Efficient Presentation of Both Cytosolic and Endogenous Transmembrane Protein Antigens on MHC Class II Is Dependent on Cytoplasmic Proteolysis

Paushali Mukherjee, Aadish Dani, Sumeena Bhatia, Nagendra Singh, Alexander Y. Rudensky, Anna George, Vineeta Bal, Satyajit Mayor and Satyajit Rath

J Immunol 2001;167;2632-2641

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Efficient Presentation of Both Cytosolic and Endogenous Transmembrane Protein Antigens on MHC Class II Is Dependent on Cytoplasmic Proteolysis[1]

Paushali Mukherjee,* Aadish Dani,† Sumeena Bhatia,* Nagendra Singh,* Alexander Y. Rudensky,‡ Anna George,* Vineeta Bal,* Satyajit Mayor,† and Satyajit Rath[2]*

Peptides from extracellular proteins presented on MHC class II are mostly generated and loaded in endolysosomal compartments, but the major pathways responsible for loading peptides from APC-endogenous sources on MHC class II are as yet unclear. In this study, we show that MHC class II molecules present peptides from proteins such as OVA or conalbumin introduced into the cytoplasm by hyperosmotic pinosome lysis, with efficiencies comparable to their presentation via extracellular fluid-phase endocytosis. This cytosolic presentation pathway is sensitive to proteasomal inhibitors, whereas the presentation of exogenous Ags taken up by endocytosis is not. Inhibitors of nonproteasomal cytosolic proteases can also inhibit MHC class II-restricted presentation of cytosolically delivered protein, without inhibiting MHC class I-restricted presentation from the same protein. Cytosolic processing of a soluble fusion protein containing the peptide epitope I-Eα_{52-68} yields an epitope that is similar to the one generated during constitutive presentation of I-Eα as an endogenous transmembrane protein, but is subtly different from the one generated in the exogenous pathway. Constitutive MHC class II-mediated presentation of the endogenous transmembrane protein I-Eα is also specifically inhibited over time by inhibitors of cytosolic proteolysis. Thus, Ag processing in the cytoplasm appears to be essential for the efficient presentation of endogenous proteins, even transmembrane ones, on MHC class II, and the proteolytic pathways involved may differ from those used for MHC class I-mediated presentation. ***The Journal of Immunology,*** **2001, 167: 2632–2641.**

T cells recognize targets via interactions of TCRs with peptide-bound MHC molecules, MHC class I or II, on APCs. Upon assembly in the endoplasmic reticulum (ER),[3] MHC class I molecules bind to peptides generated in the cytosol by proteasomal proteolysis (reviewed in Ref. 1). These peptides are transported into the ER by the TAP heterodimer, members of the ATP-binding cassette family of proteins (2). On the other hand, MHC class II molecules are prevented from binding peptides in the ER by the invariant chain (Ii), which is cleaved off in endolysosomal compartments (reviewed in Ref. 3). Binding of endolysosomally generated peptides to MHC class II takes place in these compartments, such as MIIC or CIIV (reviewed in Ref. 4), and is aided by the H-2 M (or HLA-DM) molecule (reviewed in Refs. 5 and 6). Thus, APCs are thought to present exogenous extracellular proteins on MHC class II to CD4 T cells for inducing inflammatory, allergic, or opsonizing immune responses, and endogenous cellular proteins on MHC class I to CD8 T cells for triggering cytotoxic immunity.

However, MHC class I can present peptides from exogenous proteins, either by cytosolic leakage of endosomal contents (reviewed in Ref. 7) or via direct endolysosomal processing (reviewed in Ref. 8). Conversely, MHC class II can present peptides from cytosolic proteins (reviewed in Refs. 9; see also Refs. 10 and 11). If both of these situations were exceptions, most peptides on MHC class I would still be from endogenous cellular sources and those on MHC class II from extracellular sources. Analyses of these peptide repertoires show that this is largely true for MHC class I (reviewed in Refs. 12 and 13). However, major peptides on MHC class II also appear to be mainly from cellular rather than extracellular proteins (14–16). Although this could be explained partly by endolysosomal processing of membrane proteins, many of these peptides are from cytosol-resident proteins. The mechanisms for the generation of peptides from endogenous proteins for loading on MHC class II molecules are thus poorly understood.

It has been shown recently that processing pathways can also determine the precise nature of a single peptide epitope presented by MHC class II. For example, an epitope generated from the constitutively expressed transmembrane protein I-Eα is 2 aa residues longer (aa 52–68) than the one generated by its exogenous processing via forced internalization (aa 52–66) (17). Such results suggest that the same protein may be differently processed and generate distinct peptide epitopes depending on whether it is endogenously or exogenously encountered. However, peptides from both endogenous and exogenous sources bind to MHC class II in the endolysosomal vesicles (18–21), although there are differences

*National Institute of Immunology, New Delhi, India; †National Centre for Biological Sciences, UAS-GKVK Campus, Bangalore, India; and ‡Department of Immunology, University of Washington School of Medicine, Seattle, WA 98195

Received for publication January 25, 2001. Accepted for publication July 12, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] These studies were supported in part by extramural grants from Department of Biotechnology, Government of India (to S.R.), Department of Science and Technology, Government of India (to A.G.), the Wellcome Trust (to V.B.), and U.S. Public Health Service Grant AI34206 (to A.Y.R.) and intramural support from Tata Institute of Fundamental Research (to S.M.). A.Y.R. is a Howard Hughes Medical Institute Associate Investigator. The National Institute of Immunology is supported by the Department of Biotechnology, Government of India.

[2] Address correspondence and reprint requests to Dr. Satyajit Rath, National Institute of Immunology, Aruna Asaf Ali Road, New Delhi 110067, India. E-mail address: satyajit@nii.res.in

[3] Abbreviations used in this paper: ER, endoplasmic reticulum; AAF-cmk, Ala-Ala-Phe-chloromethylketone; CA, conalbumin; Eap, I-Eα peptide; EapL, I-Eα peptide (aa 52–68); EapS, I-Eα peptide (aa 52–66); F-Dex, fluorescein-conjugated dextran; Ii, invariant chain; LLM, *N*-acetyl-leucinyl-leucinyl-methioninal; LLnL, *N*-acetyl-leucinyl-leucinyl-norleucinal; SR, scavenger receptor; TPP, tripeptidyl peptidase.

0022-1767/01/$02.00

regarding the precise characterization of this loading compartment (4). Thus, it is possible that the major processing pathway for MHC class II-restricted presentation of cellular proteins may differ from the classical endolysosomal processing pathway described for presentation of extracellular proteins on MHC class II.

In this context, we have examined the pathways of MHC class II-restricted presentation for cytosolically introduced proteins as well as a constitutively expressed transmembrane protein, and we show evidence here that the MHC class II-restricted presentation of both categories of proteins is dependent on cytosolic proteolysis, both proteasomal and nonproteasomal.

Materials and Methods

Mice

The mouse strains used, C3H/HeJ (H-2^k), C57BL/6 (H-2^b), B10.A(3R) (H-2^{i3}), and B10.A(5R) (H-2^{i5}), were bred in the small animal facility of the National Institute of Immunology (New Delhi, India) and used at 6–12 wk of age. All experiments were done with the approval of the Institutional Animal Ethics Committee.

Antigens

OVA or conalbumin (CA) (Sigma, St. Louis, MO) were maleylated with maleic anhydride (Sigma) at alkaline pH as previously described (22), dialyzed against PBS, and the degree of maleylation was estimated as the loss of free ϵ amino groups measured by 2,4,6-nitrobenzenesulfonic acid. Maleylated proteins were used only when maleylation was <90%. Demaleylation was done at pH 3.5 as described elsewhere (22) and showed regeneration of 90% of the free ϵ amino groups. A fusion protein (GST-I-Eα peptide (aa 52–68 (EapL)-myc) consisting of GST, residues aa 52–68 of the mouse I-Eα sequence, and the oligopeptide sequence of the c-myc protein recognized by the 9E10 mAb was made in *Escherichia coli* from an ampicillin-resistant plasmid with isopropyl β-D-thiogalactoside-inducible expression (data not shown) and was purified on a glutathione-Sepharose column. All Ags were dialyzed extensively against PBS to remove small degradation products, and the lack of processed fragments in all Ag preparations was confirmed in presentation assays utilizing fixed APCs.

Cell lines

The MHC class I (H-2K^b)-restricted OVA-specific T cell transfectant line B3 was a gift from Dr. M. Bevan (University of Washington, Seattle, WA). The MHC class II (I-A^b)-restricted OVA-specific T cell hybridoma 13.8 was generated from OVA-immunized C57BL/6 mice and characterized as I-A^b restricted (data not shown). The CA-specific MHC class II (I-A^k)-restricted T cell line 002 which is a transfectoma derived from the CA-specific I-A^k-restricted T cell clone D10.G4.1, and both the I-A^b-restricted I-Eα peptide (Eap)-specific T cell hybridomas, 1H3 recognizing both I-Eα peptide (aa 52–66) (EapS) and EapL, and Ea6 recognizing EapS but not EapL (17), were gifts from Dr. C. A. Janeway (Yale University, New Haven, CT) as was the B cell line E4, which is a clone of the A20 B cell line (H-2^d) transfected with I-Aα^b and I-Aβ^b.

Cytosolic Ag delivery by osmotic lysis of pinosomes

Proteins were delivered into the cytosol using osmotic lysis of pinosomes as described previously (23). Briefly, APCs were incubated in hypertonic serum-free DMEM with 0.5 M sucrose, 10% polyethylene glycol 800, and 10 mM HEPES containing antigenic protein for 10 min at 37°C, followed by washing and incubation in isotonic serum-free DMEM for 5 min. Exogenous loading of Ags was done by following the same protocol using isotonic medium. Cells were again washed and, where indicated, incubated further for 3 h to allow processing before being fixed with 1% paraformaldehyde (Sigma) for 1 min, washed, and used as APCs in T cell stimulation assays.

Fluorescence microscopy

C57BL/6 macrophages adherent on coverslip-bottom dishes were incubated with 2 mg/ml fluorescein-conjugated dextran (F-Dex) dissolved in either hypertonic medium DMEM with 0.5 M sucrose and 10% polyethylene glycol 800) or in isotonic DMEM for 15 min at 37°C. Excess F-Dex was washed off with isotonic medium and the distribution of F-Dex in cells was immediately observed on an inverted fluorescence microscope (TE-300; Nikon, Tokyo, Japan) with a ×60 oil objective with 1.4 numerical aperture. The images collected via a charge-coupled device camera (Princeton Instruments, Princeton, NJ) using Metamorph acquisition software (Universal Imaging, West Chester, PA) were processed for presentation in Adobe Photoshop (Adobe Systems, Mountain View, CA). Imaging of cells was done in the presence of 10 mM nigericin in a high potassium buffer (120 mM KCl, 5 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 20 mM HEPES, pH 7.4) to neutralize endosomal pH.

Ag presentation assays

Plastic-adherent peritoneal macrophages from thioglycolate broth-primed mice were preincubated for 30 min with various drugs, such as the lysosomotropic agents chloroquine and NH_4Cl (Sigma), the proteasome inhibitors lactacystin (Calbiochem, San Diego, CA), *N*-acetyl-leucinyl-leucinyl-norleucinal (LLnL) and *N*-acetyl-leucinyl-leucinyl-methioninal (LLM) (Sigma), or the tripeptidyl peptidase (TPP) inhibitors Ala-Ala-Phe-chloromethylketone (AAF-cmk) (Sigma) and butabindide (gift from Dr. C. Ganellin, University College, London, U.K.). Cells were then washed and either exogenously pulsed or cytosolically loaded with various Ags. They were then allowed to process Ags in the absence or presence of the inhibitors again for 3 h at 37°C and then fixed where required with 1% paraformaldehyde for 1 min, washed, and used as APCs in T cell stimulation assays.

T cell lines (1–10 × 10^4 cells/well) were stimulated with titrated concentrations of Ag-pulsed APCs in triplicate cultures in 200 μl of DMEM with 10% FCS, antibiotics, L-glutamine, and 0.5 mM 2-ME in 96-well flat-bottom plates (Falcon; Franklin Lakes, NJ) as indicated. Culture supernatants were collected 24–36 h later and used to estimate the IL-2 induced by stimulating the IL-2-dependent cell line CTLL-2 (1 × 10^4 cells/well) with them, incubating for 24 h, and pulsing the plates with 0.5 μCi of [^{3}H]thymidine/well (NEN Life Science, Boston, MA) for 12–16 h to measure the proliferative responses. Plates were harvested onto glass fiber filters for scintillation counting (Betaplate; Wallac, Turku, Finland). Data are shown as proliferation (mean ± SE) observed in triplicate CTLL-2 cultures.

Results

MHC class II-restricted presentation of proteins introduced into the APC cytosol by osmotic lysis of pinosomes

We introduced two proteins, CA or OVA, into the cytosol of mouse peritoneal macrophages by osmotic lysis of hypertonic pinosomes (23). Ag-loaded or mock-loaded H-2^k or H-2^b cells were used for CA and OVA presentation, respectively, to the appropriate T cell hybridomas, the CA-specific I-A^k-restricted line 002, and the OVA-specific I-A^b-restricted line 13.8. APCs cytosolically loaded with either Ag were well recognized by the relevant T cells (Fig. 1, *A* and *B*). Similar results were also seen with macrophage cell lines such as BMC2, splenic B cells, and B cell lines such as LB27.4 (data not shown).

To rule out the possibility of pinosomes remaining unruptured during osmotic pinosome lysis, we examined cells for residual punctate endosomal fluorescence after introducing F-Dex into cells by the osmotic lysis procedure. F-Dex fluorescence was predominantly cytosolic after osmotic lysis (Fig. 1*D*) while it was in punctate structures if endosomes were not lysed (Fig. 1*E*). We have previously shown that exogenously delivered maleylated proteins bind to scavenger receptors (SRs) on APCs and are presented to MHC class II-restricted T cells ~1000-fold better than their nonmaleylated counterparts (24). Therefore, to examine further whether the presentation of cytosolically loaded proteins was due to very low levels of residual unruptured endosomes, we determined whether maleyl proteins were presented much better than their nonmaleylated counterparts when delivered in this fashion.

Cytosolically delivered maleyl-CA or maleyl-OVA were not presented better than their native counterparts; in fact, they were very poorly presented (Fig. 1, *A* and *B*). In contrast, exogenously added maleyl-OVA was presented 300- to 1000-fold more efficiently than native OVA, and this enhanced presentation was due to SR-mediated uptake, since it could be blocked by an excess of maleyl-BSA (Fig. 1*C*). These data further confirm that the MHC class II-restricted presentation of proteins delivered via osmotic lysis of pinosomes is not due to the presence of residual unruptured

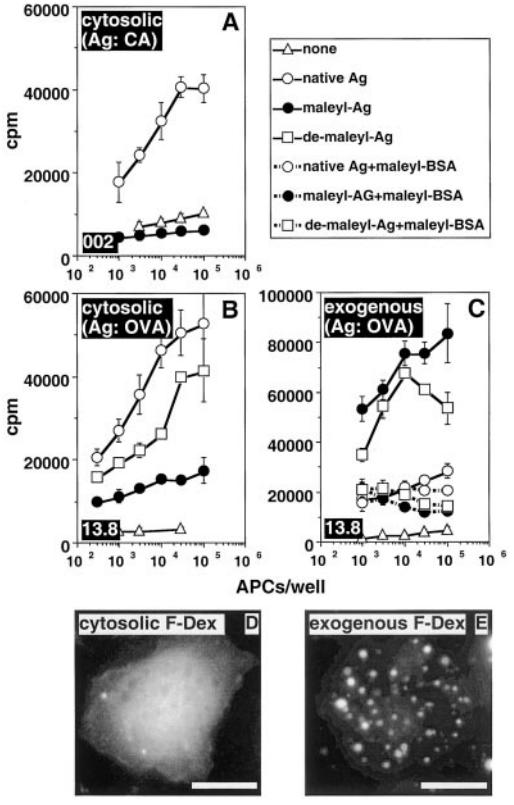

FIGURE 1. Native Ags, but not maleylated proteins, loaded into the APC cytosol by osmotic lysis of pinosomes are well presented to MHC class II-restricted T cells. *A–C,* The Ags indicated were loaded (10 mg/ml) into macrophage APCs in the form and by the route shown, and titrating numbers of these APCs were used to stimulate the appropriate T cell lines. For CA, the T cell line was 002, while for OVA, it was 13.8. *D* and *E,* Distribution of F-Dex in macrophages by fluorescent microscopy immediately upon either cytosolic loading by osmotic lysis of pinosomes (*D*) or exogenous loading (*E*) with F-Dex (2 mg/ml). Bar, 20 μm. The data are representative of five to six independent experiments.

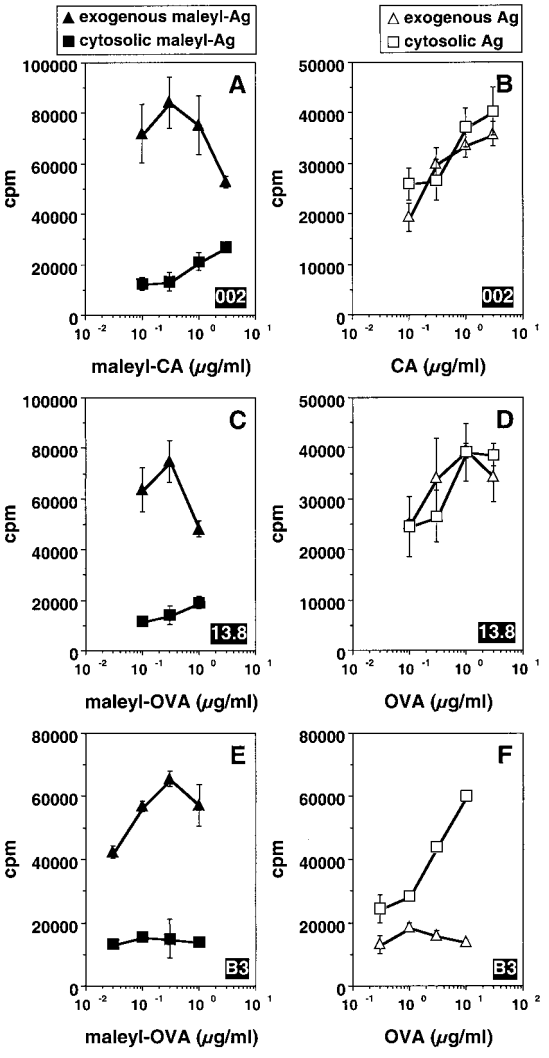

FIGURE 2. The cytosolic pathway of MHC class II-restricted presentation in highly efficient. Macrophages were pulsed with varying doses of various Ags as shown for 15 min in either hypertonic (cytosolic) or isotonic (exogenous) medium, followed by culture in isotonic medium with appropriate T cell lines. The proteins used were either native or maleylated as shown. *A* and *B,* Responses of the CA-specific I-A^k-restricted T cell line 002 to Ag-loaded C3H/HeJ macrophage APCs. *C* and *D,* Responses of the OVA-specific I-A^b-restricted T cell line 13.8 to Ag-loaded C57BL/6 macrophage APCs, while *E* and *F* show the responses of the OVA-specific H-$2K^b$-restricted T cell line B3 to Ag-loaded C57BL/6 macrophage APCs. The results are representative of four independent experiments.

pinosomes. The cytosolic pathway for MHC class II-restricted presentation observed is thus distinct from the classical pathway for presenting exogenous proteins.

Efficiency of generation of peptide-MHC class II complexes via cytosolic or endocytic pathways

We next examined the efficiency of the MHC class II-restricted presentation of cytosolic proteins by comparing the relative levels of peptide-MHC complexes generated from varying concentrations of proteins loaded either into the cytosol or in endosomes. For this, either C57BL/6 or C3H/HeJ macrophages were pulsed with varying concentrations of native or maleylated OVA or CA in either hypertonic medium or isotonic medium for 15 min, followed by culture in isotonic medium along with the appropriate T cell lines.

The efficiency of presentation of cytosolic CA or OVA on MHC class II was not very different from that of equivalent amounts of exogenous CA or OVA (Fig. 2, *B* and *D*). In contrast, cytosolic OVA was far better presented on MHC class I than exogenously delivered OVA (Fig. 2*F*). As expected, the maleyl proteins were very poorly processed and presented on either MHC class I or MHC class II if they were loaded in the cytosol, while their processing was very efficient for loading on both classes of MHC if they were given exogenously (Fig. 2, *A, C,* and *E*). To ensure that similar amounts of extracellular fluid (and proteins) were taken up by APCs during cytosolic and endocytic loading, we determined the amounts of HRP included as a tracer associated with APCs by either mode of loading, and found that ~10 μl of fluid per million cells was taken up in both cases (data not shown). These data suggest that the cytosolic Ag processing pathway for peptide loading on MHC class II is efficient even in comparison to the classical postpinocytotic endosomal processing pathway, making the mechanisms involved in this pathway of major interest.

Abolition of MHC class II-restricted presentation of cytosolic proteins by proteasomal inhibition

The poor presentation of cytosolic maleyl proteins was surprising. Maleylation blocks the free ϵ amino groups of lysine residues (22), methylation of which marks proteins for ubiquitination and proteasomal degradation (25). We have already shown that protein

maleylation inhibits their cytosolic MHC class I-restricted presentation (26). It is therefore possible that processing of cytosolic proteins for presentation via MHC class II may also depend on the availability of free ϵ amino groups in the protein. We confirmed this by demaleylating the maleyl protein, which is known to maintain the SR-binding conformation conferred by maleylation, although the lysine ϵ amino groups are reactivated (22). Demaleylated maleyl-OVA showed regeneration of free lysine ϵ amino groups (data not shown) as well as enhanced SR-mediated MHC class II-restricted presentation exogenously (Fig. 1*C*). However, unlike maleyl-OVA, demaleylated maleyl-OVA was well presented to T cells upon loading in the APC cytosol as well (Fig. 1*B*). These data show that MHC class II-restricted presentation of cytosolically introduced proteins requires free ϵ amino groups on lysine residues, suggesting the possibility that they may be processed via ubiquitin-tagged proteasomal degradation similar to that observed in the classical MHC class I-restricted presentation pathway (25).

We next addressed the proteolytic mechanisms involved in the processing of cytosolically loaded proteins for MHC class II-restricted presentation. To explore the role of proteasomes, we used various inhibitors of proteasomal degradation, lactacystin, LLnL, and LLM (27). Of the three proteasome inhibitors used, LLnL and LLM are reported to be equipotent inhibitors of cathepsin B and calpains but with marked differences in their inhibition against the proteasomes. The presence of lactacystin, LLnL, or LLM during processing of cytosolic CA or OVA inhibited their MHC class II-restricted presentation (Fig. 3, *B* and *D*). However, none of these inhibitors affected the presentation of exogenous maleyl-CA or maleyl-OVA (Fig. 3, *A* and *C*) as well as of exogenous OVA or CA (data not shown), ruling out any nonspecific inhibition mediated by their ability to inhibit nonproteasomal activities. Lactacystin also inhibited MHC class I-restricted presentation of cytosolic OVA (Fig. 3*F*), confirming that proteasome-dependent processes were affected. MHC class I-restricted presentation of exogenous maleyl-OVA was not inhibited by lactacystin (Fig. 3*E*), consistent with the extra cytosolic processing of proteins delivered via SRs for presentation on MHC class I (26). Thus, MHC class II-restricted presentation of cytosolic proteins follows the MHC class I-restricted presentation pathway in being dependent on proteasomal activity, presumably for peptide generation. However, exogenous protein presentation is proteasome independent for both classes of MHC.

Involvement of nonproteasomal cytoplasmic proteolysis in MHC class II-restricted presentation of cytosolic Ags

Proteasomal proteolysis has been implicated in the generation of most MHC class I-restricted peptides (27). However, recent data also point to the involvement of nonproteasomal proteolytic mechanisms in the cytoplasm in generating peptides for MHC class I-restricted presentation (28), perhaps in tandem with proteasomal mechanisms (29). We therefore looked at the role of nonproteasomal proteolysis in MHC class II-restricted presentation of cytosolically introduced proteins by using two inhibitors, AAF-cmk and butabindide, both of which inhibit TPPs, particularly TPP-II (30–32). Butabindide appears to be specific for TPP-II, whereas AAF-cmk inhibits other cytosolic proteases implicated in the N-terminal processing of some MHC class I-binding epitopes (33). Treatment of macrophages with as high a concentration of AAF-cmk as 25 μM during presentation of cytosolic OVA did affect the MHC class I-restricted presentation of OVA to B3 T cells somewhat (Fig. 4*B*), but inhibited its presentation to MHC class II-restricted 13.8 T cells markedly (Fig. 4*D*). However, AAF-cmk had no effect on the presentation of exogenous maleyl-OVA to either B3 or 13.8 T cells (Fig. 4, *A* and *C*), confirming the specificity of the inhibition of the cytosolic pathway. Butabindide also caused some inhibition of MHC class I-restricted presentation of cytosolic OVA, although it had no effect on the presentation of exogenous maleyl-OVA (Fig. 4). However, the inhibition of MHC class II-restricted presentation of OVA by butabindide was not as marked as that caused by AAF-cmk (Fig. 4). Taken together, these results indicate a key role played by nonproteasomal TPP proteases in the MHC class II-restricted presentation of cytosolic Ags, distinct from their role in MHC class I-mediated presentation.

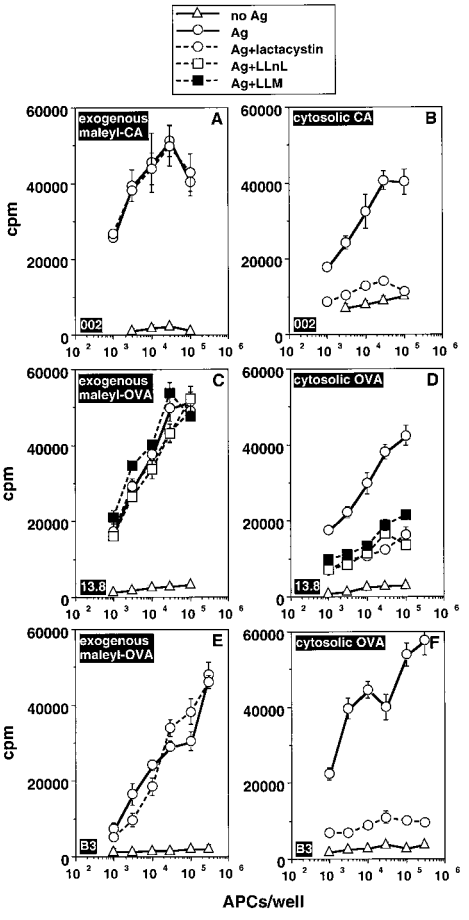

FIGURE 3. The processing of cytosolically loaded proteins for presentation on MHC class II is inhibited by proteasome inhibitors. *A* and *B*, The responses of the CA-specific I-A^k-restricted T cell line 002 to titrating numbers of C3H/HeJ macrophage APCs either mock loaded or loaded with either exogenous maleyl-CA (*A*) or cytosolic CA (*B*) in the absence or presence of 25 μM lactacystin are shown. *C* and *D*, The responses of the OVA-specific I-A^b-restricted T cell line 13.8 to titrating numbers of C57BL/6 macrophage APCs either mock loaded or loaded with either exogenous maleyl-OVA (*C*) or cytosolic OVA (*D*) in the absence or presence of 25 μM inhibitor are shown. *E* and *F*, The responses of the OVA-specific H-$2K^b$-restricted T cell line B3 to titrating numbers of C57BL/6 macrophage APCs either mock loaded or loaded with either exogenous maleyl-OVA (*E*) or cytosolic OVA (*F*) in the absence or presence of 25 μM lactacystin are shown. Macrophages were pulsed with the various Ags (native proteins, 10 mg/ml; maleyl proteins, 100 μg/ml) and paraformaldehyde fixed 3–4 h later for use as APCs. The data are representative of five to six independent experiments.

Lack of role for endosomal acidification in processing of cytosolic Ags for MHC class II- restricted presentation

Ags in the endocytic compartments of the cells are hydrolyzed to oligopeptides by acid-optimal proteases. Lysosomotropic agents

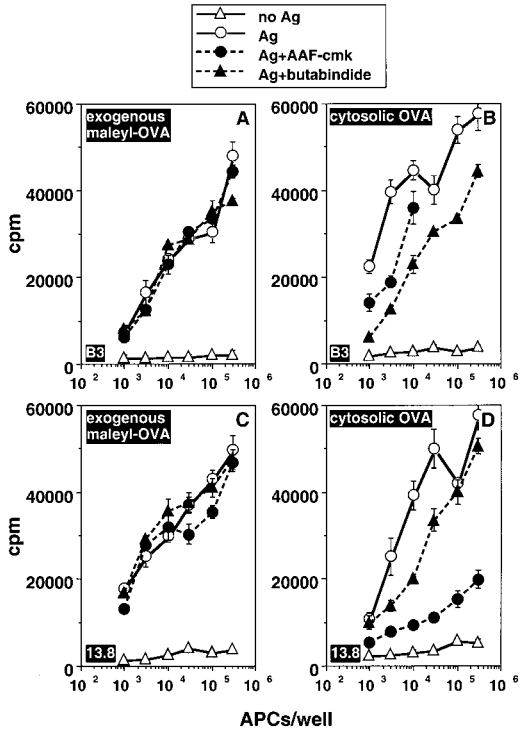

FIGURE 4. Cytosolic processing of OVA for MHC class II-mediated presentation is inhibited by AAF-cmk but not by butabindide, while neither can inhibit MHC class I-restricted presentation. The responses of either OVA^+H-$2K^b$-restricted B3 T cells or OVA^+I-A^b-restricted 13.8 T cells to C57BL/6 macrophages loaded either cytosolically with OVA (1 mg/ml) or exogenously with maleyl-OVA (100 μg/ml) in the absence or presence of either 25 μM AAF-cmk or 25 μM butabindide are shown. The data shown are representative of two independent experiments.

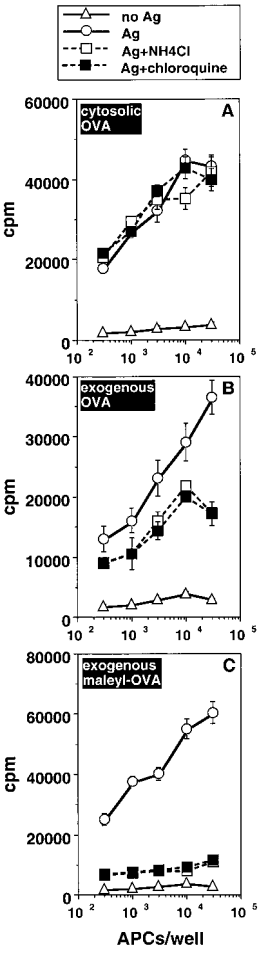

FIGURE 5. Cytosolic processing of OVA for MHC class II-mediated presentation does not require endolysosomal acidification. The responses of OVA-specific 13.8 T cells to C57BL/6 macrophages loaded with OVA (1 mg/ml) either cytosolically or exogenously, or loaded exogenously with maleyl-OVA (100 μg/ml) in the absence or presence of either 200 mM ammonium chloride or 100 μM chloroquine are shown. The data shown are representative of two independent experiments.

such as ammonium chloride and chloroquine raise the pH of the distal acidic vesicles and inhibit proteolysis in these compartments (8), inhibiting both Ag degradation and Ii proteolysis. To examine whether the presentation of cytosolic OVA also required further endolysosomal processing in addition to the cytosolic proteolysis described above, its sensitivity to chloroquine and ammonium chloride was next tested. APCs were preincubated with various concentrations of either drug for 1 h before loading with Ags. They were then loaded cytosolically with OVA in hypertonic medium or exogenously with either OVA or maleyl-OVA in isotonic medium and incubated in isotonic medium for processing for 3 h in the presence or absence of the drugs before fixation and used for stimulating T cell lines. Neither 100 μM chloroquine nor 200 mM ammonium chloride had any effect on the presentation of cytosolic OVA (Fig. 5*A*), although the presentation of exogenous fluid-phase OVA was consistently reduced by both treatments (Fig. 5*B*). The presentation of exogenous maleyl-OVA was blocked completely by chloroquine and 200 mM ammonium chloride (Fig. 5*C*). In addition, 2 mM ammonium chloride also effectively blocked presentation of maleyl-OVA on MHC class II (data not shown), in keeping with the data we have previously reported for its MHC class I-restricted presentation (26). These results demonstrate that cytosolic OVA processing is independent of the acidic environment of the endolysosomal compartments.

Generation of similar MHC class II-presented epitopes by cytosolic and constitutive endogenous pathways

The existence of a separate and efficient pathway for processing and presentation of cytosolic proteins on MHC class II suggests that it may also be used for MHC class II-restricted presentation of endogenous cellular proteins. To address this issue, we used T cell hybridomas recognizing a well-characterized peptide epitope generated constitutively from an endogenous protein source, I-Eα. The I-Eα transmembrane MHC class II polypeptide yields the peptide epitope 52–68 (Eap), from the extracytoplasmic region of the molecule, which binds to I-A^b (15). This Eap-I-A^b complex is constitutively present at very high frequencies (up to 15%) of surface I-A^b in I-Eα-expressing cells, such as in the mouse strains B10.A(3R) or B10.A(5R). Two kinds of Eap-specific T cell hybridomas have been reported. One group (such as the cell line 1H3 used here) recognizes both APCs given exogenous Eap-containing protein and APCs constitutively expressing I-A^b and I-Eα (15), while the other (such as the cell line Ea6 used here) responds only to presentation of exogenous Eap-containing protein and not to APCs expressing I-A^b and I-Eα constitutively (17). This is because exogenous processing of Eap-containing protein generates a short peptide (aa 52–66; EapS), while the constitutive endogenous Eap-containing protein yields a long peptide (aa 52–68; EapL) for presentation on I-A^b (17).

We used this difference to ask if presentation of Eap on I-A^b via the cytosolic processing pathway resembled its presentation from endogenously expressed I-Eα. To this end, we used a chimeric

fusion protein containing the EapL peptide between GST and an 11-mer c-myc tag (GST-EapL-myc). We delivered GST-EapL-myc to H-2^b macrophages either exogenously or cytosolically and tested the responses of the T cell lines 1H3 (responsive to both EapS and EapL) and Ea6 (responsive only to EapS). As previously reported (17), the EapL-I-A^b complex on B10.A(5R) APCs was recognized by 1H3 but not by Ea6 T cells (Fig. 6*A*). Similarly, cytosolically delivered GST-EapL-myc was only recognized by 1H3 and not by Ea6 T cells (Fig. 6*B*). However, exogenously processed GST-EapL-myc was well recognized by both 1H3 and Ea6 T cells (Fig. 6*C*). This result indicated that the peptide species generated from the cytosolically delivered protein resembles that generated from the processing of an endogenously expressed transmembrane protein. Furthermore, lactacystin treatment inhibited the presentation of cytosolically loaded GST-EapL-myc to 1H3, but did not affect the presentation of exogenously delivered GST-EapL-myc to 1H3 or Ea6 T cells (Fig. 6, *B* and *C*). Inhibitors of endolysosomal acidification such as chloroquine or ammonium chloride significantly inhibited the presentation of GST-EapL-myc as an exogenous protein (Fig. 7*A*), but they did not inhibit the presentation of cytosolic GST-EapL-myc (Fig. 7*B*) to 1H3 T cells. Thus, the presentation pathways of cytosolic Eap-containing protein and of the constitutive endogenous transmembrane protein I-Eα are parallel in generating a similar fine specificity of Eap.

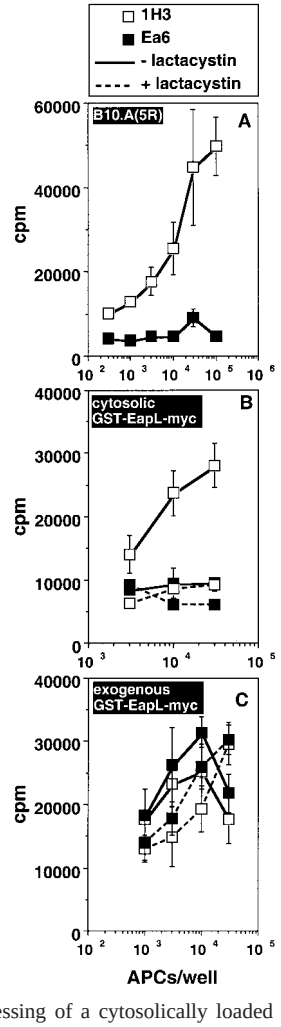

FIGURE 6. Processing of a cytosolically loaded GST-EapL-myc protein leads to the lactacystin-sensitive formation of the EapL form of the peptide which is also generated from the constitutive endogenous source. The responses of either EapL/EapS-nondiscriminating 1H3 T cells or the EapS-specific Ea6 T cells to titrating numbers of either B10. A(5R) macrophages (*A*) or of C57BL/6 macrophages loaded cytosolically (*B*; 100 μg/ml) or exogenously (*C*; 30 μg/ml) with GST-EapL-myc in the absence or presence of 25 μM lactacystin are shown. The data shown are representative of four independent experiments.

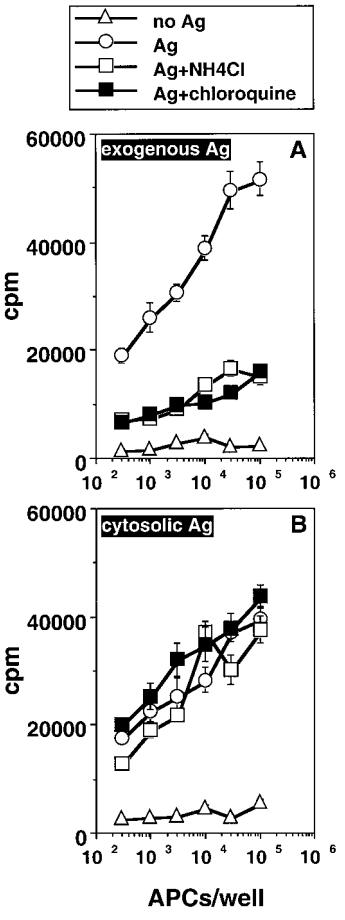

FIGURE 7. Cytosolic processing of GST-EapL-myc for MHC class II-mediated presentation does not require endolysosomal acidification. The responses of Eap-specific 1H3 T cells to C57BL/6 macrophages loaded either exogenously (30 μg/ml) or cytosolically (100 μg/ml) with GST-EapL-myc in the absence or presence of either 200 mM ammonium chloride or 100 μM chloroquine are shown. The data shown are representative of two independent experiments.

Inhibition of constitutive MHC class II-restricted presentation of endogenous transmembrane I-Eα by lactacystin and AAF-cmk

Since cytosolic GST-EapL-myc and endogenous I-Eα both appear to yield the EapL epitope, I-A^b-restricted presentation of constitutively expressed endogenous transmembrane I-Eα is likely to be mediated by this novel pathway. If this were the case, the constitutive expression of the EapL-I-A^b complex should be sensitive to inhibitors of the cytosolic pathway of MHC class II-restricted presentation. We therefore incubated macrophages from B10.A(3R) mice, which express both I-A^b and I-Eα, in lactacystin for varying periods of time before fixing them and using them as APCs for 1H3 T cells. The reactivity of 1H3 went down progressively over time when the stimulating B10.A(3R) APCs were incubated in lactacystin (Fig. 8*A*). This was a specific loss of presentation rather than one due to nonspecific inhibitor toxicity, since even after a 24-h incubation with or without lactacystin, B10.A(3R) macrophages stimulated OVA-specific 13.8 T cells comparably if exogenous maleyl-OVA was added during the last 3 h of the incubation (Fig. 8*B*). Furthermore, the loss of 1H3 reactivity of B10.A(3R)

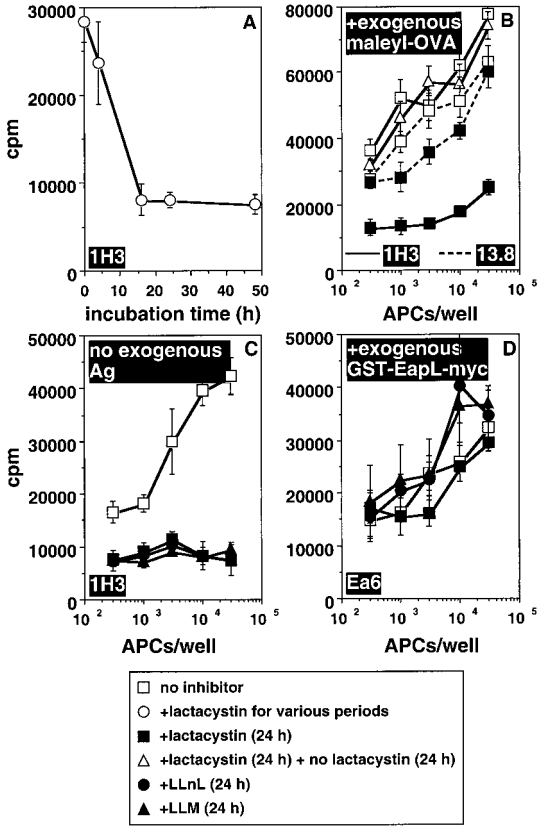

FIGURE 8. Presentation of an MHC class II-restricted peptide derived from a constitutive endogenous protein is specifically inhibited by lactacystin. *A*, Responses of the Eap-specific T cell line 1H3 to B10.A(3R) macrophages (1×10^5 APCs/well) incubated for varying periods of time as indicated in 25 μM lactacystin before being fixed and used as APCs are shown. *B*, Responses evoked by B10.A(3R) macrophages incubated for 24 h either without or with 25 μM lactacystin and pulsed for the last 3 h of the incubation with maleyl-OVA (100 μg/ml) from either Eap-specific 1H3 T cells or OVA-specific 13.8 T cells are shown. The responses of 1H3 T cells to B10.A(3R) macrophages incubated for 24 h in 25 μM lactacystin, washed, and incubated for another 24 h in lactacystin-free medium are also shown. *C* and *D*, Responses evoked from Eap-specific 1H3 T cells by B10.A(3R) macrophage APCs incubated for 24 h either without a proteasome inhibitor, or in the presence of 25 μM lactacystin, or with LLnL or LLM before being fixed and used as APCs are shown (*C*). Alternatively, these APCs were pulsed for the last 6 h of presentation with 30 μg/ml GST-EapL-myc in the continuing presence of the proteasome inhibitors before being fixed and used to stimulate Ea6 T cells, the responses of which are shown (*D*). The data shown are representative of three independent experiments.

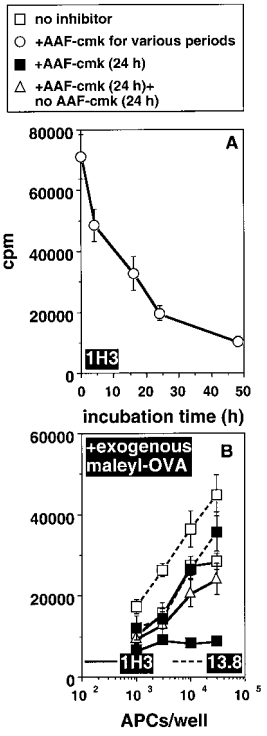

FIGURE 9. Presentation of an MHC class II-restricted peptide derived from a constitutive endogenous protein is specifically inhibited by AAF-cmk. *A*, Responses of the Eap-specific T cell line 1H3 to B10.A(3R) macrophages (1×10^4 APCs/well) incubated for varying periods of time in 10 μM AAF-cmk before being fixed and used as APCs are shown. *B*, Responses evoked by B10.A(3R) macrophages incubated for 24 h either without or with 10 μM AAF-cmk and pulsed for the last 3 h of the incubation with maleyl-OVA (100 μg/ml) from either Eap-specific 1H3 T cells (—) or OVA-specific 13.8 T cells (- - -) are shown. The responses of 1H3 T cells to B10.A(3R) macrophages incubated for 24 h in 10 μM AAF-cmk, washed, and incubated for another 24 h in AAF-cmk-free medium are also shown. Data shown are representative of two independent experiments.

macrophages incubated for 24 h with either lactacystin, LLnL, or LLM (Fig. 8*C*) was specific, since unchanged levels of EapS-I-A^b complexes recognizable by Ea6 were generated by these same B10.A(3R) macrophages from exogenous GST-EapL-myc protein added for the last 6 h of the 24-h incubation in proteasome inhibitors (Fig. 8*D*). Further incubation of the APCs for 24 h after washing out lactacystin allowed them to regain the ability to stimulate 1H3 T cells, confirming that the effect of lactacystin was not due to nonspecific toxicity (Fig. 8*B*).

The effect of AAF-cmk on the endogenous generation of EapL-I-A^b complexes was similarly tested. Incubation in 10 μM AAF-cmk over a 24-h period also led to a progressive loss of the ability of B10.A(3R) macrophages to stimulate 1H3 T cells (Fig. 9*A*). When B10.A(3R) macrophages were incubated in AAF-cmk for 24 h and pulsed for the last 3 h of this period with maleyl-OVA, AAF-cmk had no effect on their ability to present maleyl-OVA to 13.8 T cells, despite abrogating 1H3-stimulatory capacity (Fig. 9*B*). Furthermore, removal of AAF-cmk-containing medium followed by another 24 h of incubation allowed these cells to regain their ability to stimulate 1H3 T cells (Fig. 9*B*). These data confirm that nonproteasomal proteases are also specifically involved in the processing of endogenous transmembrane protein for MHC class II-restricted presentation.

To rule out the possibilities of nonspecific toxicity of protease inhibitors by yet another approach, we used a transformed B cell line expressing both I-A^b and I-Eα. E4 is a B cell line derived from the A20 B cell lymphoma line which expresses the H-2^d MHC haplotype and has been transfected with I-A^b (34). Thus, E4 cells express both I-A^b and I-E, and therefore constitutively express the Eap-I-A^b complex. When E4 cells were incubated in either lactacystin or AAF-cmk for 24 h, their ability to stimulate 1H3 T cells was significantly lost (Fig. 10). There was no significant loss of cell viability during this period (data not shown), and removal of the inhibitors followed by further incubation for 24 h allowed the cells to regain 1H3-stimulatory capacity (Fig. 10).

Discussion

We present characterization here of the proteolytic mechanisms involved in the presentation of peptide epitopes derived from endogenous proteins to MHC class II-restricted T cells. These mechanisms are operative both for proteins introduced into the cytosol

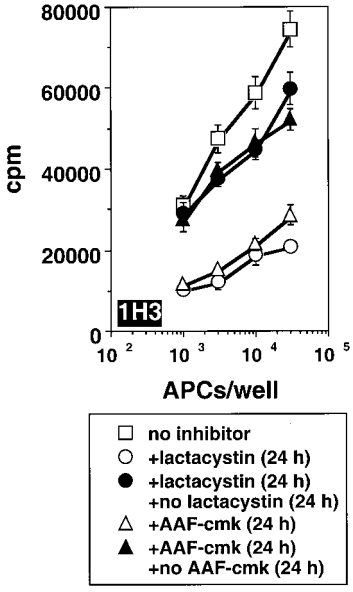

FIGURE 10. Endogenous constitutive MHC class II-mediated presentation in B lineage cells is dependent on cytosolic proteolysis. Aliquots of the E4 B cell line was incubated for 24 h in either 10 μM lactacystin or 10 μM AAF-cmk, or without inhibitor. Some aliquots were further incubated without the inhibitors in fresh medium for an additional 24 h. All samples were fixed and used as APCs in titrating numbers for the Eap-specific T cell line 1H3, responses of which are shown. The data are representative of two independent experiments.

by osmotic lysis of pinosomes and for a constitutive endogenous transmembrane protein and can be found in both macrophage and B cell lineages. These mechanisms are distinct from the ones needed for exogenous Ag presentation and do not require endosomal acidification. They are dependent on cytosolic proteasomal proteolysis, similar to those mediating MHC class I-restricted presentation of cytosolic proteins. However, nonproteasomal protease activities are also involved in this pathway, in a fashion distinct from their role in MHC class I-mediated presentation. The approach we have used so far has relied on pharmacological inhibitors, and it remains possible that noncanonical effects of the inhibitors used may have contributed to their effects, although both the consistency of results from a variety of inhibitors and the specificity of the effects observed argue together strongly against such a possibility.

Most reported examples of MHC class II-restricted presentation of endogenous cellular proteins involve either viral proteins (35, 36) or proteins encoded by gene constructs transfected into APC lines (37). We have taken a different approach by loading preformed proteins into the cytosol via the osmotic lysis of pinosomes. The method allows us to make quantitative comparisons between the presentation of similar quantities of the same protein delivered either endogenously or exogenously. As against this, the disadvantage is the possibility that some pinosomes may escape osmotic lysis and Ag contained in them may be processed conventionally. This would have been a problem of potential seriousness only if the method did not reveal any major differences between the two pathways. Since our data clearly demonstrate a number of such differences, this is not a significant problem. In further support of this, we have demonstrated that the delivery of a maleylated protein exogenously to macrophages leads to a greatly improved efficiency of MHC class II-mediated presentation as compared with the native form of the protein, and yet the same maleyl protein is presented very poorly if at all by the cytoplasmic route. This confirms that the MHC class II-mediated presentation seen after cytosolic loading of APCs is not due to conventional processing of the contents of residual unruptured pinosomes. Although the loading of proteins via osmotic lysis of pinosomes is an unphysiological mode of delivery, the relevance of the conclusions drawn from such an approach is validated by the fact that a constitutive endogenous transmembrane protein, I-Eα, is also proteasome dependent for its MHC class II-restricted presentation.

There are conflicting reports about the efficiency of MHC class II-mediated presentation of cytosolic proteins. For example, cytosolically expressed H-2L^d (37) or viral nucleoprotein (36) are poorly presented on MHC class II, but cytosolic influenza virus matrix protein is well presented (10). We find that the introduction of various soluble proteins (OVA, CA, or GST-EapL-myc) into the cytosol by osmotic lysis of pinosomes allows their efficient MHC class II-restricted presentation. The evidence for generation of subtly different peptidic species (EapL vs EapS) from the same protein, depending on whether it is processed endogenously or exogenously, suggests a possible explanation for reported failures to detect MHC class II-mediated presentation of proteins from endogenous sources. In most of these studies, T cells generated by exogenously processed forms of the proteins were used to detect endogenous processing, which may generate either different fine specificities of the same peptide epitope as in the studies here or even completely different peptide epitopes from the same protein (38).

A number of findings suggest that this proteasome-dependent endogenous pathway is far from being minor or inefficient. First, there is no major difference in the efficiency of presentation of the same protein as exogenous fluid-phase protein or as introduced cytosolic protein. Second, the fine specificity of a peptide epitope generated via this cytosolic pathway is the same as that generated at high density from a constitutive endogenous protein and different from that seen upon exogenous processing. Third, the constitutive expression of this high-density epitope from its constitutive cellular source, I-Eα, is also specifically inhibited by inhibitors of cytosolic proteolysis. This pathway is thus likely to play a major role in the shaping of the peptide repertoire of MHC class II on APCs in vivo.

It is increasingly evident that different APC lineages have significant differences in their pathways of Ag processing and presentation (37, 39, 40). We have therefore used normal ex vivo peritoneal macrophage APCs rather than transformed cell lines or mixed APC populations. However, we have also obtained similar results using freshly isolated B cells as well as transformed cell lines of other lineages (data not shown), suggesting that the pathway identified here is a general mechanism rather than a monocytic lineage-specific one.

Proteasomal proteolysis is crucial for generating peptide epitopes from cytoplasmic proteins for presentation on MHC class I (reviewed in Ref. 1). The role of proteasomal activity in MHC class II-restricted presentation of endogenous cellular proteins has also been previously suggested. The lack of presentation of a variant Ag with a short cytosolic half-life has been used to argue against the involvement of proteasomes (10). However, inhibitors of proteasomal activity such as LLnL and lactacystin have recently been shown to inhibit MHC class II-restricted presentation of proteins such as hen egg lysozyme (11) and glutamate decarboxylase (41) from endogenous but not exogenous sources. Similarly, our results here show that cytosolic presentation is inhibited by the blocking of ϵ amino groups on the Ag by maleylation, indicating that free ϵ amino groups are required for the process involved, consistent, among other possibilities, with an effect mediated by ubiquitination, which is a signal for proteasomal degradation (1).

Furthermore, lactacystin, LLnL, and LLM inhibit the presentation of cytosolically loaded proteins, establishing that proteasomal degradative activity is essential for MHC class II-mediated presentation of cytoplasmic proteins.

Crucially, our results further show that the MHC class II-restricted presentation of an endogenous transmembrane protein, I-Eα, is also inhibited by proteasome inhibitors. Prolonged treatment with proteasome inhibitors may have consequences in addition or further to the inhibition of proteasomal activity, such as global inhibition of protein synthesis. Although this formal possibility remains, global changes such as inhibition of protein synthesis do not explain the specific inhibition of MHC class II-restricted presentation of cytosolic proteins, since during the same period, presentation of exogenously added maleyl-OVA, which requires new protein synthesis (being cycloheximide sensitive (data not shown)), is not affected at all. Thus, these data suggest that MHC class II-restricted presentation of endogenous transmembrane I-Eα protein is proteasome dependent. This may be related to the retrotranslocation of membrane and secretory proteins during their assembly and the role of proteasomes in the degradation of their poorly folded forms (42–46). An alternative explanation may lie in the generation of cytosolic forms of I-Eα as defective ribosomal products due to inefficient translocation or folding for a variety of reasons (47–49).

Although our results show that proteasomes are required for the generation of peptides for this pathway, they do not suggest that proteasomes are sufficient for peptide generation. It is quite possible that proteasomal degradation is one intermediate event in a chain of degradative steps. Although there have been previous suggestions that some of these steps may in fact be endosomal (18–21), we find no evidence that this pathway is dependent on endosomal acidification. Nonetheless, it remains possible that translocation of large postproteasomal intermediates could be followed by further processing in the endolysosomal compartments, explaining why the presentation of some peptide-MHC class II epitopes from endogenous sources is sensitive to agents that disrupt lysosome function (30).

Furthermore, we find that nonproteasomal protease activities are likely to play a critical role in the presentation of endogenous proteins on MHC class II, and that this may be different from their role in processing of peptides for loading on MHC class I. There is controversy about the role of nonproteasomal cytosolic enzymatic systems in the generation of peptides loaded on MHC class I. Although cells selected in proteasome inhibitors have been shown to possess normal levels of surface MHC class I suggesting such a possibility (28), proteasomal inhibition in such situations has been shown to be incomplete (50). Since the presentation of cytosolic proteins on MHC class II is completely lactacystin inhibitable, our data do not suggest a proteasome-independent pathway of peptide generation in this event.

However, there have also been suggestions that amino-terminal trimming of proteasomally generated intermediates may be required before loading on MHC class I and that this trimming may be extraproteasomal (29, 33). A number of possibilities have been suggested for the identity and location of these nonproteasomal trimming peptidases. Some of these have been postulated as ER-based mechanisms (29), whereas others possibilities involve cytosolic enzymes such as cytosolic leucine amino peptidase (51) and the more recently identified puromycin-sensitive aminopeptidase and bleomycin hydrolase (33). Our data suggest that such an extraproteasomal degradatory component is also likely be involved in the processing of cytosolic proteins for loading on MHC class II.

It has been reported that the presentation of SIINFEKL, the peptide recognized by H-2K^b-restricted B3 T cells, on OVA-transfected APCs is inhibited by 50 μM AAF-cmk (33). AAF-cmk causes only marginal inhibition of MHC class I-mediated presentation of cytosolically loaded OVA at 25 μM in our experiments. The difference may be due either to the lower concentrations we have used and/or to the possibility that the newly synthesized nascent OVA polypeptide may be handled differently in cytosolic degradation from loaded, preformed mature OVA. However, MHC class II-mediated presentation is inhibited drastically, not only by 25 μM AAF-cmk, but also by 5 μM AAF-cmk (data not shown). This ability of AAF-cmk to inhibit the presentation of OVA on MHC class II even when it does not inhibit it for MHC class I suggests that extraproteasomal protease requirements may indeed be more stringent for peptides destined for MHC class II than for MHC class I. The identity and location of this essential TPP activity identified by AAF-cmk is still unclear. It is possible that TPP-II is not the only activity involved, since butabindide, an inhibitor of TPP-II (31, 32), causes less drastic inhibition of the presentation of cytosolic protein on MHC class II. Whether other related enzymatic activities such as TPP-I, puromycin-sensitive aminopeptidase, or bleomycin hydrolase are also involved remains to be investigated. Although our data do not address the possibility, calpain has also been implicated in the processing of cytosolic Ags for MHC class II-mediated presentation (41).These major differences between the cytosolic pathways for MHC class I and MHC class II may also depend on the fact that there may be multiple categories of proteasomes, perhaps in separate intracellular locations (52) or generating various kinds (perhaps differing lengths) of peptides differentially available for the transport pathways. The identity of these transport mechanisms carrying cytosolically generated peptide fragments into vesicular compartments for loading on MHC class II molecules is thus now an intriguing question, and we have data indicating that a novel cytosol-to-endosome pathway of peptide transport may be involved in the MHC class II-mediated presentation via this endogenous pathway (P. Mukherjee S. Bhatia, A. Dani, A. George, A. Y. Rudensky, S. Mayor, and S. Rath, manuscript in preparation).

There are major implications of such a distinct cytosolic proteolytic pathway for the presentation of endogenous proteins on MHC class II. The rules for generation of these peptides would be different in the two pathways even for the same protein, resulting in nonidentical peptide repertoires for the same self-protein depending on whether it is processed as a cellular protein or an extracellular protein during tissue damage. This in turn may be a factor modulating the education of the T cell repertoire in terms of self-nonself discrimination as well as its failure in autoimmune situations.

Acknowledgments

We thank Dr. C. Ganellin for reagents, Dr. M. Bevan for gifts of cell lines, and Dr. C. A. Janeway for cell lines and critical discussions. S.M. thanks Morven Roberts for help in establishing some techniques and F. F. Bosphorus and K. Belur for inspiration.

References

1. York, J., and K. L. Rock. 1996. Antigen processing and presentation by the class I major histocompatibility complex. *Annu. Rev. Immunol. 14:369.*
2. Townsend, A., and J. Trowsdale. 1993. The transporters associated with antigen presentation. *Semin. Cell Biol. 4:53.*
3. Cresswell, P. 1996. Invariant chain structure and MHC class II function. *Cell 84:505.*
4. Neefjes, J. 1999. CIIV, MIIC and other compartments for MHC class II loading. *Eur. J. Immunol. 29:1421.*
5. Jensen, P. E., D. A. Weber, W. P. Thayer, L. E. Westerman, and C. T. Das. 1999. Peptide exchange in MHC molecules. *Immunol. Rev. 172:229.*
6. Kropshofer, H., G. J. Hammerling, and A. B. Vogt. 1999. The impact of the non-classical MHC proteins HLA-DM and HLA-DO on the MHC class II molecules. *Immunol. Rev. 172:267.*

7. Rock, K. L. 1996. A new foreign policy: MHC class I monitor the outside world. *Immunol. Today 17:131.*
8. Harding, C. V. 1996. Class I MHC presentation of exogenous antigens. *J. Clin. Immunol. 16:90.*
9. Lechler, R., G. Aichinger, and L. Lightstone. 1996. The endogenous pathway of MHC class II antigen presentation. *Immunol. Rev. 151:51.*
10. Gueguen, M., and E. O. Long. 1996. Presentation of a cytosolic antigen by major histocompatibility complex class II molecules requires a long-lived form of an antigen. *Proc. Natl. Acad. Sci. USA 93:14692.*
11. Bonifaz, L. C., S. Arzata, S., and J. Moreno. 1999. Endogenous and exogenous forms of the same antigen are processed from different pools to bind MHC class II molecules in endocytic compartments. *Eur. J. Immunol. 29:119.*
12. Pamer, E., and P. Cresswell. 1998. Mechanisms of MHC class I-restricted antigen processing. *Annu. Rev. Immunol. 16:323.*
13. Rock, K. L., and A. L. Goldberg. 1999. Degradation of cell proteins and the generation of MHC class I-presented peptides. *Annu. Rev. Immunol. 17:739.*
14. Rudensky, A. Y., P. Preston-Hurlburt, S.-C. Hong, A. Barlow, and C. A. Janeway, Jr. 1991. Sequence analysis of peptides bound to MHC class II molecules. *Nature 353:622.*
15. Rudensky, A. Y., S. Rath, P. Preston-Hurlburt, D. B. Murphy, and C. A. Janeway, Jr. 1991. On the complexity of self. *Nature 353:660.*
16. Chicz, R. M., R. G. Urban, J. C. Gorga, D. A. Vignali, W. S. Lane, and J. L. Strominger. 1993. Specificity and promiscuity among naturally processed peptides bound to HLA-DR alleles. *J. Exp. Med. 178:27.*
17. Barlow, A. K., X. He, and C. A. Janeway, Jr. 1998. Exogenously provided peptides of a self-antigen can be processed into forms that are recognized by self-T cells. *J. Exp. Med. 187:1403.*
18. Harding, C. V., and H. J. Geuze. 1993. Immunogenic peptides bind to class II MHC molecules in an early lysosomal compartment. *J. Immunol. 151:3988.*
19. Rudensky, A. Y., M. Maric, S. Eastman, L. Shoemaker, P., DeRoos, and J. S. Blum. 1994. Intracellular assembly and transport of endogenous peptide-MHC class II complexes. *Immunity 1:585.*
20. Amigorena, S., J. R. Drake, P. Webster, and I. Mellman. 1994. Transient accumulation of new class II MHC molecules in a novel endocytic compartment in B lymphocytes. *Nature 369:113.*
21. Ferrari, G., A. M. Knight., C. Watts, and J. Pieters. 1997. Distinct intracellular compartments involved in invariant chain degradation and antigenic peptide loading of major histocompatibility complex MHC class II molecules. *J. Cell Biol. 139:1433.*
22. Haberland, M. E., and A. M. Fogelman. 1985. Scavenger receptor-mediated recognition of maleyl bovine plasma albumin and the demaleylated protein in human monocyte macrophages. *Proc. Natl. Acad. Sci. USA 82:2693.*
23. Moore, M. W., F. R. Carbone, and M. J. Bevan. 1988. Introduction of soluble proteins into the class I pathway of antigen processing and presentation. *Cell 54:777.*
24. Singh, N., S. Bhatia, R. Abraham, S. K. Basu, A. George, V. Bal, and S. Rath. 1998. Modulation of T cell cytokine profile and peptide-MHC complex availability in vivo by delivery to scavenger receptors via antigen maleylation. *J. Immunol. 160:4869.*
25. Michalek, M. T., E. P. Grant, and K. L. Rock. 1996. Chemical denaturation and modification of ovalbumin alters its dependence on ubiquitin conjugation for class I antigen presentation. *J. Immunol. 157:617.*
26. Bansal, P., P. Mukherjee, S. K. Basu, A. George, V. Bal, and S. Rath. 1999. MHC class I-restricted presentation of maleylated proteins binding to scavenger receptors. *J. Immunol. 162:4430.*
27. Fenteany, G., R. F. Standant, W. S. Lane, S. Choi, E. J. Corey, and S. L. Schreiber. 1995. Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification of lactacystin. *Science 268:726.*
28. Glas, R., M. Bogya, J. S. McMaster, M. Gaczynska, and H. L. Pleogh. 1998. A proteolytic system that compensates for loss of proteasome function. *Nature 392: 618.*
29. Paz, P., N. Broouwenstijn, and N. Shastri. 1999. Discrete proteolytic intermediates in the MHC class I antigen processing pathway and MHC class I-dependent peptide trimming in the ER. *Immunity 28:241.*
30. Geier, E., G. Pfeifer, M. Wilm, M. Lucchiari-Hartz, W. Baumeister, K. Eichmann, and G. Neidermann. 1999. A giant protease with potential to substitute for some functions of the proteasome. *Science 283:978.*
31. Rose, C., F. Vargas., P. Facchinetti., P. Bourgeat, R. B. Bambal, P. B. Bishop, S. M. T. Chan, A. N. J. Moore, C. R. Ganellin, and J.-C. Schwartz. 1996. Characterization and inhibition of a cholecystokinin-inactivating serine peptidase. *Nature 380:403.*
32. Ganellin, C. R., P. B. Bishop, R. B. Bambal, S. M. Chan, J. K. Law, B. Marabout, P. M. Luthra, A. N. Moore, O. Peschard, P. Bourgeat, et al. 2000. Inhibition of tripeptidyl peptidase II. 2: generation of the first novel lead inhibitor of cholecystokinin-8 inactivating peptidase: a strategy for the design of peptidase inhibitors. *J. Med. Chem. 43:664.*
33. Stoolze, L., M. Scirle, G. Schwartz, C. Schroter, M. W. Thompson, L. B. Hersh, H. Kalbacher, S. Stevanovic, H.-G. Rammensee, and H. Schild. 2000. Two new proteases in the MHC class I processing pathway. *Nat. Immunol. 1:413.*
34. Ben-Nun, A., E. Choi, K. R. McIntyre, S. A. Leeman, D. J. McKean, J. G. Seidman, and L. H. Glimcher. 1985. DNA-mediated transfer of major histocompatibility class II I-A^b and I-A^{bm12} genes into B lymphoma cells: molecular and functional analysis of introduced antigens. *J. Immunol. 135:1456.*
35. Jaraquemada, D., M. Marti, and E. O. Long. 1990. An endogenous processing pathway in vaccinia virus-infected cells for the presentation of cytoplasmic antigens to class II-restricted T cells. *J. Exp. Med. 172:947.*
36. Oxenius, A., M. F. Bachmann, P. G. Ashton-Rickardt, S. Tonegawa, R. M. Zinkernagel, and H. Hengartner. 1995. Presentation of endogenous viral proteins in association with major histocompatibility complex class II: on the role of intracellular compartmentalization, invariant chain and the TAP transporter system. *Eur. J. Immunol. 25:3402.*
37. Loss, G. E., C. G. Elias, P. E. Fields, R. K. Ribando, M. McKisic, and A. J. Sant. 1993. Major histocompatibility complex class II-restricted presentation of an internally synthesized antigen displays cell type variability and segregates from the exogenous class II and endogenous class I presentation pathways. *J. Exp. Med. 178:73.*
38. Michalek, M. T., B. Benaceraff, and K. L. Rock. 1992. The class II-presentation of endogenously synthesized ovalbumin displays clonal variation, requires endosomal/lysosomal processing, and is up-regulated by heat shock. *J. Immunol. 148:1016.*
39. Kovats, S., P. E. Whiteley, P. Concannon, A. Y. Rudensky, and J. S. Blum. 1997. Presentation of abundant endogenous class II DR-restricted antigens by DM-negative B cell lines. *Eur. J. Immunol. 27:1014.*
40. Robadey, C., H. J. Wallny, and S. Demotz. 1996. Cell type-specific processing of the I-E^d-restricted hen egg lysozyme determinant. *Eur. J. Immunol. 26:1656.*
41. Lick, J. D., J. F. Elliot, and J. S. Blum. 2000. Cytoplasmic processing is prerequisite for presentation of an endogenous antigen by major histocompatibility complex class II proteins. *J. Exp. Med. 191:1513.*
42. Hiller, M. M., A. Finger, M. Schweiger, and D. H. Wolf. 1996. ER degradation of a misfolded luminal protein by the cytosolic ubiquitin-proteasome pathway. *Science 273:1725.*
43. Hughes, E. A., C. Hammond, and P. Cresswell. 1997. Misfolded major histocompatibility complex class I heavy chains are translocated into the cytoplasm and degraded by the proteasome. *Proc. Natl. Acad. Sci. USA 94:1896.*
44. Huppa, J. B., and H. L. Ploegh. 1997. The α chain of the T cell antigen receptor is degraded in the cytosol. *Immunity 7:113.*
45. Dusseljee, S., R. Wubbolts., D. Verwoerd, A. Tulp, H. Janssen, J. Calafat, and J. Neefjes. 1998. Removal and degradation of the free MHC class II β chain in the endoplasmic reticulum requires proteasomes and is accelerated by BFA. *J. Cell Sci. 111:2271.*
46. O'Hara, T., G. D. Wiens, E. A. Whitcomb, C. A. Enns, and M. B. Rittenberg. 1999. Proteasome involvement in the degradation of unassembled Ig light chains. *J. Immunol. 163:11.*
47. Hegde, R. S., and V. R. Lingappa. 1999. Regulation of protein biogenesis at the endoplasmic reticulum membrane. *Trends Cell Biol. 9:132.*
48. Reits, E. A., J. C. Vos, M. Gromme, and J. Neefjes. 2000. The major substrates for TAP in vivo are derived from newly synthesized proteins by proteasomes. *Nature 404:774.*
49. Schubert, U., L. C. Anton, J. Gibbs, C. C. Norbury, J. W. Yewdell, and J. R. Bennick. 2000. The rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature 200:770.*
50. Princiotta, M. F., U. Scbubert, W. Chen, J. R. Bennick, J. Myung, C. M. Crews, and W. Yewdell. 2001. Cells adapted to the proteasome inhibitor 4-hydroxy-5-iodo-3-nitrophenylacety-leu-leu-leucinal-vinyl sulfone requires enzymatically active proteasomes for continued survival. *Proc. Natl. Acad. Sci. USA (e-pub ahead of print).*
51. Beninga, J., K. L. Rock, and A. L. Goldberg. 1998. Interferon-γ can stimulate post-proteasomal trimming of the N-terminus of an antigenic peptide by inducing leucine aminopeptidase. *J. Biol. Chem. 273:18734.*
52. Rivett, A. J. 1998. Intracellular distribution of proteasomes. *Curr. Opin. Immunol. 10:110.*