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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

Major histocompatibility complex (MHC) class II-associated antigen presentation is mainly linked to processing of exogenous antigens upon cellular uptake by endocytosis, but has also been observed for endogenously sythesized antigens. We have studied the MHC class II-associated presentation of the endogenously synthesized membrane associated glycoprotein (GP) and the cytosolic nucleoprotein (NP) of lymphocytic choriomeningitis virus (LCMV) in professional antigen presenting cells (APC) of mice. Since LCMV is a noncytopathic virus and minimally affects cellular protein synthesis, it is a convenient virus for the study of antigen presentation. In contrast, most other studies assessing class II-associated presentation of endogeneously synthesized viral antigens used cytolytic viruses such as vaccinia, measles and influenza virus, which drastically interfere with host cell functions. In addition, most studies were performed using non-professional APC. We found that class II-associated presentation of endogenously synthesized membrane associated LCMV-GP was efficient and could not be inhibited by chloroquine or leupeptin. Neither the transporter associated with processing (TAP) system nor the invariant chain (Ii) were significantly involved in this process. In contrast, MHC class II-associated presentation of endogenously synthesized cytosolic LCMV-NP was not observed even in Ii-deficient APC. Thus, MHC class II loading of endogenously synthesized LCMV-GP apparently does not require processing in acidic endosomal compartments as defined by chloroquine and leupeptin insensitivity. Furthermore, although the TAP molecules transport peptides of up to 15 amino acids in length, which potentially could bind to MHC class II molecules in the endoplasmic reticulum, such a process apparently does not occur for either the glycoprotein or the nucleoprotein. Therefore, the subcellular localization of an endogenously synthesized protein influences crucially whether or not MHC class II loading can occur independently of the acidic compartments usually involved in MHC class II loading.

1 Introduction

The T cell receptor of $CD8^+$ and $CD4^+$ T cells recognizes short peptides derived from nominal antigens in association with MHC class I or II molecules, respectively [1–4]. Despite the structural and functional similarity between the MHC class I and class II molecules [5, 6], they exhibit a different cellular distribution and follow different intracellular trafficking to the cell surface. Classically, MHC class I molecules present endogenous antigens (*i.e.* endogenously synthesized proteins), whereas MHC class II molecules present antigen fragments generated after uptake of exogenous antigens by endocytosis [7–11].

MHC class I molecules consist of a heavy chain that associates with β2-microglobulin in the endoplasmic reticulum (ER) where this heterodimer is loaded with peptides, mainly originating from endogenously synthesized proteins [12–14]. These bound peptides are generated in the cytoplasm most probably by the proteolytic action of the proteasome complex [15]. They are transported into the ER via the TAP transporter molecule [3]. Whether, and to what extent, the peptides are additionally trimmed within the ER to fit into the peptide-binding groove of MHC class I is unclear [16–18].

The heterodimeric MHC class II molecules consist of an α and β chain which associate in the ER with the invariant chain (Ii) [19–25]. The invariant chain is thought to prevent the loading of newly synthesized class II molecules with peptides in the ER to some extent, to contain an endosomal targeting signal, and thus to be involved in separating MHC class II intracellular traffic from distinct class I pathways [26–29]. MHC class II molecules are loaded primarily, but not exclusively [30], with peptides

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Abbreviations: NP: Nucleoprotein **GP:** Glycoprotein **B-GP:** LCMV-GP expressed by recombinant baculovirus **B-NP:** LCMV-NP expressed by recombinant baculovirus **ER:** Endoplasmic reticulum **HEL:** Hen egg lysozyme **Ii:** Invariant chain **MOI:** Multiplicity of infection **TAP:** Transporter associated with processing

Key words: Class II / Viral antigens / Endogenous / Invariant chain / TAP

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within the recently described endosome-related acidic MIIC compartment which was characterized in B cells and in MHC class II$^+$ melanoma cells [31, 32]. These peptides are generated by acidic proteolysis of endocytosed proteins in endosomal compartments.

Although this MHC class discrimination by the immune system has been found to be very efficient, it has proven less stringent than initially assumed. There have been many reports describing MHC class I loading by exogenous antigens *in vivo* such as for ovalbumin, hepatitis B surface antigen, HIV-1 envelope protein, SV40 large T antigen, vesicular stomatitis virus nucleoprotein and LCMV glycoprotein and nucleoprotein [33–37]. On the other hand, many endogenously synthesized proteins, such as immunoglobulin light chains, measles virus matrix protein, influenza A matrix protein vaccinia recombinant proteins, hen egg lysozyme (HEL), herpes surface antigen and myelin basic protein were found to be presented by MHC class II molecules [33, 38–46]. While some of these endogenous antigens were shown to be presented by an endosomal compartment-associated pathway defined by chloroquine sensitivity [33, 43, 44], some other endogenous antigens seemed to be processed by other or additional pathways for MHC class II loading [40, 42].

We investigated MHC class II-associated presentation of two endogenously synthesized viral antigens with different subcellular localizations in professional APC. These cells are suitable to study class II-associated antigen presentation, since they possess the MIIC compartment which is involved in MHC class II loading [31, 32]. LCMV is neither cytolytic nor does it inhibit host cell protein synthesis significantly, and LCMV replication only minimally affects the host cell [47, 48]. Therefore, LCMV-infected professional APC represent an ideal experimental system to analyze class II-associated presentation of endogenously synthesized viral antigens since subcellular compartments remain fully intact during infection. This confers an advantage of the LCMV model infection as compared to the cytopathic influenza, vaccinia and measles viruses used so far to study class II loading pathways of endogenously synthesized antigens.

I-A^b-associated presentation of LCMV-glycoprotein (GP) and nucleoprotein (NP)-derived peptides was assessed by measuring induced IL-2 secretion of a GP- and a NP-specific CD4$^+$ T cell hybridoma, respectively. These peptides are involved in a primary anti-LCMV immune response as shown by both induction of peptide specific CD4$^+$ T cells *in vivo* upon LCMV infection and by activation of the T cell hybridomas by APC infected *in vivo*. We found that only endogenously synthesized LCMV-GP could load MHC class II molecules independently of proteolysis in acidic compartments as defined by chloroquine and leupeptin insensitivity, whereas endogenously synthesized LCMV-NP was not presented independently of proteolysis in endocytic compartments even in the absence of Ii. These data indicate that processing and MHC class II loading of the endogenously synthesized membrane-associated glycoprotein is likely to take place in the ER or pre-Golgi compartment. They suggest further that Ii may not compete with peptide binding to newly synthesized class II molecules in the ER, but may possibly interfere with the binding of whole proteins.

2 Materials and methods

2.1 Mice

C57BL/6 mice were obtained from the breeding colony of the Institut für Zuchthygiene, Zürich. The generation of the mice deficient for the invariant chain and deficient for the TAP transporter system, respectively, has been described [49, 50]. Mice were bred in a conventional mouse facility.

2.2 Viruses

The LCMV isolate WE was originally provided by Dr. F. Lehmann-Grube, Hamburg, Germany and grown on L929 cells with a low multiplicity of infection (MOI). The recombinant baculoviruses expressing the LCMV-GP or NP have been described [51]. All recombinant baculoviruses were derived from nuclear polyhedrosis virus and were grown at 28 °C in *Spodoptera frugiperda* cells in TC-100 medium (Gibco, Basel, Switzerland). Proteins were produced as described [37]. Recombinant vaccinia viruses expressing LCMV-GP, LCMV-NP and GP- and NP-proteins truncated at their C termini, respectively, have been described [52, 53].

2.3 Generation of LCMV-specific Th cell hybridomas

2.3.1 LCMV-NP-specific T cell hybridoma VE8

CD8-deficient mice [54] were immunized with 20 µg purified, UV-inactivated LCMV-WE in incomplete Freund's adjuvant subcutaneously. After 14 days, lymph node cells were restimulated *in vitro* with 5 µg/ml purified UV-inactivated LCMV-WE and irradiated C57BL/6 spleen cells. After 4 days, activated T cells were fused with the BW$^{\alpha-\beta-}$ cell line [55]. The specificity of hybridoma VE8 was demonstrated by IL-2 secretion by the hybridoma, measured upon stimulation with irradiated spleen cells and different exogenous antigens such as UV-inactivated LCMV-WE, recombinant baculovirus-derived LCMV-GP or -NP. IL-2 in the supernatant was determined after 24 h by the IL-2-sensitive cell line CTLL-2 followed by AlamarBlue™ (Biosource, International) color reaction as described in Sect. 2.6. VE8 is LCMV-NP-specific and I-A^b-restricted. Peptide mapping of the VE8 hybridoma was performed in two steps. In a first step, the protein region containing the VE8 epitope was defined using as exogenous antigens five different LCMV-NP of different lengths truncated at their C-termini expressed by recombinant vaccinia viruses [53]. This experiment allowed us to define the region containing the epitope to about 100 amino acids. Finally, peptide mapping was performed with a series of overlapping 20-mer peptides. Peptide P61 recognized by VE8 consists of amino acids 309–328 of the NP of LCMV-WE.

2.3.2 LCMV-GP-specific T cell hybridoma 5A1

C57BL/6 mice were infected intravenously with 5×10^6 pfu of a vaccinia LCMV-GP recombinant virus. After 12 days, spleen cells were restimulated *in vitro* with 5 µg/ml UV-inactivated, purified LCMV-WE for 4 days, fol-

lowed by fusion with the $BW^{\alpha-\beta-}$ cell line [55]. 5A1 specificity was determined as described for the VE8 hybridoma. 5A1 is I-A^b-restricted and specific for the LCMV glycoprotein. Initial epitope mapping was again performed using a series of LCMV-GP of different lengths truncated at their C-termini, expressed by recombinant vaccinia virus [52], as exogenous antigens. Final peptide mapping with synthetic overlapping peptides identified peptide P13, consisting of amino acids 61–80 of LCMV-GP.

2.4 T cell proliferation

C57BL/6 mice were infected with 200 pfu LCMV-WE intravenously. After 13 days, $CD4^+$ T cells were purified from a spleen cell suspension by magnetic cell sorting. $CD4^+$ T cells (1×10^5) were incubated in 96 well plates with threefold serial dilutions of either purified, UV-inactivated LCMV (highest concentration: 5 μg/ml), P13, or P61 (highest concentration: 5 μg/ml) or medium only, in the presence of 7×10^5 iradiated (2000 cGy) C57BL/6 spleen cells for 3 days. Proliferation was assessed by incorporation of [^{3}H] thymidine (25 μCi/well).

2.5 Peptide presentation by APC infected *in vivo*

C57BL/6 mice were immunized with 2×10^6 pfu LCMV, and 6 days later, spleen cells from infected, from naive and from LCMV carrier mice were irradiated (2500 cGy) and incubated in threefold serial dilutions (highest concentration: 3×10^6 cells/well) together with 4×10^4 5A1 or VE8 T cell hybridomas, respectively, for 20 h. IL-2 concentration in the supernatant was determined as described in Sect. 2.6.

2.6 MHC class II presentation of viral antigens in the presence or absence of chloroquine or leupeptin

In the absence of drugs, 2×10^5 lymphocytes from either C57BL/6, Ii-deficient or TAP-deficient mice were incubated with threefold serial dilutions of live LCMV (MOI = 2 at the highest concentration), UV-inactivated LCMV (same amount as live LCMV), recombinant baculovirus-derived LCMV-GP or LCMV-NP (3 μg/ml at the highest concentration), P13 (1 μg/ml at the highest concentration) or P61 (1 μg/ml at the highest concentration) in 96-well plates in a total volume of 50 μl. After 4 h, 4×10^4 hybridoma cells per well were added in 150 μl for 13 h. Then, 50 μl supernatant were transferred into a new 96-well plate and 1×10^4 CTLL cells were added per well for 15 h. Quantification of viable CTLL cells was perfomed by AlamarBlue™ color reaction (Biosource, International) and measured by fluorescence emission at 590 nm using the CytoFluor™ 2350 (Millipore) fluorimeter.

If the antigen presentation assay was performed in the presence of chloroquine or leupeptin, the APC were incubated with the same serial dilutions of antigens in the same volume as above, but containing 150 μM chloroquine or 0.5 mM leupeptin for 4 h. Hybridoma cells (4×10^4) were added in 150 μl for 13 h, lowering the chloroquine concentration to 38 μM. In the leupeptin experiments, 4×10^4 hybridoma cells were added in 150 μl containing 0.5 mM leupeptin. IL-2 concentration in the supernatant was determined as described above.

2.7 MHC class II presentation of regurgitated antigens

Aliquots of 200 μl of the same supernatants tested for IL-2 content as described above or 5 μg/ml peptide, were incubated for 3 h with 7×10^6 C57BL/6 spleen cells at 37 °C. Subsequently, the cells were washed three times and incubated overnight with 4×10^4 hybridoma cells. IL-2 concentration in the supernatant was determined as described in Sect. 2.6.

3 Results

3.1 Generation and characterization of two I-A^b restricted T cell hybridomas

Immunization of naive mice with UV-inactivated, purified LCMV or recombinant vaccinia virus expressing LCMV-GP and subsequent restimulation *in vitro* with purified, UV-inactivated LCMV resulted in two LCMV-specific T cell hybridomas, 5A1 and VE8, which recognized LCMV-GP and LCMV-NP, respectively (Fig. 1). Epitope mapping identified GP-derived peptide P13 (amino acids 61–80 of LCMV-GP) to be recognized by SA1 and NP-derived peptide P61 (amino acids 309–328 of LCMV-NP) to be recognized by VE8 (Fig. 1A).

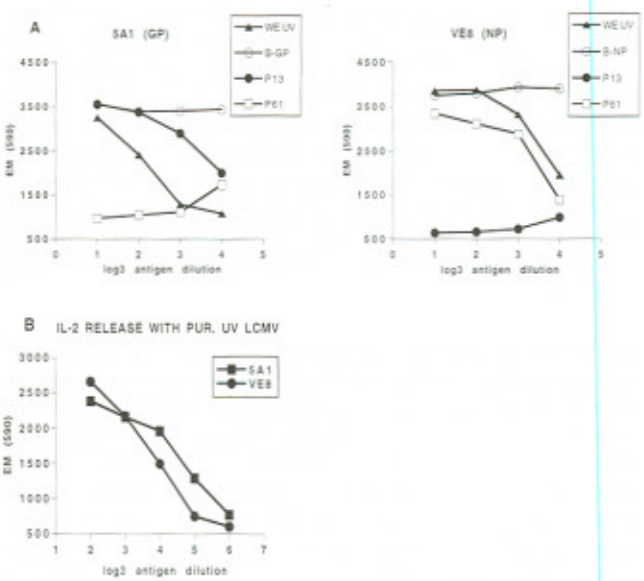

Figure 1. (A) Antigen specificity of T cell hybridomas 5A1 and VE8. C57BL/6 spleen cells were pulsed with different exogenously supplied antigens [UV-inactivated purified LCMV, baculovirus recombinant LCMV-GP or -NP and LCMV peptides P13 (GP derived) and P61 (NP derived)]. Activation of the hybridomas was measured by secretion of IL-2 using the IL-2-dependent cell line CTLL. The AlamarBlue™ color reaction was used for quantification of viable CTLL cells. Exact concentrations of the stimulating antigens are described in Sect. 2.3. (B) The sensitivities of both hybridomas for exogenously supplied LCMV antigen are similar and were compared using the same threefold serial dilutions of UV-inactivated purified LCMV and C57BL/6 spleen cells as APC.

within the recently described endosome-related acidic MIIC compartment which was characterized in B cells and in MHC class II$^+$ melanoma cells [31, 32]. These peptides are generated by acidic proteolysis of endocytosed proteins in endosomal compartments.

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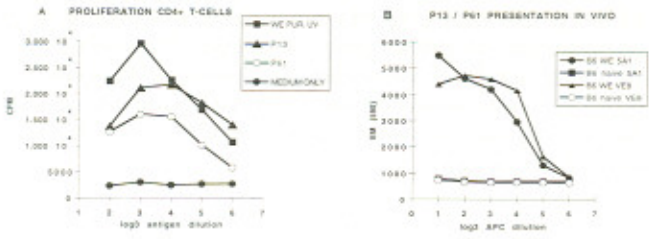

Figure 2. (A) T cell proliferation *ex vivo*. Purified CD4$^+$ T cells from an LCMV-infected mouse proliferated in the presence of either purified UV-inactivated LCMV or the peptides P13 or P61, respectively. (B) Presentation of P13 or P61 *in vivo*. Irradiated spleen cells from a LCMV-infected mouse were used as APC to activate the T cell hybridomas 5A1 or VE8.

The range of sensitivity for exogenously supplied LCMV-antigen of both hybridomas were comparable, shown by the equal IL-2 production upon stimulation with graded amounts of purified, UV-inactivated LCMV (Fig. 1B).

3.2 Presentation of P13 and P61 *in vivo*

We analyzed whether P13 or P61 represented epitopes recognized during a normal LCMV-specific immune response. T cell proliferation of purified CD4$^+$ Th cells 13 days post-infection of C57BL/6 mice with 200 pfu LCMV clearly showed that both P13- and P61-specific Th cells are induced *in vivo* (Fig. 2A), demonstrating that during the natural time-course of an LCMV infection, both P13 and P61 are presented and recognized.

Additionally, we measured peptide presentation *in vivo* using in irradiated spleen cells from LCMV-infected C57BL/6 mice 6 days post-infection as APC. Activation of both hybridomas was observed using these APC generated *in vivo* (Fig. 2). Thus, both hybridomas 5A1 and VE8 recognize peptides that are naturally generated *in vivo* during LCMV infection.

3.3 Activation of the GP- and NP-specific hybridomas using replicating versus inactivated LCMV

We examined the capacity of either LCMV-infected C57BL/6 spleen cells or spleen cells loaded with exogenously supplied UV-inactivated LCMV to activate the GP-

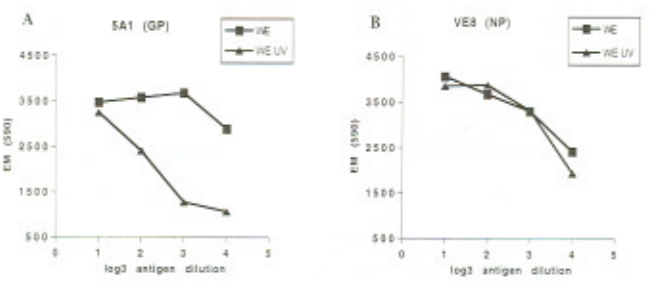

Figure 3. Hybridoma responsiveness to live versus UV-inactivated LCMV. In the presence of C57BL/6 APC, live and UV-inactivated LCMV virus were compared in their activation capacity for the GP-specific 5A1 (A) or the NP-specific VE8 (B) T cell hybridomas.

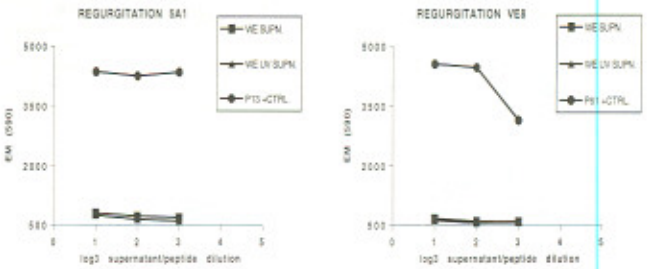

Figure 4. Regurgitation and class II presentation of viral antigens. The same supernatants analyzed in Fig. 3 for IL-2 content or newly supplied peptides were used to pulse fresh C57BL/6 spleen cells. After extensive washing, these spleen cells were used as APC to stimulate the T cell hybridomas. IL-2 production was determined with the IL-2 dependent cell line CTLL.

specific hybridoma 5A1 or the NP-specific hybridoma VE8 (Fig. 3). While the NP-specific hybridoma VE8 exhibited the same activation capacity with both live and UV-inactivated LCMV, live virus showed an at least tenfold enhanced presentation capacity as compared to UV-inactivated virus in the case of the GP-specific hybridoma 5A1 (Fig. 3). Thus, LCMV-NP seemed to be only presented via the classical endocytic pathway, whereas LCMV-GP was apparently presented by an additional class II presentation pathway. A possible explanation for the enhanced MHC class II loading by LCMV-GP derived from live virus as compared to UV-inactivated virus could be that the membrane-associated GP was shed and degraded outside the cell surface. To rule out external class II loading by GP fragments, fresh APC were incubated with overnight culture supernatant from LCMV-infected APC, from APC pulsed with UV-inactivated LCMV, or with freshly supplied peptide. Thereafter, the cells were washed extensively and used as APC to activate the T cell hybridomas (Fig. 4). Only spleen cells incubated with newly added peptide were able to activate the T cell hybridomas. Neither culture supernatant from LCMV-infected APC nor supernatant from APC pulsed with UV-inactivated LCMV could provide sufficient amounts of protein fragments for class II loading, as evaluated by specific IL-2 release by sensitive T cell hybridomas. Therefore, the enhanced class II presentation of LCMV-GP derived from live virus could not be explained by regurgitation of presentable GP-derived protein fragments by the APC.

The possibility that the enhanced presentation of LCMV-GP was due to progeny virus that entered the endocytic pathway could be excluded because the same effect should have been observable in that case for LCMV-NP presentation. In addition, virus titers were determined in the APC supernatants and within the infected APC at the end of the assay, and were found to be less than about 1/60th of the initial virus dose (data not shown).

3.4 Chloroquine resistence of MHC class II presentation of endogenously synthesized LCMV-GP

Both a chloroquine-insensitive pathway for class II presentation of endogenously synthesized antigens (measles virus, influenza matrix and λ-chain presentation [38, 40, 42]) and a chloroquine-sensitive pathway (vaccinia-HA

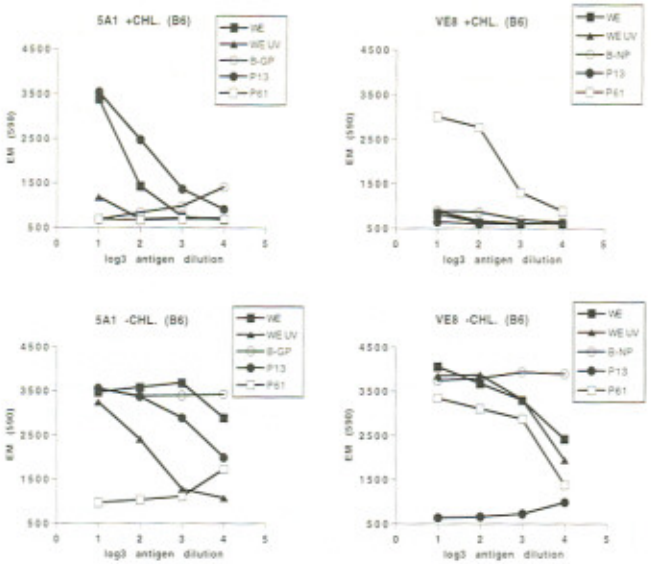

Figure 5. Chloroquine sensitivity of antigen presentation. The T cell hybridomas 5A1 and VE8 were stimulated in presence (upper panel) or absence (lower panel) of chloroquine with the same antigens examined in Fig. 1 (live LCMV, UV-inactivated LCMV, baculovirus recombinant LCMV-GP and -NP, P61 and P13) in the presence of C57BL/6 spleen cells as APC. IL-2 production was determined using the IL-2-dependent cell line CTLL.

recombinant viruses and vaccinia-hepatitis B surface antigen recombinant virus [33, 43, 44, 45]) have been described. We therefore analyzed the chloroquine sensitivity of class II presentation of endogenously synthesized LCMV-GP (Fig. 5). In LCMV-infected C57BL/6 spleen cells, endogenously synthesized LCMV-GP was effciently presented in the presence of chloroquine, whereas exogenously supplied antigens were not presented (as shown here for the recombinant LCMV-NP, GP and UV-inactivated

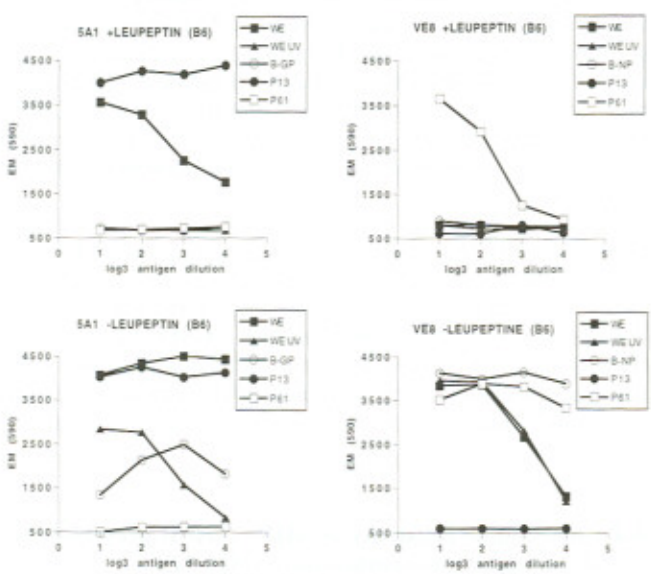

Figure 6. Leupeptin sensitivity of antigen presentation. Using C57BL/6 spleen cells as APC, the T cell hybridomas 5A1 and VE8 were stimulated in the presence (upper panel) or absence (lower panel) of leupeptin with the same antigens described in Fig. 5. IL-2 production was determined with the IL-2-dependent cell line CTLL.

LCMV). In contrast, LCMV-NP was not presented at al the presence of chloroquine, suggesting that NP is ot presented via the classical endocytic pathway. Presentatic.. of exogenously added soluble LCMV-GP and LCMV-NP-derived peptides was only marginally affected by the presence of chloroquine.

The possibility that presentation of endogenously synthesized LCMV-GP is due to a much higher intracellular GP concentration or a higher sensitivity of the GP-specific T cell hybridoma could be ruled out, since the same virus-infected APC were used for the presentation of endogenous LCMV-GP and NP, LCMV-NP is the first synthesized viral protein after infection [56], and LCMV-NP is present at higher intracellular concentrations (75 % of the viral protein mass) compared to the GP (25 % of the viral protein mass) [57]. Finally, both T cell hybridomas exhibited a comparable sensitivity towards exogenously supplied LCMV antigen (Fig. 1). These findings suggest that subcellular compartmentalization plays a critical role for the presentation of endogenously synthesized antigens.

3.5 Leupeptin resistance of MHC class II presentation of endogenously synthesized LCMV-GP

Leupeptin has been shown to inhibit intracellular degradation of polypeptides in acidic compartments and thereby to deplete – at least to some extent – the endocytic pathway of peptides available for class II binding [58]. Additionally, proteolytic removal of Ii is inhibited by leupeptin, as well as surface deposition of newly synthesized class II molecules [59]. By analogy to the chloroquine experiments, only endogenously synthesized LCMV-GP was able to load MHC class II molecules in the presence of leupeptin,

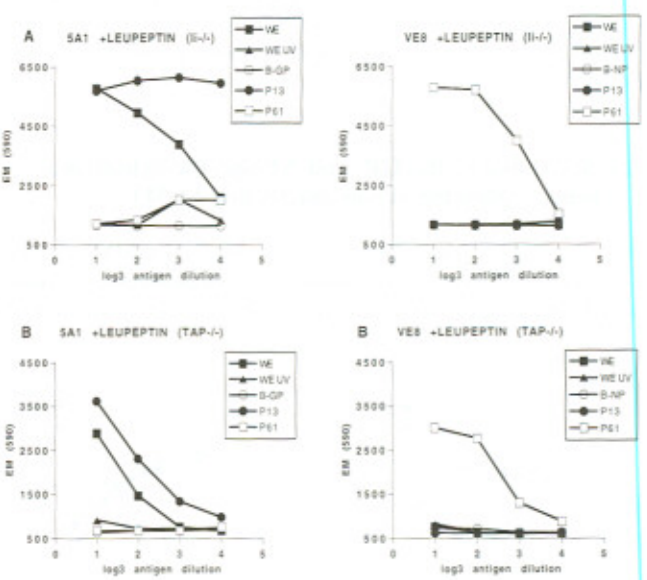

Figure 7. Antigen presentation in Ii-deficient and TAP-deficient APC. (A) Ii-deficient spleen cells as APC: The same antigens described in Fig. 5 were used to stimulate the T cell hybridomas 5A1 and VE8 using spleen cells from Ii-deficient mice as APC in the presence of leupeptin. (B) TAP-deficient spleen cells as APC: The same antigens described in Fig. 5 were used in the presence of leupeptin and spleen cells from TAP-deficient mice as APC to activate the T cell hybridomas 5A1 and VE8.

Table 1. MHC class II-associated presentation of endogenously synthesized self or neoself proteins, their intracellular localization and the nature of the antigen presenting cell

Self proteins	Presentation	Localization	Chloroquine[a)]	BFA[b)]	Ii[c)d)]	APC	Reference
HLA-B/C	+	membrane	insensitive	sensitive		B cell	Chen et al. [66]
Ii	+	ER/endosomes				B lymphocyte	Rudensky et al. [64, 65]
MHC I-E^b	+	membrane				B lymphocyte	
MuLV gp70	+	membrane				B lymphocyte	
tm proteins	+	membrane			inhibitory	B lymphocyte	Newcomb and Cresswell [67]
HSP	+	cytosol			inhibitory	B lymphocyte	
secreted protein	+	ER/secreted			inhibitory	B lymphocyte	
V-CH3 secreted	+	ER/secreted			independent	myeloma	Bikoff [71]
V-CH3 cytosol	−	cytosol			inhibitory	myeloma	
HEL soluble	+	ER/secreted				B lymphocyte	Brooks et al. [46]
HEL ER	+	ER				B lymphocyte	
HEL membrane	+	membrane				B lymphocyte	
HEL aa 1–80	+/−	membrane				fibroblast	Moreno et al. [69]
HEL aa 1–80	+	membrane				B lymphocyte	
$\lambda 2^{215}$ membrane	+	membrane				B lymphocyte	Weiss and Bogen [38]
$\lambda 2^{215}$ ER	+	ER				B lymphocyte	
$\lambda 2^{215}$ cytosol	−	cytosol				B lymphocyte	
$\lambda 2^{215}$ nuclear	−	nucleus				B lymphocyte	
HEL membrane	+/−	membrane			depend./ indiff./inhibitory[d)]	spleen cells	Bodmer et al. [61]
HEL secreted	+/−	ER/secreted			depend./ indiff./inhibitory[d)]	spleen cells	
Measles matrix	+	cytosol	insensitive			fibroblast	Jacobson et al. [41]
Measles NP	+	cytosol	insensitive			fibroblast	

a) Chloroquine sensitivity of class II presentation indicated only when examined.
b) Brefeldin A sensitivity of class II presentation indicated only when examined.
c) Effect of the absence of Ii on class II presentation indicated only when examined.
d) Dependence upon, indifference of, or inhibition by Ii depends on the epitope analyzed.

but neither exogenous LCMV-GP, LCMV-NP antigen, nor endogenously synthesized NP were able to load class II molecules in the presence of leupeptin (Fig. 6).

3.6 Invariant chain is neither needed nor inhibitory for presentation of endogenously synthesized LCMV-GP

The invariant chain has been shown to be involved in the presentation of exogenously supplied antigens, and is thought to prevent MHC class II loading with peptides in the ER [28, 60, 61]. Therefore, we considered the possibility that LCMV nucleoprotein degraded in the cytosol and transported into the ER by the TAP transporter might be presented in the absence of Ii, or that the LCMV-GP might be more efficiently presented on MHC class II in the absence of Ii. Antigen presentation experiments were performed using spleen cells from Ii-deficient mice as APC (Fig. 7A). To analyze only class II presentation of endogenous proteins, the experiments were performed in the presence of leupeptin. MHC class II presentation of endogenously synthesized GP by both C57BL/6 spleen cells and Ii-deficient spleen cells was comparable, if not marginally enhanced in the Ii-deficient APC. Similar results were obtained with these cells using non-saturating peptide concentrations (not shown). In contrast, no MHC class II presentation of endogenously synthesized cytoplasmic nucleoprotein could be detected in Ii-deficient APC in the presence of leupeptin (Fig. 7A). As expected, no significant presentation of LCMV-NP was observed in the absence of leupeptin (not shown). Therefore, we conclude that LCMV-NP protein fragments that might be present in the ER in C57BL/6 spleen cells were not able to bind to MHC class II in the absence of competing Ii.

3.7 TAP transporter is not needed for presentation of endogenous LCMV glycoprotein

It has previously been suggested that MHC class II loading with peptides derived from endogenously synthesized pro-

Table 2. MHC class II-associated presentation of endogenously synthesized viral proteins upon infection, their intracellular localization and the nature of the antigen-presenting cell

Viral antigens	Presentation	Localization	Chloroquine[a]	BFA[b]	Ii[c]	TAP[d]	APC	Reference
vacc-HIVgp160	+	membrane					B lymphocyte	Polydefkis et al. [72]
vacc-HIVgp160	−	cytosol					B lymphocyte	
vacc-HIVgp160	−	secreted					B lymphocyte	
vacc-H3	+	membrane	sensitive			independent	B lymphocyte	Malnati et al. [43]
vacc-cytoH3	+	cytosol	sensitive			independent	B lymphocyte	
vacc-miniH3	+	cytosol	sensitive			dependent	B lymphocyte	
vacc-influenza matrix	+	cytosol	sensitive	insensitive			B lymphocyte	Jaraquemada et al. [44]
vacc-HBsAG	+	membrane	sensitive				PBL	Jin et al. [33]
influenza HA	+/−	membrane			inhibitory		fibroblast	Dodi et al. [45]
influenza matrix	+	cytosol	insensitive	sensitive	independent		B lymphocyte	Nuchtern et al. [42]
measles virus	+	?	insensitive		independent		fibroblast	Sékaly et al. [40]
measles tmF	+	membrane	insensitive				B lymphocyte	van Binnendijk et al. [75]
LCMV GP	+	membrane	insensitive		independent	independent	spleen cells	this report
LCMV NP	−	cytosol					spleen cells	this report

a) Chloroquine sensitivity of class II presentation indicated only when examined.
b) Brefeldin A sensitivity of class II presentation indicated only when examined.
c) Effect of the absence of Ii on class II presentation indicated only when examined.
d) Effect of the absence of TAP on class II presentation indicated only when examined.

3.7 TAP transporter is not needed for presentation of endogenous LCMV glycoprotein

It has previously been suggested that MHC class II loading with peptides derived from endogenously synthesized proteins in the ER requires functional TAP transporters [62]. Therefore, we analyzed whether the absence of the TAP transporter influenced presentation of endogenous LCMV-GP or -NP by using spleen cells from TAP-deficient mice in the presence of leupeptin as APC (Fig. 7B). Presentation of endogenously synthesized LCMV-GP was not impaired in spleen cells from TAP-deficient mice demonstrating that the import into the ER of GP (-fragments) presentable by MHC class II is not dependent on a functional TAP transporter, but most probably occurred cotranslationally. Presentation of endogenously synthesized NP was not affected by the absence of TAP transporter (Fig. 7B), consistent with the finding that cytosolic LCMV-NP was not presented at all by a class II loading pathway independent of proteolysis in acidic compartments.

4 Discussion

This study describes TAP- and Ii-independent, as well as chloroquine- and leupeptine- insensitive MHC class II presentation of endogenously synthesized membrane-associated GP in LCMV-infected professional APC. In contrast, endogenously synthesized LCMV-NP, a cytosolic protein, was not presented by this chloroquine- and leupeptin-insensitive class II loading pathway. Since the peptides studied are involved in a primary anti-LCMV immune response, and since the noncytopathic LCMV does not interfere measurably with the cell physiology [47, 48, 63], these results are biologically relevant. They indicate that alternative, albeit less efficient, processing pathways exist, allowing class II molecules to be loaded with endogenously synthesized proteins [62].

Many studies on MHC class II presentation of endogenously synthesized antigens have been performed, leading to apparently contradictory results concerning the mechanism(s) of intracellular MHC class II loading. These results are summarized in Tables 1 and 2 and are discussed in relation to our present findings. In Table 1, MHC class II presentation studies of endogenous self-antigens as well as of endogenous neo-self antigens are listed. MHC class II-associated presentation in professional APC is almost exclusively observed for endogenously synthesized proteins either residing in or passing through the ER [38, 36, 61, 64–69]. In contrast, endogenously synthesized proteins which do not have natural access to the ER are not found to be presented on MHC class II molecules. As an exception, endogenous measles virus-derived matrix and nucleocapsid proteins were found to be presented on MHC class II molecules [41]. In some studies, the presence of Ii

proved to be inhibitory for MHC class II loading by endogenous antigens [61, 67], whereas in other studies, the presence of Ii seemed to be irrelevant for MHC class II loading by endogenously synthesized antigens [11, 13, 34]. Thus, certain antigens might not be able to compete efficiently with Ii in the ER for binding to newly synthesized class II molecules, and certain other endogenous antigens are still somehow able to load class II molecules via the classical endosomal class II loading pathway. The notion that only endogenous proteins residing or passing through the ER are able to load class II molecules is corroborated by the findings of Weiss and Bogen [38], who targeted an endogenous λ-chain to different subcellular compartments. Only the transmembrane or ER-targeted proteins were presented on MHC class II, whereas the cytosolic or nuclear proteins were not. The same is true for differentially targeted endogenous HEL antigens, where only soluble, transmembrane or ER-targeted HEL were able to load MHC class II [46]. Additionally, it has been demonstrated in one example [66] that MHC class II-associated presentation of the endogenous self antigen HLA-B/C is insensitive to treatment with chloroquine, and therefore apparently does not require proteolysis in acidic compartments.

Class II presentation of infectious virus-derived endogenous antigens has been analyzed (Table 2). These data are not, however, easily generalizable. It is difficult to compare these results, first, since different and often non-professional APC were used, and second, the different characteristics of the various viral systems do not permit valid comparisons. The importance of the nature of the APC has been revealed by two examples. First endogenous cytosolic V-CH3 was presented by fibroblasts but not by myeloma cells, whereas a secreted V-CH3 protein was presented by both fibroblasts and myeloma cells [70, 71], and second certain epitopes of transfected, membrane-associated HEL were presented by professional APC, but not by fibroblasts [69].

MHC class II presentation of recombinant vaccinia virus-derived endogenously synthesized proteins seems generally to be chloroquine sensitive, regardless of the subcellular localization of the proteins, as shown for vaccinia-influenza matrix [44], for vaccinia-H3, vaccinia-cytoH3, vaccinia-miniH3 [43] and vaccinia-hepatitis B surface antigen [33]. This chloroquine-sensitive class II loading by endogenously synthesized membrane as well as cytosolic proteins might be explained by the fact that vaccinia virus infection leads to a major shut-down in cellular protein synthesis, resembling treatment with Brefeldin A or cycloheximide [63]. Therefore, membrane proteins such as influenza HA, which are naturally transported into the ER, might not be able to load MHC class II molecules outside of endosomal compartments in the presence of chloroquine because no newly synthesized αβ class II heterodimers are available. Thus, only recycled MHC class II molecules from the surface would be available to be loaded with recycled HA from the membrane under these circumstances, a process which should be inhibitable by chloroquine. This recycling pathway may also explain the data of Polydefkis et al. [72]: using recombinant vaccinia viruses, they found that only the endogenous membrane-associated form of HIV gp160 is presented on class II, whereas the secreted or cytosolic forms of HIV gp160 were not presented.

Cytoplasmic proteins derived from vaccinia virus recombinant proteins appear to gain access to endosomal compartments, possibly by degradation-promoting pathways [15], by association with heat shock proteins [73] or as a result of the large intracellular aggregates formed by vaccinia that might fuse with endosomal compartments [63]. Since vaccinia virus and influenza virus are both cytolytic, it is conceivable that they disrupt intracellular compartmentalization. This makes it very difficult, if not impossible, to compare the processing of differently targeted recombinant proteins, since the proteins might finally be uniformly distributed within the virus-infected cell.

Our results, which resemble data obtained with transfected cell lines and by elution and analysis of natural self peptides bound to class II molecules [38, 46, 61, 65–67, 70, 72] therefore suggest that the contradictory results obtained with different viral systems do not reflect distinct processing properties of professional APC, but more likely reflect the different effects of the viruses on host cell membrane compartments, protein synthesis and processing.

Upon LCMV infection of professional APC, endogenously synthesized LCMV-GP was found to be loaded onto MHC class II molecules in a chloroquine- and leupeptin-insensitive manner. Additionally, equivalent class II presentation of endogenous LCMV-GP was observed in the absence of both TAP and Ii – if not even enhanced in the absence of Ii. Since leupeptin and chloroquine may not abolish all lysosomal/endosomal proteolysis, our results do not formally prove that processing of endogenous GP and class II loading by endogenous GP occur outside endosomal compartments. Nevertheless, taken together, the chloroquine and leupeptin insensitivity and the non-involvement of Ii indicate that endogenous LCMV-GP is processed and loaded onto class II molecules outside endosomal compartments, and most probably within the ER. Upon cotranslational import of the LCMV-GP into the ER, the unfolded polypeptide may associate directly with newly synthesized MHC class II αβ heterodimers. To form this trimolecular complex, LCMV-GP must efficiently compete with the binding of Ii, since binding of peptide and Ii have been shown to be mutually exclusive [62]. Within this trimolecular complex LCMV-GP could be trimmed either in the ER or in a pre-Golgi compartment. Indeed, it has been shown that proteolytic activity may exist in the ER [16–18]. These MHC class II-peptide complexes would then be transported directly to the cell surface by the secretory pathway [62].

Cytosolic LCMV-NP synthesized upon LCMV-infection, however, is not loaded onto class II molecules in a manner comparable to LCMV-GP. Two different explanations may account for this finding: first, it is conceivable that the protease machinery in the cytoplasm generates a different peptide repertoire than the proteases in the acidic endosomal compartments, as shown by Bodmer et al. [61, 69]. Thus, the NP peptide recognized by VE8 may not be generated by cytoplasmic proteases, but is generated within the endosomal compartments. Second, it may be that NP-peptide are generated in the cytosol and transported into the ER via the TAP system (it has been shown that the transporter molecules accept peptides of up to 15 amino acids in length [74]), but that MHC class II molecules in the ER might not be able to bind these free peptides. We

consider this second possibility to be more likely: This implies that Ii may not really prevent peptide binding to free MHC class II molecules in the ER, but rather interferes with the binding of whole proteins.

The class II presentation pathway of endogenously synthesized LCMV-GP, but not of LCMV-NP described here, corroborates previous findings that at least some endogenously synthesized proteins that naturally have access to ER/pre-Golgi compartments are able to load MHC class II molecules, and suggests that this most probably occurs without the involvement of processing in endosomal compartments. This pathway may be relevant for T helper cell induction *in vivo*. Virus-infected professional APC may be able to initiate an antiviral immune response even before the infected cells are lysed – a pathway that may be particularly important for non-cytolytic viruses – and before viral antigens are liberated to enter the classical exogenous class II pathway, to activate T-helper cells.

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