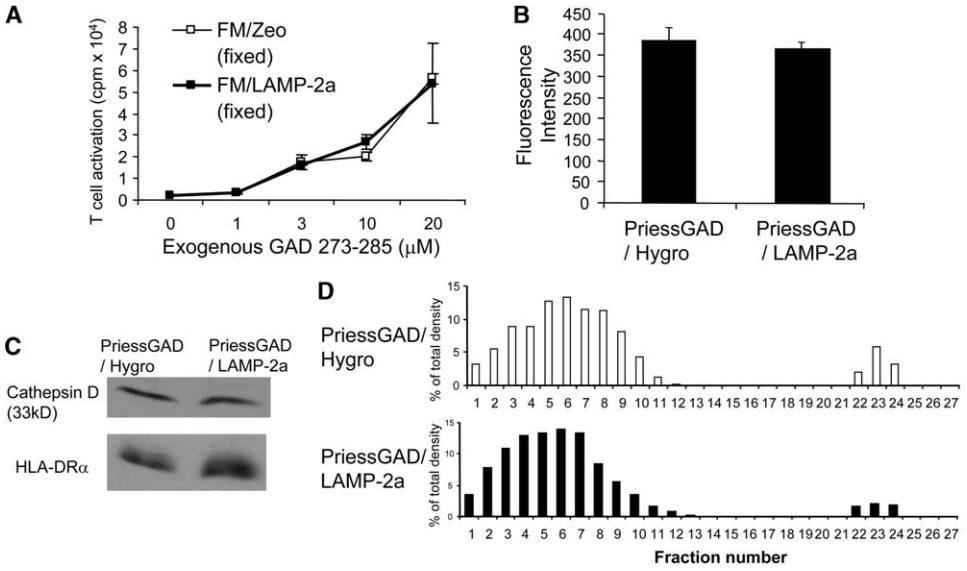

Figure 5. Alterations in Antigen Presentation Observed in Lamp-2a Overexpressing APC Were Not Due to the Possible Roles of Lamp-2 Protein in Cell Adhesion and Lysosomal Function

(A) Presentation of surface-loaded $GAD_{273–285}$ was not affected by enhanced plasma membrane Lamp-2a. APCs were fixed with 1% paraformaldehyde, incubated with increasing concentrations of $GAD_{273–285}$ peptide for 12 hr, and then assayed for T cell activation. Similar results were obtained with live cells and exogenous peptide. Error bars indicate the mean ± the standard deviation.

(B) Unaltered autophagosome labeling in Lamp-2a-overexpressing APC. PriessGAD/Hygro and PriessGAD/LAMP-2a cells were incubated with 0.05 mM MDC at 37°C for 10 min. After extensive washing, cells were lysed and fluorescence of MDC was measured. Error bars indicate the mean ± the standard deviation.

(C) Cathepsin D maturation was unaltered in APCs overexpressing Lamp-2a. Cell lysates from PriessGAD/Hygro or PriessGAD/LAMP-2a were subjected to immunoblot analysis with antibodies specific for human cathepsin D or the HLA-DR α chain.

(D) Subcellular distribution of cathepsin D was unaltered in APC overexpressing Lamp-2a. Membrane organelles prepared from PriessGAD/Hygro and PriessGAD/LAMP-2a cells were placed on a continuous sucrose gradient for fractionation. Samples were collected and resolved on SDS-PAGE. Cathepsin D was detected by immunoblotting. Lysosomes are found in gradient fractions 1–4 with prelysosomes and/or late endosomes in fractions 5–9 and early endosomes in fractions 23–25.

compartments accessible by MHC class II molecules. In control studies, the 60 kDa peptide complexes were not detected in peptide-electroporated cells lacking class II molecules (Figure 7C). Also, immunoprecipitation of cell lysates with antibodies specific for class II molecules resulted in the depletion of these peptide-class II complexes, again confirming the specificity of this assay (Figure 7D). Loading of cytoplasmic GAD peptides onto MHC class II was a slow process, with complex formation readily detectable 8 hr after electroporation (Figure 7E). These results demonstrate a role for Lamp-2a in facilitating the delivery of peptides from the cytoplasm to MHC class II molecules.

Discussion

Biochemical and functional studies of professional and nonprofessional APC have identified epitopes derived from cytoplasmic antigens presented in the context of both murine and human MHC class II molecules (Chicz et al., 1993; Lich et al., 2000; Malnati et al., 1993, 1992; Rudensky et al., 1991). These epitopes are drawn from a diverse pool of cytosolic antigens including metabolic enzymes, cytoskeletal proteins, viral and tumor antigens, misfolded proteins, and molecules bound to the cytoplasmic face of endosomes and lysosomes (Chicz et al., 1993; Malnati et al., 1992; Rudensky et al., 1991; Wang et al., 1999). The identification of antiviral as well as tumor-specific CD4 T cells that are reactive with cytoplasmic antigens in humans suggests a potential role for this pathway in host defense. Yet, little is known concerning the molecular events that modulate this novel pathway for class II antigen presentation. Studies indicate that cytoplasmic epitopes derived from autoantigens reach class II molecules via an endogenous pathway, with cytosolic proteases playing a role in this processing pathway (Lich et al., 2000; Malnati et al., 1992). Here, specific mechanisms regulating the intersection of cytoplasmic self peptides with MHC class II molecules were explored by using human B lymphoblastoid cells. These studies failed to reveal a key role for TAP in facilitating cytoplasmic peptide delivery to MHC class II molecules. Yet, a lysosomal protein, Lamp-2a, and its accessory chaperone, hsc70, were found to modulate the efficiency of cytoplasmic peptide and antigen presentation by class II proteins in these professional APC.

Encoded within the class II region of the MHC and inducible by IFN-γ, TAP is well established as a transporter that brings peptides from the cytoplasm to nascent MHC class I molecules resident in the ER. TAP facilitates the delivery of a broad spectrum of peptides, which are 8–25 amino acids in length, from the cytoplasm into the ER (Androlewicz and Cresswell, 1994).

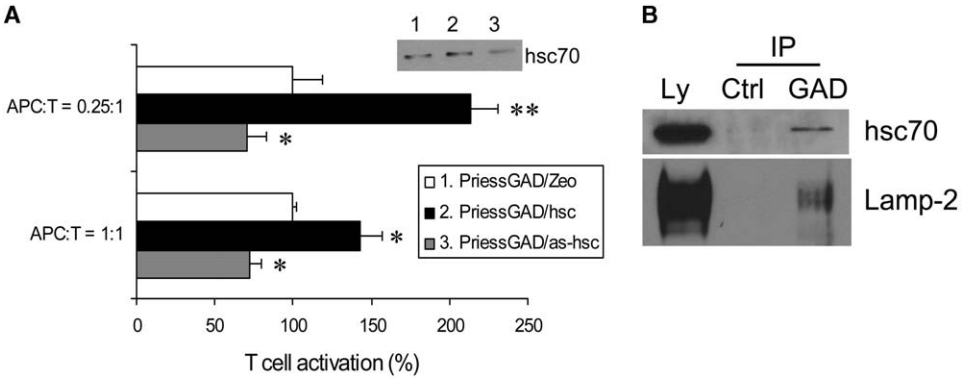

Figure 6. Manipulation of hsc70 Levels in APC Altered Class II Cytoplasmic Antigen Presentation

(A) PriessGAD cells were transfected with a control vector pcDNA3.1/Zeo(−) or vectors encoding sense or antisense human hsc70 cDNA. PriessGAD/Zeo (control), PriessGAD/hsc (hsc sense), and PriessGAD/as-hsc (hsc antisense) cells were incubated with $GAD_{273–285}$-specific 33.1 T cells at the ratios indicated for 24 hr. T cell activation was measured as described. Insert: Cell lysates ([1], PriessGAD/Zeo; [2], PriessGAD/hsc; [3], PriessGAD/as-hsc; 50 μg protein/lane) were resolved on 9% SDS-PAGE and analyzed by immunoblotting for hsc70 levels. *$p < 0.05$; **$p < 0.01$, Student's t test. Results are representative of three to five individual experiments. Error bars indicate the mean ± the standard deviation.

(B) Coprecipitation of hsc70 and Lamp-2 with GAD antigen. Cell lysate was prepared from PriessGAD cells. Immunoprecipitation was performed with GAD mAb or control Ab. After SDS-PAGE, the presence of hsc70 and Lamp-2 was detected with specific Abs by immunoblotting.

Whereas MHC class I molecules incorporate only short ligands transported via TAP, it remained possible that newly synthesized MHC class II molecules might also access the pool of peptides transported by TAP. Human TAP function was selectively disrupted by constitutive expression of the viral protein ICP47 in B cells. Class I protein surface expression and antigen presentation were diminished by greater than 50%, confirming ICP47 function. Yet, there was no detectable change in the presentation of three distinct epitopes by MHC class II molecules, and there was no difference in overall class II expression observed. These findings are in agreement with earlier studies by Long and colleagues with human B cells, which suggested that class I null APC lacking segments of the MHC encoding the genes for TAP remains capable of presenting cytoplasmic viral antigens in the context of class II molecules (Malnati et al., 1992). Studies with murine TAP-deficient melanomas transfected to express class II I-A^k and cytoplasmic or ER-targeted hen egg lysozyme also failed to detect a role for TAP in class II presentation (Dissanayake et al., 2005). Yet, class II presentation of epitopes encoded within two transmembrane viral glycoproteins from infectious influenza was reduced in studies with TAP-deficient murine fibroblasts and dendritic cells (Tewari et al., 2005). Whether these differences are related to how antigens are targeted or distributed within the cytoplasm, or possibly altered mechanisms for class II presentation of cytoplasmic antigens in distinct professional and nonprofessional APC, is unknown.

First identified during studies of serum starvation-induced stress, Lamp-2a and its cofactor hsc70 have been proposed to function in transporting proteins or fragments of protein from the cytoplasm into the lysosomes for proteolysis (Chiang et al., 1989; Cuervo and Dice, 1996). This process has been termed chaperone-mediated autophagy due to a requirement for heat shock cognate protein hsc70, which is found both in the cytoplasm and associated with endosomes and lysosomes. Overexpression of Lamp-2a or hsc70 in human B cells resulted in enhanced presentation of multiple cytoplasmic epitopes via class II molecules. Similarly, a reduction in hsc70 expression led to diminished class II presentation of cytoplasmic antigens. Lamp-2 and hsc70 have been observed to bind cytoplasmic proteins destined for transport and proteolysis. Here, both Lamp-2a and hsc70 could be detected complexed with GAD. Changes in exogenous peptide or antigen presentation were not observed with overexpression of Lamp-2a or manipulation of cellular hsc70 levels. Yet, class II presentation of peptides delivered into the cytoplasm by electroporation could be enhanced by overexpression of Lamp-2a. These latter studies suggest that Lamp-2a can function after partial processing of antigens to facilitate epitope delivery to class II molecules.

Lamp-2 molecules reside primarily in lysosomes and prelysosomes (or mature endosomes) but can also be found at very low levels in early endosomes and at the plasma membrane (Gough and Fambrough, 1997). The overexpression of Lamp-2a in B lymphoblastoid cells resulted in only a very minimal increase in the surface expression of this protein, with studies ruling out any potential role for these surface molecules in regulating APC-T cell interactions. Tumor cell expression of cell surface Lamp-2 has been proposed to regulate adhesion and possibly metastasis (Ohannesian et al., 1994; Saitoh et al., 1992). Cells transfected to overexpress Lamp-2a did not show any alterations in subcellular morphology or organelle content, as assessed by transmission electron microscopy, and changes were not detected in the sorting or maturation of a lysosomal protease, cathepsin D, in these APC. Consistent with our failure to detect such changes in lysosomes, functional assays with multiple exogenous antigens (GAD, human serum albumin, and tetanus toxoid) as well as

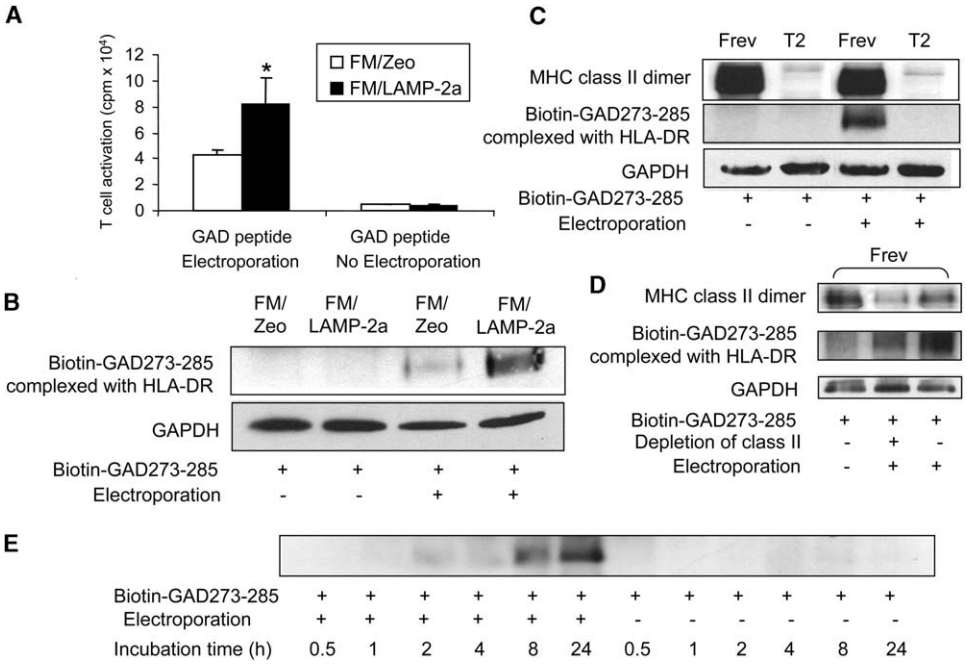

Figure 7. Overexpression of Lamp-2a Promoted the Translocation of GAD Peptides from the Cytoplasm to MHC Class II Molecules

(A) Overexpression of Lamp-2a increased the presentation of cytoplasmic $GAD_{273–285}$ peptide. FM/Zeo and FM/LAMP-2a cells were incubated with $GAD_{273–285}$ (20 μM) for 5 min on ice followed by electroporation to deliver this peptide into the cytoplasm. Control cells were not subjected to electroporation. T cell activation by these APC was measured. *$p < 0.05$, Student's t test. Error bars indicate the mean ± the standard deviation.

(B) Overexpression of Lamp-2a increased the translocation of $GAD_{273–285}$ peptide from the cytoplasm to MHC class II molecules. FM/Zeo and FM/LAMP-2a cells were incubated with biotin-$GAD_{273–285}$ for 5 min on ice followed by electroporation and extensive washing as described in (A). After culturing for 16 hr, cells were lysed and resolved on SDS-PAGE and transferred to nitrocellulose for detection of biotin-peptide complexed with class II DR. Streptavidin-HRP was used to detect biotin-$GAD_{273–285}$ migrating with the MHC class II dimer at 60 kDa. GAPDH was blotted with anti-GAPDH mAb as a loading control.

(C) The 60 kDa streptavidin-reactive band was only detected in MHC class II-expressing cells. Biotin-$GAD_{273–285}$ peptides were introduced into Frev or T2 (MHC class II negative) cells by electroporation. Western blots were performed by using streptavidin-HRP, an antibody specific for MHC class II dimer and GAPDH.

(D) Depletion of MHC class II dimer from cell lysate decreased the density of the streptavidin-reactive band. After electroporation with biotin-$GAD_{273–285}$ and incubation, Frev cell lysates were incubated with anti-HLA-DR antibody (middle lane) or control antibody (right lane) followed by incubation with protein G sepharose. After depletion by protein G Sepharose, the lysates were subjected to SDS-PAGE for detection of biotin, MHC class II dimer, or GAPDH. Representative data are shown from three to five separate studies.

(E) After electroporation with biotin-$GAD_{273–285}$ and extensive washing, Frev cells were incubated at 37°C for variable periods of time as indicated. Cells were lysed for the detection of biotin-peptide complexed with class II DR.

secreted human IgG did not reveal any differences in class II presentation by cells overexpressing Lamp-2a. Each of these antigens requires processing within acidic, mature endosomal and lysosomal compartments for efficient class II antigen presentation (Haque et al., 2002; Lich et al., 2000; Pathak et al., 2001). These results strongly suggest that Lamp-2a uniquely regulates the pathway for cytoplasmic antigen presentation by MHC class II molecules.

Hsc70 has been reported to serve multiple functions in cells. As a chaperone, hsc70 can bind to numerous proteins to mediate both folding and degradation (Bercovich et al., 1997; Terada et al., 1995). Cytoplasmic GAD was readily detected bound to hsc70 in B lymphoblasts expressing this antigen, in line with the potential role of hsc70 as a cytoplasmic chaperone. Overexpression of hsc70 in macrophages reportedly enhanced exogenous antigen presentation, yet the mechanism was not investigated (Panjwani et al., 1999). Studies suggest that this chaperone protein may be found within endosomes and lysosomes in addition to residing within the cytoplasm, with a proposal that this dual localization may be important in moving proteins into lysosomes (Agarraberes et al., 1997). The requirement for hsc70 observed in the current studies of cytoplasmic antigen presentation suggests that peptide delivery to class II molecules is not via conventional autophagy. In the latter, components from the cytoplasm are indiscriminately engulfed by membrane vacuoles derived from the ER. These vacuoles later fuse with or give rise to lysosomes (Wang and Klionsky, 2003). Conventional autophagy can be inhibited by treating cells with the drug 3-methyl adenine (Seglen and Gordon, 1982). By using several T cells specific for cytoplasmic antigens, no change in class II presentation was detected with APC exposed to this drug (data not shown). However, because of the connection between autophagosomes and lysosomes, it remains possible that conventional

autophagy may, under some circumstances, facilitate cytoplasmic epitope delivery to class II molecules. Disruption of autophagy in APC either with this drug or by other means was reported to inhibit class II presentation of an expressed bacterial cytoplasmic antigen and a viral nuclear antigen (Nimmerjahn et al., 2003; Paludan et al., 2005).

Interestingly, Lamp-2b has been suggested to play a role in regulating conventional autophagy and lysosome biogenesis (Nishino et al., 2000). Patients lacking functional Lamp-2b (Danon disease) accumulate intracellular vacuoles in skeletal and cardiac muscle. In the current study, overexpression of Lamp-2b failed to influence endogenous or exogenous antigen presentation by APC. Yet in APC treated with antisense DNA to reduce both Lamp-2a and Lamp-2b isoform expression, exogenous as well as cytoplasmic antigen presentation by class II molecules was disrupted. Lamp-2a and Lamp-2b share 94% sequence homology. Structural differences in Lamp-2a and Lamp-2b are confined to their transmembrane and cytoplasmic domains. How these domains contribute to the distinct functions of Lamp-2 isoforms remains unclear.

Recent studies suggest MHC class I and class II molecules cross over in their access and surveillance of intracellular and extracellular compartments for antigenic peptides derived from pathogens and self. Class I molecules predominantly display cytoplasm peptides that have been transported into the ER. Yet, in specialized APC such as dendritic cells, these MHC molecules may also access antigens internalized and processed within endosomes, lysosomes, and phagosomes (Ackerman and Cresswell, 2004; den Haan et al., 2000). Likewise, class II molecules were traditionally seen as the primary vehicle for activating immune responses against extracellular pathogens or self molecules accessing the endocytic pathway. Yet, the ability of class II molecules to promote CD4 T helper and cytolytic cell responses to cytoplasmic antigens derived from viruses and tumors may, in some cases, be central to promoting long-lasting, durable protective immunity (Armstrong et al., 1998; Jacobson et al., 1989). Remarkably, many viruses and tumors have evolved mechanisms to disrupt class I presentation via blocking the translocation of antigenic epitopes by TAP (Ahn et al., 1997; Fruh et al., 1995; Hengel et al., 1997; Hill et al., 1995; Lehner et al., 1997). Analysis of Lamp-2a messenger RNA from a wide variety of professional and nonprofessional APC revealed a broad pattern of constitutive expression (data not shown). Thus, the ability of class II molecules to access cytoplasmic peptides via the Lamp-2a:hsc70 complex may represent an important mechanism by which host cellular responses can counter viral and tumor immune evasion of the class I pathway.

Experimental Procedures

Cells

PriessGAD, a B-LCL constitutively expressing GAD, was generated from Priess (HLA-A2, DR4w4) by retroviral transduction with GAD65 cDNA (Lich et al., 2000). FM cells expressing SMA, a mutant Ig κ light chain, were generated from the B cell Frev (DR1, DR4w4) by transfection with a plasmid encoding SMA (Dul et al., 2001). All B-LCL were cultured in Iscove's MEM with 10% heat-inactivated calf serum. The T cell hybridomas recognizing HLA-DR4 epitopes 33.1 (specific for $GAD_{273–285}$), 2.18 (specific for Ig $\kappa_{188–203}$), 1.21 (specific for Ig $\kappa_{145–159}$), 17.9 (specific for $HSA_{64–76}$), and 49.23.2 (specific for an epitope of tetanus toxin) were cultured in RPMI 1640 with 10% fetal calf serum (FCS) and 50 μM β mercaptoethanol. The CD8 T cell hybridoma 4VA1 specific for HLA-A2 and influenza A/PR/8/34 matrix M1 (residues 58–66) was maintained in DMEM with 10% FCS (Canaday et al., 2003). Human CTLs specific for EBV BMRF1 antigens were maintained for cytolytic assays as described (Lautscham et al., 2001).

Plasmids and Cell Transfection

The pMCFR-PAC plasmid encoding ICP47 was provided by Dr. P. Cresswell (Yale University). Human Lamp-2a cDNA and hsc70 cDNA were provided by Drs. J.F. Dice and A.M. Cuervo (Tufts University). Lamp-2a cDNA was transferred into pcDNA/Hygro$^{(+)}$ and pcDNA/Zeo$^{(-)}$ (Invitrogen). The Lamp-2 antisense vector was generated by inserting Lamp-2a cDNA (1–658) in an inverted direction into pcDNAhygro$^{(+)}$. The hsc70 sense vector was generated by inserting hsc70 cDNA into pcDNAZeo$^{(-)}$. The hsc70 antisense vector was generated by inserting hsc70 cDNA (1042–2187) in an inverted orientation into pcDNAZeo$^{(-)}$. The plasmid encoding SMA cDNA was a gift from Dr. Y. Argon (University of Pennsylvania). B-LCL, PriessGAD, or FM was transfected with linearized plasmids (10–20 μg/ml) by electroporation (250 V, 950 μF). For Lamp-2 isoforms and hsc70, cells were transfected in three to five independent assays to ensure reproducible alterations in gene expression and correlation with functional assays. Independently transfected cell lines were tested by immunoblotting for Lamp-2 and hsc70 protein steady-state expression, with titrations of 5–100 μg of total cell protein analyzed by immunoblotting with densitometric analysis. In B cells transfected with Lamp-2 antisense DNA, densitometry revealed that Lamp-2 protein levels in treated cells were 58% of that of controls (n = 6). Cells transfected for overexpression of Lamp-2 on average contained 1.48-fold more of this protein compared with control cells (n = 6). After cell transfection with antisense DNA for hsc70, residual levels of this protein were 43% compared with controls (n = 4). Cells transfected for hsc70 overexpression on average contained 1.38-fold more hsc70 protein than controls (n = 5). All transfectants were checked for class II steady-state and surface expression prior to functional analysis.

Antibodies

Anti-ICP47 rabbit serum was a gift from Dr. D.C. Johnson (Oregon Health Science University), and commercial antibodies used were as follows: α-Lamp-2 mAb (H4B4, Developmental Studies Hybridoma Bank), α-hsc70 mAb (Maine Biotechnology Service), human cathepsin D antibody (Calbiochem), anti-GAPDH mAb (Chemicon) and GAD-specific mAb (Sigma). The mAb DA6.147 recognizing HLA-DR4 α was provided by Dr. P. Cresswell (Yale University). The mAb w6/32 recognizes HLA-ABC, and L243 recognizes HLA-DR.

Flow Cytometry

Cells were incubated with w6/32, L243, H4B4, or isotype control mAbs followed by FITC-conjugated rabbit anti-mouse IgG (Jackson ImmunoResearch). Samples were analyzed by flow cytometry with Cell Quest software.

Immunoblotting

Cells were lysed in 10 mM Trizma Base, 150 mM NaCl, and 1% Triton X-100 buffer. Samples were resolved on 9% SDS-PAGE and transferred onto nitrocellulose membrane (Osmonics, Inc.). After probing with specific antibodies, proteins were visualized by incubation with horseradish peroxidase-conjugated goat-anti-mouse or goat-anti-rabbit antibodies (Jackson ImmunoResearch Laboratories, Inc.) and ECL (Amersham Pharmacia Biotech).

Immunoprecipitation

Cells were lysed in 10 mM Trizma Base, 150 mM NaCl, 1% octyl glucoside, 0.2 mM PMSF, 0.1 mM TLCK [pH 7.5] followed by preclearing with rabbit serum and protein G Sepharose. Antibodies were added to cell lysates at 4°C for 2 hr or overnight, followed by

protein G Sepharose. Antigen-antibody complexes were collected and washed prior to elution and analysis by SDS-PAGE and Western blotting.

Antigen Presentation Assay

For the cytoplasmic antigen presentation assay, variable numbers of APC were incubated with 2×10^4 specific T cells for 24 hr at 37°C. For exogenous antigen presentation, APC were preincubated with serially diluted antigens for 16 hr at 37°C. 2.5×10^4 APC were then incubated with 5×10^4 specific T cells for 24 hr. For MHC class I-mediated antigen presentation assays, 1×10^5 APCs were infected with live influenza A/PR/8/34 allantoic fluid (Charles River) by using serial dilutions from 1:50 through 1:400 for 16 hr at 37°C, followed by coculture with T cells for 24 hr. An IL-2-dependent cell line, HT-2, was used to measure IL-2 production following T cell activation. CTL assays to assess class I presentation of EBV antigens were completed as described (Lautscham et al., 2001).

Electroporation

Cells were washed with cold phosphate-buffered saline (PBS) twice and incubated with $GAD_{273–285}$ peptide or biotin-$GAD_{273–285}$ (20 μM) for 5 min on ice. Electroporation (270 V, 125 μF, pulse twice) was performed with Bio-Rad Gene Pulser II. Control cells were incubated with peptides without electroporation. All cells were extensively washed and cultured at 37°C for 16 hr. Cells were used in T cell assays, SDS-PAGE, and/or Western blots to detect biotin-$GAD_{273–285}$:class II complexes.

Monodansylcadaverine labeling

Cells were incubated with 0.05 mM MDC (Sigma) in PBS at 37°C for 10 min (Munafo and Colombo, 2001), washed with PBS, and lysed in buffer (10 mM Tris-HCl, 1% Triton X-100 [pH 7.5]). The fluorescence intensity was measured at excitation wavelength 380 nm and emission wavelength 525 nm for triplicate samples. Serum starvation was used as a control to demonstrate increased cellular autophagy and MDC labeling.

Subcellular Fractionation

Sucrose-gradient centrifugation was performed as described (Maric et al., 1994). Samples (2×10^8 cells) were homogenized at 4°C, centrifuged at 900 × g for 10 min, and the supernatant was spun at 200 × g above a 1.58 M sucrose cushion for 15 min to remove nuclei and mitochondria. Lysates were then centrifuged on continuous sucrose gradients (0.47–1.71 M) at 80,000 × g for 20 hr at 4°C. Fractions were collected and analyzed by SDS-PAGE and Western blot. Specific markers for organelles such as early endosomes and lysosomes were detected by immunoblotting (Maric et al., 1994).

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