Brief Definitive Report

Epitope-specific Enhancement of Antigen Presentation by Invariant Chain

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Summary

The MHC class II–associated invariant chain (Ii) is involved in the intracellular sorting of class II molecules to the endocytic pathway where peptides from processed exogenous antigens are bound, and thereby Ii is thought to enhance antigen presentation. Here we demonstrate that presentation of only one out of five epitopes of a given antigen is augmented by Ii. We have compared the presentation of five different epitopes derived from hen egg white lysozyme (HEL) to A^k-restricted T hybridomas by rat-2 fibroblasts transfected with A_α^k and A_β^k (RKK) and RKK cells supertransfected with the mouse invariant chain (RKKI). Only the presentation of the HEL epitope 46-61 was enhanced whereas the presentation of the HEL epitopes 25-43, 34-45, 112-124, and 116-129 was unchanged or even slightly diminished in RKKI cells. The presentation of the epitopes 25-43 and 34-45 was virtually insensitive to the lysosomotropic reagent chloroquine. Brefeldin A (BFA), which inhibits protein egress from the endoplasmic reticulum, blocked the presentation of all epitopes tested in RKKI cells. In contrast, in Ii-negative RKK cells only the presentation of the epitope HEL(46-61) was inhibited by BFA and the presentation of the epitopes 25-43 and 34-45 was only slightly impaired. These findings suggest that Ii may target class II molecules to selected endosomal subcompartments involved in the processing of different peptides derived from an endocytosed antigen. As a result, the enhancement of the class II–restricted presentation in Ii expressing cells appears to be epitope specific rather than antigen specific.

The MHC class II–associated invariant chain has been ascribed a number of different functions. One proposed role is to provide a sorting signal for the intracellular transport of class II molecules (1). In HeLa cells, transport of class II molecules to the endocytic compartment was observed only when Ii was coexpressed (2, 3). Ii probably directs class II molecules to a late endosomal compartment (4). A second role has been suggested by biochemical studies indicating that Ii can block the peptide binding to class II $\alpha\beta$ dimers (5, 6). Thus, Ii may prevent peptide loading onto class II molecules in preendosomal compartments. A third possible role of Ii is to act as a chaperone in the assembly and folding of class II molecules, and in their egress from the endoplasmic reticulum (7, 8).

These proposed functions suggest that Ii may facilitate the class II–restricted presentation of exogenous antigens to T cells. For Ltk^- cells transfected with mouse class II genes in the presence or absence of Ii, Ii expression was reported to enhance the presentation of some antigens like the complement component C5 (9), hen egg lysozyme or measles virus hemagglutinin (10), but not of other antigens like ovalbumin or pork insulin (11). The crucial role of Ii in the presentation of certain antigens was further demonstrated with splenocytes from mice lacking the Ii gene (12). Recently, it was shown that the alternatively spliced Ii gene product p41 plays a role in the enhanced presentation of some antigens (13). Utilizing rat-2 fibroblasts expressing A^k in the presence or absence of Ii, we previously reported that presentation of HEL but not of ribonuclease A (RNase) was enhanced in the presence of Ii (14). Extending these findings we now show that the facilitating function of Ii is restricted to only one out of five epitopes derived from processing of HEL, suggesting that Ii may be involved in the fine tuning of the immune response to exogenous antigens.

Materials and Methods

Transfectants. Rat-2 fibroblasts transfected with cDNA con-

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/10/1453/06 $2.00
Volume 178 October 1993 1453–1458

structs coding for the α and β chain of A^k (RKK) have been described (14). Supertransfection with genomic DNA coding for murine Ii was performed as described (14). RKKI clone 4 which expresses high amounts of Ii was used in this study. Ii expression was determined by cytoplasmic immunoperoxidase staining and by immunoprecipitation. The T cell hybridomas 2B6.31 (15), 1C5.1, 2D4.1 (16), 3A9 (17), 2C8.4 (18), and TS1.2 (19) have been described. The T cell hybridomas 3B11.1, 4G4.1, 1G5.1, 2B5.1, and 1B9.1 were generated for the present study and characterized as described (18).

Immunoprecipitations. Immunoprecipitations were performed as described (14). Briefly, 3×10^6 cells were labeled for 1 h with 100 μCi of a mixture of 80% [^{35}S]methionine and [^{35}S]cysteine (ICN-Flow, Cleveland, OH). The adherent cells were harvested from the dishes with TBS lysis buffer containing 1% NP-40. The lysates were precleared with Protein A-soluble (Sigma Chemical Co., St. Louis, MO) and a mixture of normal rat and mouse sera. For immunoprecipitations, Protein A–Sepharose beads (Pharmacia, Inc., Piscataway, NJ) preabsorbed with monoclonal antibodies were added to the lysates for 2 h. A^k molecules were precipitated with mAb H116-32. To precipitate Ii, mAb In1 was used together with the protein A binding anti-rat kappa chain antibody MAR18.5 (for references see 13). The immunoprecipitates were analyzed on 12% SDS-PAGE.

Cytofluorometry, Peptide Binding Assay. Surface immunofluorescence staining for A^k was performed with mAb H116-32 and FITC-conjugated goat anti-mouse Ig (GIBCO-BRL Life Technols, Inc., Gaithersburg, MD). For intracellular staining of Ii, cells were permeabilized with 0.3% saponin (Sigma Chemical Co.) and stained with mAb In1 followed by FITC-conjugated goat anti-rat Ig (Southern Biotechnology, Inc., Birmingham, AL).

Peptide binding to cell surface A^k molecules was measured essentially as described (20). RKK and RKKI cells were mixed with varying concentrations of biotinylated HEL peptide 46-61 and incubated at 37°C for 3.5 h. Subsequently the cells were stained with successive layers of fluoresceinated avidin D, biotinylated goat anti-avidin D antibody, and again fluoresceinated avidin D (Vector Labs, Inc., Burlingame, CA). Staining was at 4°C for at least 30 min per layer followed by two washes. Class II expression was determined in the same experiment using saturating amounts of FITC-conjugated mAb H116-32. 5000 cells per sample were analyzed for median fluorescence channel (MFC) intensity on a FACScan flow cytometer (Becton Dickinson and Co., Mountain View, CA). After background subtraction, the effects of variations in A^k expression between cells was eliminated by calculating MFC ratios [(MFC with peptide – MFC without peptide)/(MFC with H116-32-FITC – MFC with control Ab)].

Cell Cultures. Antigen presentation assays were performed as described (13, 14). Briefly, cultures containing 2.5×10^4 presenting cells and 5×10^4 T hybridoma cells were set up in microtiter plates with antigen in 200 μl RPMI 1640 supplemented with 10% FCS. After 24 h of culture, 50 μl of supernatants were assayed for the presence of IL-2 by culture with 10^4 CTLL-2 cells for 24 h. During the final 4 h of culture, the CTLL-2 cells were pulsed with 1 μCi [^{3}H]-methyl-thymidine (Amersham Intl., Buckinghamshire, England). Each point in the graphs of Figs. 2–4 represent the mean from triplicate cultures.

RKK and RKKI cells were preincubated with graded doses of chloroquine (Sigma Chemical Co.) for 30 min or with brefeldin A (BFA) (Sandoz) for 15 min in complete medium followed by the addition of antigen for 4 h as described (19). Subsequently, the cells were fixed in 0.05% glutaraldehyde for 30 s on ice, quenched in 0.2 M glycine, and washed three times before T hybridoma cells were added.

Results and Discussion

The influence of Ii on the presentation of helper epitopes derived from HEL was studied using A^k-transfected Ii-negative rat-2 fibroblasts (RKK), or RKK cells supertransfected with genomic DNA coding for mouse Ii (RKKI). In RKKI cells a high level of Ii expression in excess of A^k molecules was shown by sequential immunoprecipitation with monoclonal antibodies against A^k and Ii (Fig. 1 *a*). The expression of Ii in RKKI cells was homogenous as determined by intracellular cytofluorometry (Fig. 1 *b*). The level of A^k cell surface expression was slightly lower in the RKKI clone used in this study than in RKK parental cells (Fig. 1 *b*), possibly due to intrinsic clonal variability of these cells. Although Ii is supposed to facilitate the egress of class II molecules from

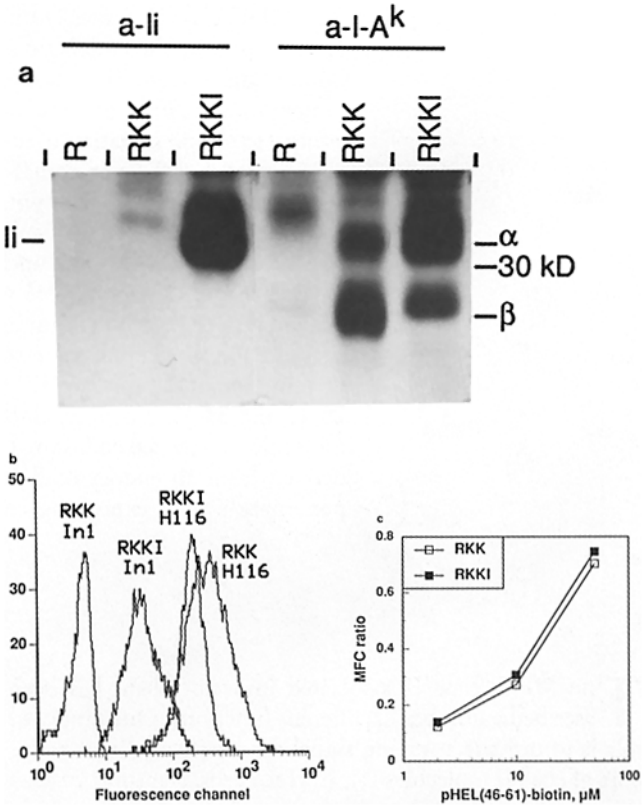
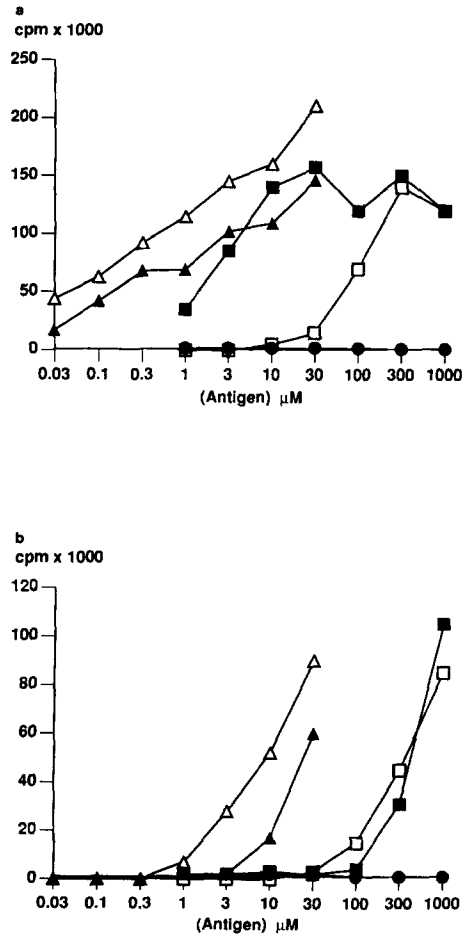


Figure 1. (*a*) Immunoprecipitation of A^k and Ii molecules from metabolically labeled RKK and RKKI cells. For control, untransfected rat-2 cells (R) were used. First A^k was precipitated with mAb H116-32 (*anti-I-A^k*), then Ii was precipitated from the same lysates with mAb In1 (*anti-Ii*). The positions of the α and β chains of A^k and of the p31 form of Ii are indicated. Ii coprecipitates with A^k in RKKI cells but is absent in RKK cells. As visible for the β chain of A^k, class II molecules are more rapidly processed into higher glycosylated forms in the presence of Ii. After clearing of class II molecules, high amounts of free Ii were recovered from RKKI cells. (*b*) Surface immunofluorescence staining of RKK and RKKI cells for A^k with mAb H116-32. Ii was intracellularly stained with mAb In1 after permeabilization of cells with saponin. (*c*) Indistinguishable binding of biotinylated peptide HEL(46-61) to RKK and RKKI cells. Cells were incubated with graded amounts of biotinyl-HEL(46-61) and stained with two layers of avidin-FITC sandwiched by biotinylated anti-avidin Ab. The MFC ratios shown represent relative peptide binding, normalized for differences in class II expression that were determined in the same experiment (see Materials and Methods).

Table 1. *Activation of A^k-restricted T Cell Hybridomas by Ii-negative RKK Cells and by Ii-positive RKKI Cells*

Hybridoma	Peptide	Restriction	EC_{50} for protein (μM) RKK	RKKI	ratio EC_{50} RKK/RKKI	EC_{50} for peptide (μM) RKK	RKKI
2B6.31	HEL 25-43	A^k	220	270	0.82	5.00	13.00
3B11.1	HEL 34-45	A^k	60	100	0.60	3.00	6.00
1C5.1	HEL 46-61	A^k	150	2	75.00	0.05	0.13
4G4.1	HEL 46-61	A^k	45	12	3.75	0.50	1.10
1G5.1	HEL 46-61	A^k	170	40	4.25	0.04	0.05
2B5.1	HEL 46-61	A^k	200	7	28.57	0.30	0.80
3A9	HEL 46-61	A^k	100	3	33.33	0.50	0.80
2D4.1	HEL 112-124	A^k	500	600	0.83	0.10	0.40
2C8.4	HEL 116-129	A^k	500	500	1.00	0.13	0.50
1B9.1	HEL 116-129	A^k	150	300	0.50	0.07	0.30
TS1.2	RNase 43-56	A^k	0.5	2	0.25	1.50	2.00

The responsiveness of T cell hybridomas to protein antigen (HEL or RNase) or to the indicated synthetic peptide presented by RKK or RKKI cells was estimated by the antigen concentration (μM) giving 50% of the maximal response (EC_{50}). A representative experiment out of three performed with similar results is shown.



the endoplasmic reticulum we have observed similar levels of surface class II on a large variety of class II transfectants such as rat-2, HeLa, and RMA cells in the absence and presence of Ii. This is in contrast to splenocytes from Ii knockout mice, which express only low levels of class II on the cell surface (12). The reason for this discrepancy is not clear. In any case, when differences in I-A^k expression were taken into account, the relative ability of surface class II molecules to bind peptides was similar in RKK and RKKI cells (Fig. 1 *c*).

10 T cell hybridomas with specificity for five A^k-restricted HEL epitopes were used to study the effect of Ii on the presentation of individual epitopes. In Table 1, a quantitative evaluation of the response curves is given. The ratio of the antigen concentrations yielding 50% of the maximal responses (EC_{50}) for RKK vs. RKKI cells was greater than 3.0 for all five T cell hybridomas recognizing the HEL peptide 46-61, namely 1C5.1, 4G4.1, 1G5.1, 2B5.1, and 3A9, indicating a clear enhancement of the presentation of this epitope in the presence of Ii. A typical example is presented in Fig. 2 *a*. It can be seen that RKKI cells stimulated 3A9 T hybridoma

Figure 2. Efficiency of the presentation of the A^k-restricted HEL epitopes 46-61 and 25-43 by Ii-negative and Ii-positive rat-2 cells. RKK cells (*open symbols*) or RKKI cells (*closed symbols*), 2.5 × 10^4/well, were incubated for 24 h with graded concentrations of HEL (*squares*) or the appropriate peptide (*triangles*) and 5 × 10^4/well T hybridoma cells. For control fixed RKKI cells (*circles*) were incubated with HEL. In (*a*) hybridoma 3A9 (HEL 46-61) and in (*b*) hybridoma 2B6.31 (HEL 25-43) was used. IL-2 production was determined by [^{3}H]thymidine incorporation into CTLL-2 cells. Data are presented as mean cpm from triplicate cultures.

cells at an about 30-fold lower HEL concentration than RKK cells, but that at high HEL concentrations RKK stimulated almost as well as RKKI. For the hybridomas 1C5.1, 2B5.1, and 3A9, the EC_{50} ratios were about 10 to 20 times greater than for the hybridomas 4G4.1 and 1G5.1. This difference did not correlate with the sensitivity of the hybridomas as determined by their responses to exogenously added peptide (Table 1). Exogenously added peptide was always presented somewhat more efficiently by RKK cells than by RKKI cells. This difference probably reflects the slightly higher level of A^k expression in RKK because the degree of loading with peptide was similar for both cell lines (Fig. 1 *c*). The fine specificities of these hybridomas for shorter peptides within the sequence 46-61 have not been compared. Thus, it seems possible that the differences in the EC_{50} ratios are due to differentially enhanced presentation of subdeterminants of epitope 46-61.

Using the hybridomas 2B6.31 (Fig. 2 *b*) and 3B11.1 recognizing the overlapping HEL epitopes 25-43 and 34-45, respectively, an EC_{50} ratio close to unity was found indicating that Ii did not facilitate the presentation of these epitopes. The hybridomas 2D4.1, 2C8.4, and 1B9.1 recognizing the overlapping COOH-terminal epitopes HEL 112-124 and 116-129, respectively, showed EC_{50} ratios ≤ 1.0 demonstrating the lack of enhancement for these epitopes by Ii. For comparison, the hybridoma TS1.2 specific for the RNase epitope 43-56 was included (Table 1). RKK cells were clearly superior to RKKI cells in the presentation of RNAse (EC_{50} ratio 0.25).

The lysosomotropic reagent chloroquine interferes with the acidification of the endosomal compartment. For selected

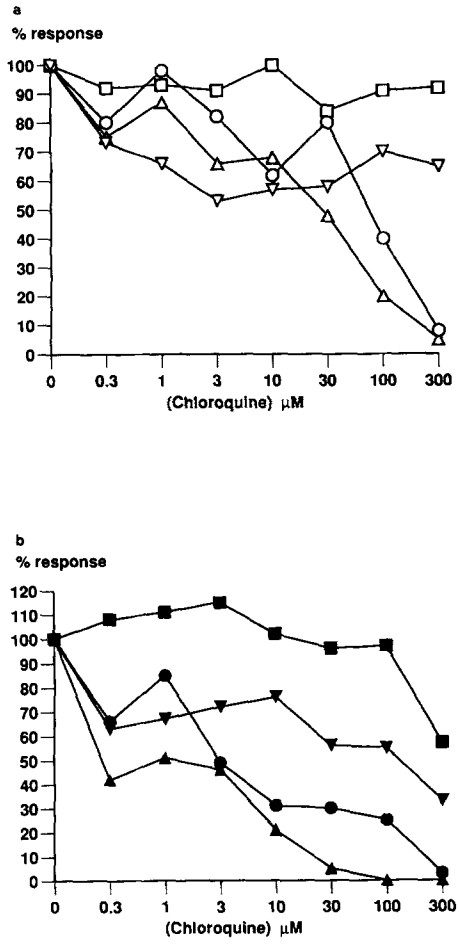


Figure 3. Chloroquine sensitivity of the presentation of HEL by RKK and RKKI cells. RKK cells (*a*) and RKKI cells (*b*), 2.5 × 10^4/well, were incubated 30 min before and during a 4-h pulse with 1 mM HEL in the presence of graded doses of chloroquine. The presenting cells were fixed and 5 × 10^4/well T hybridoma cells, 2B6.31 (peptide 25-43) (▽), 3B11.1 (peptide 34-45) (□), 4G4.1 (peptide 46-61) (△), and 1B9.1 (peptide 116.129) (○), respectively, were added for 24 h. The control responses in the absence of chloroquine were (*a*), 189263 cpm (▽), 347265 cpm (□), 300111 cpm (△), 108263 (○), and (*b*), 65958 (▽), 106663 (□), 114726 (△), 12581 (○).

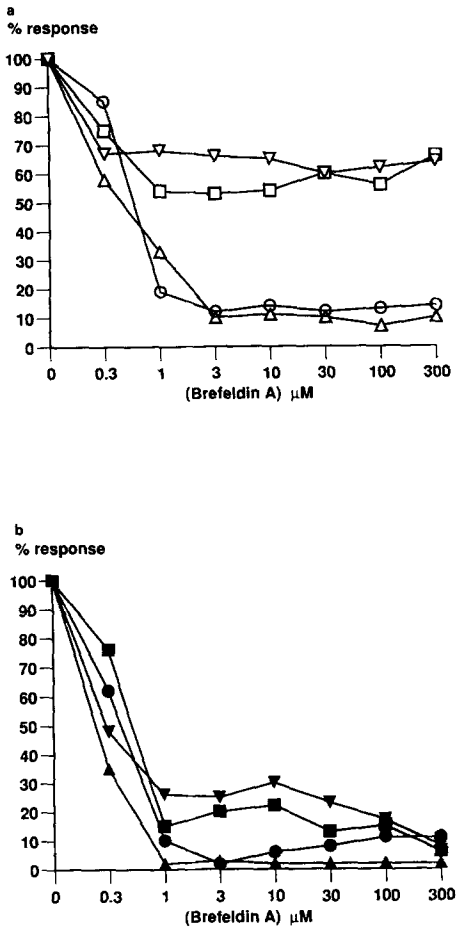


Figure 4. Brefeldin sensitivity of the presentation of HEL by RKK and RKKI cells. RKK cells (*a*) and RKKI cells (*b*), 2.5 × 10^4/well, were incubated 15 min before and during a 4-h pulse with 500 μM HEL in the presence of graded doses of BFA. The presenting cells were fixed and 5 × 10^4/well T hybridoma cells, 2B6.31 (▽), 3B11.1 (□), 4G4.1 (△), and 1G5.1 (○), respectively, were added for 24 h. The control responses in the absence of BFA were (*a*), 166822 cpm (▽), 180794 cpm (□), 322707 cpm (△), 103109 (○), and (*b*), 53502 (▽), 39600 (□), 97765 (△), 17434 (○).

hybridomas we studied the effect of chloroquine on the presentation of HEL epitopes by RKK and RKKI cells (Fig. 3). Interestingly, the presentation of the epitope 34-45 was not inhibited by titrated doses of chloroquine in RKK cells, and showed a slight inhibition only at 300 μM in RKKI cells. Similarly, increasing doses of chloroquine had little effect on the presentation of epitope 25-43. In contrast, chloroquine clearly inhibited the presentation of the epitopes 46-61 and 116-129 in both RKK and RKKI cells. For the four epitopes studied, presentation by RKKI cells was always somewhat more sensitive to chloroquine than presentation by RKK cells (see Fig. 3 *b*). This is in agreement with the observation that chloroquine inhibits the proteolytic removal of Ii from class II $\alpha\beta$ dimers before their transport to the cell surface (21).

The fungal metabolite brefeldin A blocks the vesicular transport from the endoplasmic reticulum. In addition, BFA was reported to induce the fusion of early endosomes with the *trans*-Golgi network (22). However, the extent of impairment induced by BFA on the transport and the degradation of endocytosed proteins is controversial (22, 23). We have studied the effect of BFA on the presentation of the HEL epitopes 25-43, 34-45, and 46-61 (Fig. 4). When RKK cells were used as APC, the presentation of the epitopes 25-43 and 34-45 was inhibited by only 30-40% even at the highest BFA doses used, whereas the presentation of epitope 46-61 was almost completely blocked. In a previous study presentation of epitope 116-129 by LK-35.2 B cells was also inhibited by BFA (24). In RKKI cells, the presentation of the three epitopes tested was clearly inhibited by high doses of BFA, although the presentation of epitopes 25-43 and 34-45 was inhibited to a lesser extent at concentrations lower than 30 μM BFA.

Out of the five A^k-restricted HEL epitopes studied here, only the presentation of the epitope 46-61 was enhanced in the presence of an invariant chain. This may indicate that the Ii-mediated targeting of the class II/Ii complex to a prelysosomal compartment (4) is responsible for the selective enhancement in the presentation of HEL(46-61). The presentation of this epitope, and of 116-129, was clearly more sensitive to chloroquine than the epitopes 25-43 and 34-45. In line with these results are observations showing that the chloroquine-sensitive environment of lysosomes is required for the initial reduction of disulfide bonds in HEL, and subsequent presentation of the epitope 46-61 (25, 26). The HEL epitopes 34-45 and 25-43 are virtually insensitive to chloroquine, suggesting processing in early endocytic compartments, where protein breakdown may be less dependent on acidic pH, or processing by endoproteinases relatively resistant to increased pH.

Alternatively, the loading of peptides onto class II molecules, rather than their cleavage, could be differentially influenced by chloroquine. This would be consistent with the observation that the binding of peptide 34-45 to fixed A^k-expressing cells is unchanged between pH 5.0 to pH 7.4, whereas the binding of peptide 46-61 shows an optimum at pH 5.5 (27). Utilizing purified A^k molecules, Sette et al., found a binding optimum for HEL(46-61) at pH 4.5 (28).

In RKK cells, the presentation of peptide 46-61 was significantly more sensitive to BFA treatment than the presentation of the peptides 25-43 and 34-45. In line with previous observations (24, 29) this may imply that the peptide 46-61 binds to A^k in an endosomal subcompartment fed with newly synthesized class II molecules which are retained in the ER in the presence of BFA. In contrast, the peptides 25-43 and 34-45 seem to have access to a pool of preexisting class II molecules that may arise from internalization of unloaded molecules from the surface. In RKKI cells the presentation of the three HEL peptides, 25-43, 34-45, and 46-61, was clearly sensitive to BFA, indicating presentation by newly synthesized class II molecules.

In conclusion, it appears that the augmenting effect of Ii is actually epitope specific rather than simply antigen specific (9, 10, 14). In addition, distinct pathways of presentation for different HEL epitopes exist. The epitope 46-61 is exclusively or predominantly bound by newly synthesized class II molecules and its presentation is highly chloroquine sensitive, whereas in the absence of Ii the epitopes 25-43 and 34-45 can be presented by a pool of preexisting class II or, in the presence of Ii, also by newly synthesized class II molecules. The precise rules controlling preferential presentation of particular epitopes from the same protein antigen by different pathways remain to be explored.

This work was supported by DFG grant Mo 389/2-1.

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Received for publication 25 January 1993 and in revised form 14 June 1993.

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