The EMBO Journal vol.15 no.24 pp.6910–6920, 1996

Distinct antigen MHC class II complexes generated by separate processing pathways

Robert Lindner and Emil R.Unanue

Center for Immunology and Department of Pathology, Washington University School of Medicine, 660 S.Euclid Avenue, St Louis, MO 63110, USA

The peptide binding site of MHC class II molecules is open at both ends and, therefore, does not restrict the length of the bound ligand. Here we show that a partially folded protein antigen (*HEL) spontaneously formed SDS-unstable complexes with the purified MHC class II molecule I-A^k (A^k). These complexes were also detected on the surface of antigen-presenting cells (APCs) where they stimulated T cells. However, they rapidly disappeared after endocytosis. Intracellular processing of *HEL gave rise to SDS-stable, long-lived A^k complexes containing *HEL peptides and, unexpectedly, full-length *HEL. Both SDS-stable products were formed in low pH compartments and then transported to the plasma membrane. In contrast to *HEL peptides, the stable association of *HEL occurred in an alternative pathway that required mature class II molecules and did not involve HLA-DM or proteases. SDS-stable *HEL–A^k complexes were formed by a reaction of endosomal A^k with endocytosed *HEL, but not by direct conversion of SDS-unstable complexes derived from the plasma membrane. Our work establishes a fundamental difference between the two MHC class II loading pathways and for the first time demonstrates a full-length protein as a product of antigen processing.

Keywords: antigen processing/HLA-DM/MHC class II/ protein antigen/proteolysis

Introduction

Major histocompatibility complex (MHC) class II molecules are heterodimeric membrane glycoproteins that present peptide determinants of exogenous protein antigens to CD4+ T cells (reviewed by Germain and Margulies, 1993; Cresswell, 1994; Wolf and Ploegh, 1995). Unlike MHC class I molecules, their peptide binding region is open at both ends and, therefore, does not limit the length of the bound ligand (Allen and Unanue, 1984; Sette *et al.*, 1989; Luescher and Unanue, 1990a; Brown *et al.*, 1993). Binding to MHC class II molecules requires a straight, extended conformation of the interacting peptide segment (Stern *et al.*, 1994). For this reason, antigenic determinants in most proteins are usually not able to interact with MHC class II molecules, but need to be converted into a binding-competent conformation first. Furthermore, antigenic determinants bound to MHC class II molecules need to be accessible to a T-cell receptor (TCR) to trigger an immune response. Studies using inhibitors of organelle acidification (Ziegler and Unanue, 1982) or *in vitro* proteolytic digestion of protein antigens (Allen *et al.*, 1984) demonstrated a critical role for the intracellular fragmentation of exogenous protein antigens into peptides, which usually possess enough conformational flexibility to interact with MHC class II molecules. The role of peptides in the MHC class II-restricted presentation was confirmed later by the isolation of naturally processed antigens from MHC class II molecules. So far, peptides ranging from nine to 28 amino acids have been found, containing a short MHC binding 'core' determinant with N- and C-terminal extensions of varying lengths (reviewed by Engelhard, 1994).

In antigen-presenting cells (APCs), the association of proteolytically processed antigenic fragments with MHC class II molecules is believed to take place in a specialized, late endocytic compartment termed MIIC (reviewed by Schmid and Jackson, 1994; Wolf and Ploegh, 1995). This compartment is accessed by newly synthesized MHC class II molecules that are occupied with proteolytic fragments of the invariant chain (Tulp *et al.*, 1994; Amigorena *et al.*, 1995). MIIC has been demonstrated to contain HLA-DM (Sanderson *et al.*, 1994; Pierre *et al.*, 1996), a recently identified cofactor required for the SDS-stable association of proteolytic antigen fragments with MHC class II (Denzin *et al.*, 1994; Fling *et al.*, 1994; Karlsson *et al.*, 1994; Morris *et al.*, 1994). HLA-DM has been shown to destabilize SDS-unstable, short-lived complexes of MHC class II molecules and peptides like the invariant chain-derived peptide CLIP, thereby allowing their exchange for peptides that form long-lived, SDS-stable complexes (Denzin and Cresswell, 1995; Sherman *et al.*, 1995; Sloan *et al.*, 1995). For this reason, HLA-DM has been suggested not only to catalyze the loading of exogenous antigen fragments but also to 'edit' the peptide repertoire on MHC class II molecules for SDS-stable, long-lived interactions (Roche, 1995; Sloan *et al.*, 1995).

Some protein antigens do not utilize the HLA-DM-dependent loading pathway outlined above: antigenic determinants of RNase A, influenza virus hemagglutinin and myelin basic protein were presented on mature MHC class II molecules (Nadimi *et al.*, 1991; Pinet *et al.*, 1994, 1995). Their MHC class II association was independent of the invariant chain and HLA-DM, but required internalization of surface MHC class II into a chloroquine-sensitive compartment.

Proteins with antigenic determinants in conformationally flexible regions, like fibrinogen (Lee *et al.*, 1988) or denatured proteins (Allen and Unanue, 1984; Sette *et al.*, 1989), are presented to T cells by chemically fixed APCs and are thought to bind directly to surface class II molecules in a way which can be detected by a TCR. To date, little information is available about how protein antigens as opposed to peptide antigens interact with MHC class II molecules and how APCs handle these events.

© Oxford University Press

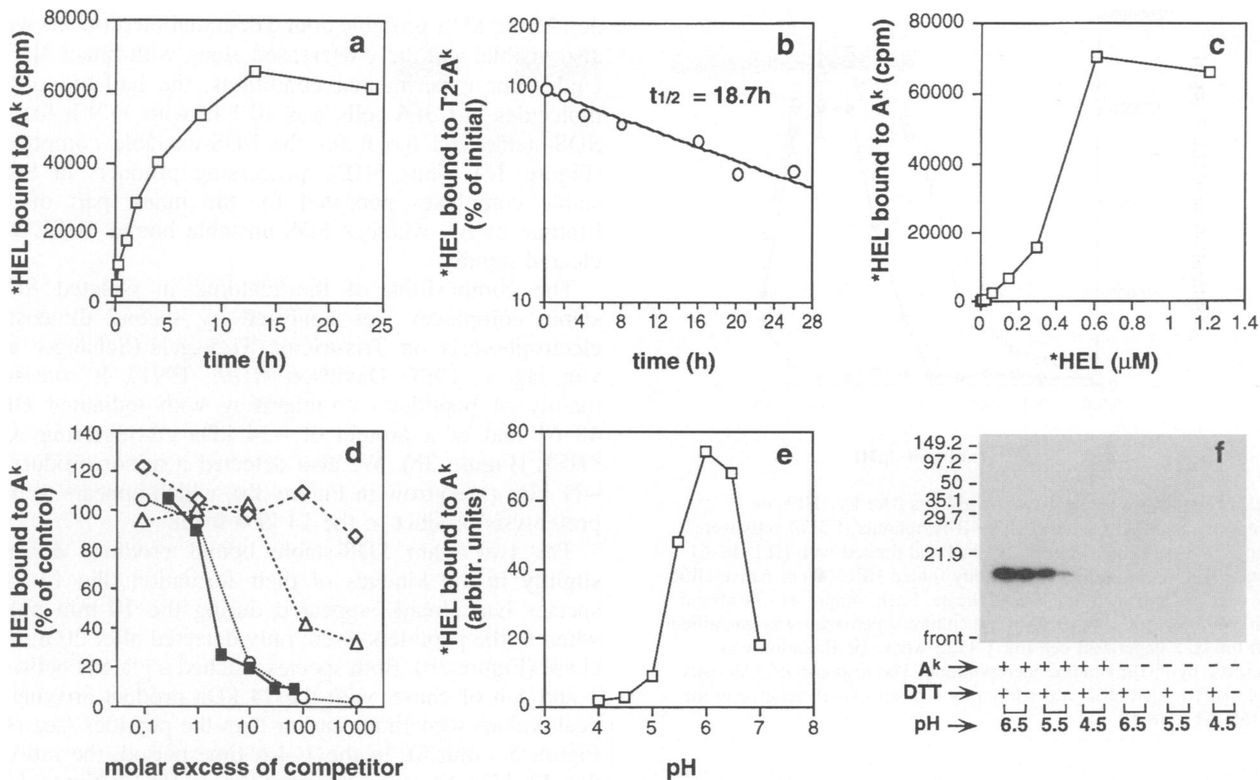


Fig. 1. Interaction of *HEL with purified A^k. (**a**) Kinetics of association. A^k was incubated with *HEL at 37°C for the times indicated. The specific activity of *HEL was 1.2×10^5 c.p.m./pmol. Bound *HEL was immunoprecipitated with anti-A^k and quantified by γ-counting. Graphed values are means of duplicates minus control (incubations without A^k usually resulted in <2% of signal). Note the slow on-rate of *HEL. (**b**) Kinetics of dissociation. Pre-formed *HEL–A^k complexes were mixed with a 1000-fold excess of HEL 48–61 peptide over *HEL, incubated, and quantified as in (a). The deduced half-life of the complex was 18.7 h. (**c**) Binding titration. A^k (3 pmol) was titrated with *HEL (0.2–40 pmol), incubated, and bound *HEL was quantified. Half-maximal binding of *HEL occurred at ~0.45 μM *HEL. (**d**) Competition experiments. A^k, *HEL and increasing amounts of 'cold'-iodinated *HEL (ciHEL, ■), HEL 48–61 peptide (○), native HEL (△) or irrelevant ovalbumin 323–339 peptide (◇) were mixed, incubated, and *HEL–A^k complexes quantified. (**e**) pH dependence of binding. A^k and *HEL were incubated at pH 4.0–7.0, immunoprecipitated with anti-A^k and bound *HEL was quantified after electrophoresis. Control precipitations with mAb 30-5-7 ruled out any pH-induced aggregation of *HEL (not shown). (**f**) Lack of SDS stability of *HEL–A^k complexes formed *in vitro*. A^k was incubated with *HEL at pH 4.5, 5.5 or 6.5 ± 2 mM dithiothreitol. *HEL–A^k complexes were isolated and analyzed in 15% Tris-glycine SDS gels without boiling the samples. Control immunoprecipitations with mAb 40F in the absence of A^k ruled out non-specific aggregation of *HEL. Note the positive effect of dithiothreitol on the binding of *HEL at suboptimal pH.

A direct approach to study the processing and the MHC class II association of antigens is to label MHC epitopes in a protein and to follow their fate within an APC. For this purpose, we used hen egg white lysozyme (HEL), a well studied protein antigen with three tyrosine residues (Y20, Y23, Y53) available for radioiodination. Y53 is located in the peptide HEL 48–61/62, the major natural processing product of HEL isolated from A^k after acid elution (Nelson *et al.*, 1992). HEL 48–61/62 forms SDS-stable, high affinity complexes with A^k (Nelson *et al.*, 1993), a propensity that correlates with a long *in vivo* half-life of the peptide–A^k heterotrimers (Nelson *et al.*, 1994). Y20 and Y23 are contained in a low affinity A^k binding peptide, HEL 18–33, that forms SDS-unstable complexes with A^k (our unpublished data). The presence of iodine on Y53, Y20 or Y23 does not interfere with the A^k binding of the HEL 48–61 peptide (Nelson *et al.*, 1993) or the HEL 18–33 peptide (our unpublished data), respectively.

Results

*HEL interacts with A^k without processing

In an attempt to radiolabel the tyrosine residues present in HEL efficiently, the native protein was unfolded in 6 M guanidinium hydrochloride, radioiodinated in the presence of the denaturant and refolded by rapid dilution (Radford *et al.*, 1992). The resulting product, *HEL, was labeled at a specific activity of $\sim2\times10^8$ d.p.m./nmol. *HEL was soluble without any tendency to aggregate, and its Stokes' radius was similar to that of native HEL. However, it showed a higher protease sensitivity than native HEL, suggesting a more open, partially folded structure (data not shown).

First we tested if *HEL was able to associate with purified A^k *in vitro* (Figure 1): *HEL bound to A^k similarly to peptide ligands of MHC class II molecules with respect to its slow association and dissociation kinetics and its affinity (Figure 1a–c) (Roof *et al.*, 1990). The association of *HEL with A^k was competed to the same extent by HEL 48–61 peptide and 'cold' iodinated *HEL (ciHEL) (Figure 1d). Native HEL competed very weakly (at an ~100-fold higher concentration than the HEL 48–61 peptide), whereas a non-A^k binding peptide did not interfere (Figure 1d). This suggested that *HEL specifically bound to the peptide binding site of A^k with an affinity comparable to that of the HEL 48–61 peptide.

Unlike the HEL 48–61 peptide, *HEL did not form SDS-stable complexes with A^k *in vitro*. Variation of the

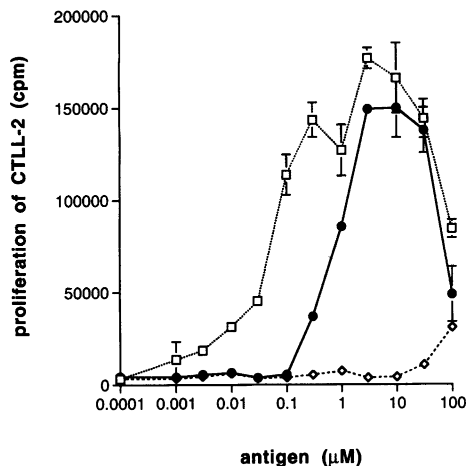


Fig. 2. Stimulation of the T-cell hybridoma 3A9 by *HEL on chemically fixed APCs. M12.C3.F6 B-lymphoma (C3F6) cells were fixed in 1% paraformaldehyde, washed and titrated with HEL 48–61 peptide (□), 'cold'-iodinated, partially folded HEL (●) or native HEL (◇) over the indicated range in triplicate. Each sample was incubated with 3A9 cells for 16 h. IL-2 in the culture supernatant was quantified with the IL-2-dependent cell line CTLL, whose proliferation was measured by [^{3}H]thymidine incorporation. The response of 3A9 cells to partially folded HEL was shifted by a factor of ~10 relative to the peptide HEL 48–61.

pH and/or the presence of reducing agents had marked effects on the binding of *HEL but did not induce SDS stability (Figure 1e and f). The pH optimum for the SDS-unstable interaction of *HEL with A^k was at pH 6.0–6.5, with almost no binding detectable at pH 5.0 and lower (Figure 1e). Thus, in an APC, *HEL would be expected to bind to A^k at the plasma membrane and in an early endocytic organelle rather than in a late endosome or a lysosome.

*HEL stimulated the T-cell hybridoma 3A9 when bound to surface A^k molecules of chemically fixed M12.C3.F6 B-lymphoma cells (C3F6) (Nabavi *et al.*, 1989). 3A9 recognizes A^k-bound peptides containing the core epitope HEL 52–61 (Allen *et al.*, 1984). Figure 2 shows the dose–response curves obtained by titrating paraformaldehyde-fixed C3F6 cells with increasing concentrations of either native HEL, ciHEL or HEL 48–61 peptide and incubating them with 3A9 cells. *HEL triggered a response of 3A9, but required an ~10-fold higher concentration to be as efficient as the HEL 48–61 peptide. Native HEL hardly triggered any response at all. Thus, at least some, if not all, *HEL molecules were bound to A^k via the 48–61 peptide segment.

*Products and kinetics of the cellular processing of *HEL*

In the absence of antigen processing, the only type of complex formed between *HEL and A^k was SDS-unstable. By contrast, SDS-stable complexes were formed after feeding *HEL to an APC. Pulse–chase experiments with C3F6 cells showed a delayed generation of SDS-stable complexes that was preceded by SDS-unstable binding of *HEL to A^k (Figure 3a). SDS-stable complexes were most abundant after 1–4 h of chase and disappeared with a half-life of 8.8 h (see Figure 3c). By contrast, SDS-unstable *HEL–A^k complexes were formed early (0 h chase) and disappeared with a half-life of 2.4 h (Figure 3c). Minor SDS-unstable bound degradation products were also visible, and these decreased along with intact *HEL. Under our experimental conditions, the half-life of A^k molecules in C3F6 cells was 10.1 h, with 11.9 h for the SDS-stable and 6.6 h for the SDS-unstable component (Figure 3d). Thus *HEL processing products in SDS-stable complexes persisted for the major part of the lifetime of A^k, whereas SDS-unstable bound *HEL was cleared rapidly.

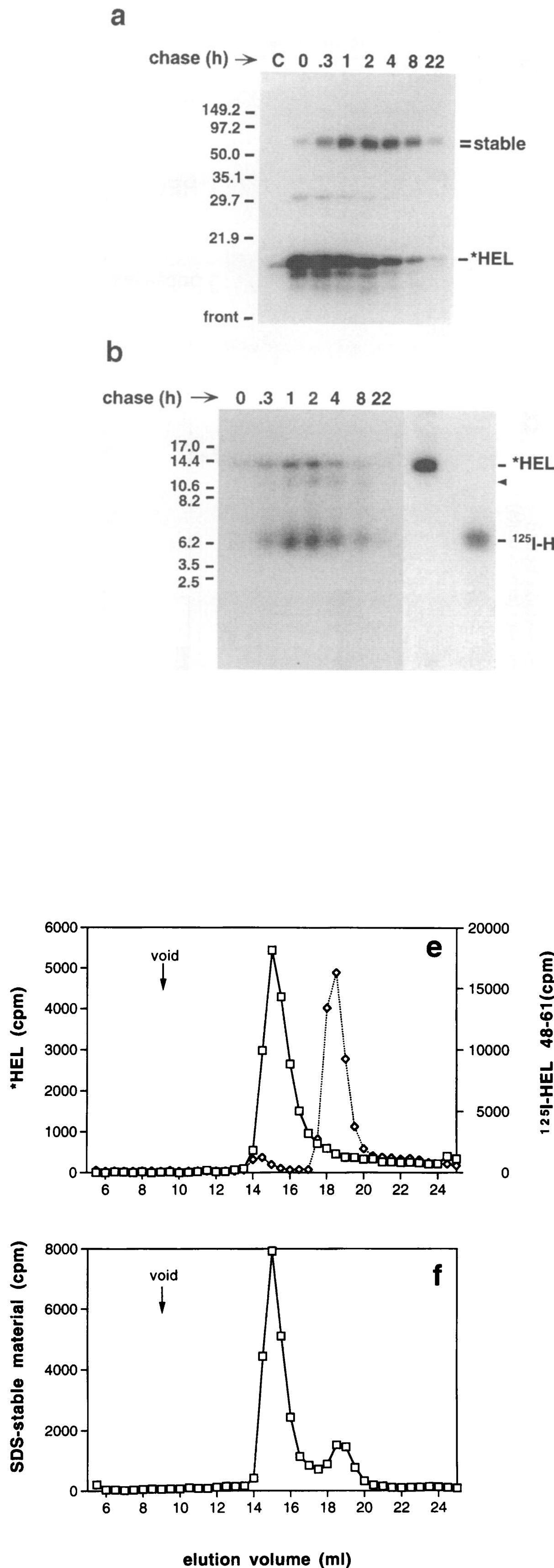
The composition of the material in isolated SDS-stable complexes was analyzed by second dimension electrophoresis on Tris-tricine SDS gels (Schägger and von Jagow, 1987; Davidson *et al.*, 1991). It consisted mainly of peptide(s) co-migrating with iodinated HEL 48–61 and of a protein of ~14 kDa co-migrating with *HEL (Figure 3b). We also detected a minor product at ~11 kDa (see arrow in Figure 3b), which appears to be a proteolysis product of the 14 kDa form.

The two main SDS-stable bound products differed slightly in the kinetics of their formation: the 14 kDa species had already appeared during the 10 min pulse, whereas the peptide(s) were only detected after 20 min of chase (Figure 3b). Both species reached a plateau between 1 and 4 h of chase, with the 14 kDa product arriving at peak values sometimes earlier than the peptides (see also Figure 5, control). In the 1–4 h time period, the ratio of the 14 kDa form to peptides was ~0.5 (estimated by radioactivity). This ratio was dependent on the pulse length, with the 14 kDa form becoming more prominent after longer pulses (data not shown). After the 1–4 h time period, the SDS-stable bound peptides disappeared with a half-life of 8.8 h. The 14 kDa product decreased more rapidly with a half-life of 5.4 h (Figure 3c). This apparent discrepancy in the half-lives of the two stable bound species was probably caused by proteolytic conversion of the 14 to the 11 kDa form (see arrow in Figure 3b). If this product is taken into account, the combined half-life for both 'long' forms is virtually identical to that of stable bound peptides (see dotted line in Figure 3c).

Some peptides behave anomalously in SDS–PAGE, not migrating according to their molecular weight (Schägger and von Jagow, 1987). In our analysis, the iodinated HEL 48–61 peptide migrated with an apparent mol. wt of ~6 kDa, whereas its true mol. wt was <2 kDa. We therefore examined the molecular size of the two principal SDS-stable processing products by an alternative method. To obtain a large quantity of radiolabeled material on A^k, C3F6 cells were pulsed with *HEL for 1 h and then chased for 3 h. SDS-stable A^k complexes were isolated and the radiolabeled material was dissociated and eluted into a small volume of a denaturing buffer (see Materials and methods). This sample was chromatographed on an FPLC-coupled Superose 12 gel filtration column equilibrated in the same buffer. Radiolabeled material eluted from the column at two positions matching the elution positions of denatured *HEL and [^{125}I]HEL 48-61 peptide (Figure 3e and f). The two principal SDS-stable processing products of *HEL in C3F6 were thus confirmed to be small peptides (<2 kDa) and *HEL (14.3 kDa).

*Intracellular formation and surface delivery of SDS-stable *HEL processing products*

To distinguish between surface and intracellular events in the MHC class II association and processing of *HEL,



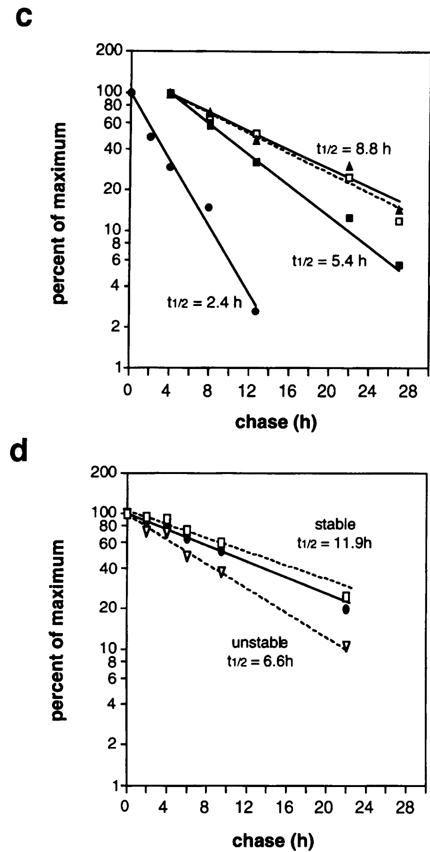


Fig. 3. Products and kinetics of the cellular processing of *HEL. (**a**) C3F6 cells were pulsed with *HEL for 10 min and then chased for the times indicated. A^k-bound material was analyzed on 15% Tris-glycine SDS gels without boiling of the samples. Lane C denotes a control immunoprecipitation of a 1 h chase sample with the mAb P4H5 (anti-Ii). (**b**) SDS-stable complexes (see a) were isolated, dissociated and their constituents electrophoresed in Tris-tricine SDS gels. *HEL and [^{125}I]HEL 48–61 were run as standards (right lanes). Note that [^{125}I]HEL 48–61 migrated more slowly than predicted by its molecular weight. Arrowhead: ~11 kDa degradation product of *HEL. (**c**) Half-life of SDS-stable and -unstable complexes. C3F6 cells were pulsed with *HEL for 30 min, chased for the indicated times and the three principal A^k-bound products (see a and b) quantified. Time points before the maximum (= 100%) were omitted. The dotted line represents the combined half-life of SDS-stable *HEL and the ~11 kDa degradation product. SDS-unstable bound *HEL (●), SDS-stable bound *HEL (■), sum of SDS-stable bound *HEL and ~11 kDa degradation product (□), SDS-stable bound *HEL peptides (▲). (**d**) Half-life of A^k molecules in C3F6 cells. A^k from surface-iodinated C3F6 cells was isolated at various times of re-culture, analyzed by SDS–PAGE and quantified. Depicted values are the means of duplicates. SDS-unstable A^k (△), SDS-stable A^k (□), total A^k (●). (**e**) Calibration of a Superose 12 gel filtration column equilibrated in 0.5 M Tris, pH 7.0, 8 M urea, 0.1% SDS, 10 mM DTT (buffer E). The markers were *HEL (□) and [^{125}I]HEL 48–61 (◇), both of which were pre-treated by boiling in SDS sample buffer. (**f**) Superose 12 gel filtration chromatography of material contained in SDS-stable A^k complexes. A^k molecules were immunoprecipitated from C3F6 cells that had been pulsed with *HEL for 1 h and then chased for 3 h. SDS-stable A^k complexes were isolated from SDS gels, boiled and the constituents eluted in buffer E. The material was chromatographed as in (e). Note that the ratio of *HEL to peptide(s) was shifted towards *HEL due to the long pulse. The main peak co-eluted with *HEL and the minor peak with [^{125}I]HEL 48–61.

the endocytosis of C3F6 cells was inhibited by energy depletion (Schmid and Carter, 1990). In this condition, very little radioactivity was associated with SDS-stable complexes and this minimal amount did not increase during the chase (Figure 4a and b). By contrast, in endocytosis-inhibited cells, SDS-unstable *HEL–A^k com-

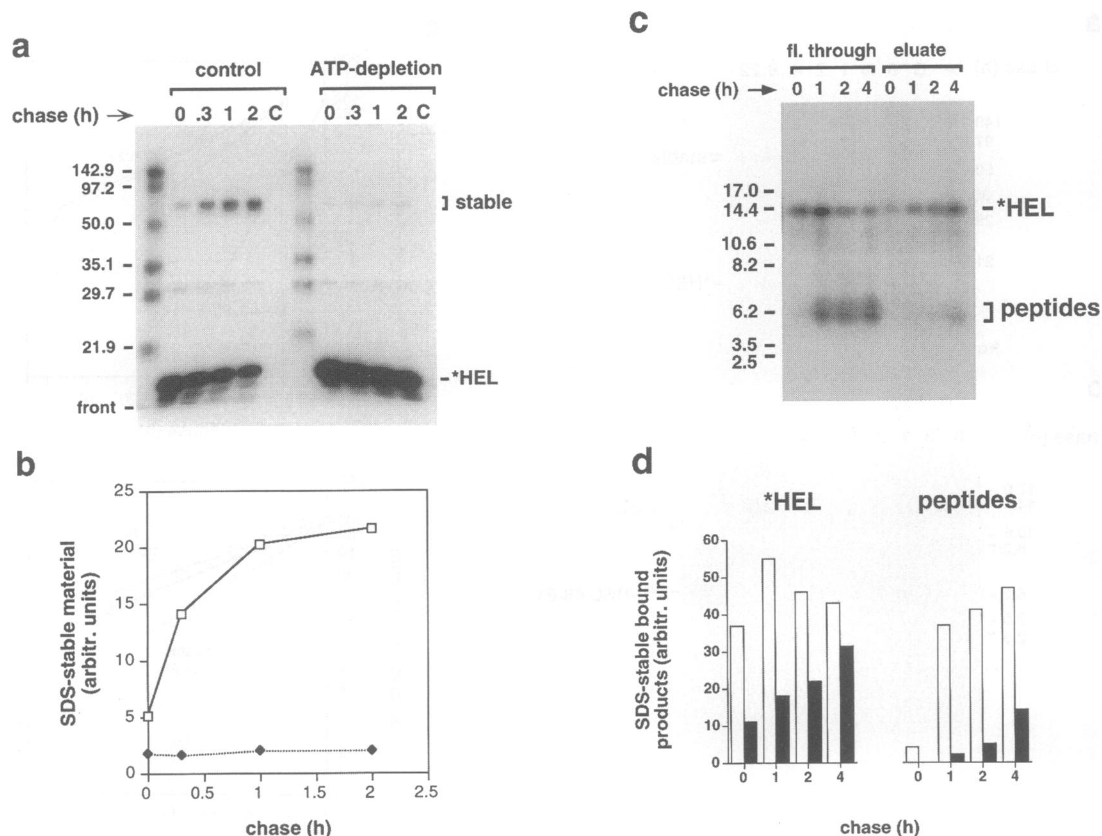


Fig. 4. Intracellular formation and surface delivery of stable *HEL processing products. (**a**) Endocytosis inhibition experiments. C3F6 cells were ATP-depleted, pulsed with *HEL for 10 min and then chased for the times indicated in the presence of the inhibitors. Untreated cells were handled identically in medium without inhibitors (control). A^k-bound material was analyzed on 15% Tris-glycine SDS gels. Lane C denotes non-specific immunoprecipitation at 2 h chase using the irrelevant mAb 30-5-7. (**b**) Quantification of SDS-stable complexes depicted in (a). Untreated cells (□), ATP-depleted cells (◆). (**c**) Surface delivery of SDS-stable *HEL processing products. C3F6 cell were pulsed with *HEL for 20 min, chased for the indicated times and then surface-biotinylated on ice. After lysis, biotinylated material (eluate) was separated from non-biotinylated material (flow through) by monomeric avidin–Sepharose. A^k was immunoprecipitated from the respective pools and the material in SDS-stable A^k complexes was analyzed by second dimension Tris-tricine SDS–PAGE. (**d**) Quantification of SDS-stable bound *HEL processing products depicted in (c). Open bars: total amount (sum of 'flow through' and 'eluate'); filled bars: surface-biotinylated complexes ('eluate').

plexes were more abundant and persistent than in untreated control cells (Figure 4a). Therefore, both the generation of SDS-stable processing products and the elimination of SDS-unstable *HEL–A^k complexes occurs after endocytosis into intracellular compartments.

We developed a surface delivery assay to determine if SDS-stable A^k complexes containing *HEL peptides or *HEL were transported from their intracellular sites of generation to the plasma membrane. C3F6 cells were surface biotinylated on ice and the biotinylated proteins were retrieved from a lysate by monomeric avidin–Sepharose (see Materials and methods). The interaction of biotin and monomeric avidin is reversible, and bound ligands can be eluted with 1 mg/ml biotin. No intracellular forms of A^k metabolically labeled by a brief pulse of [^{35}S]methionine were retrieved by this procedure (data not shown). After a 20 min pulse with *HEL and a chase from 0 to 4 h, both SDS-stable processing products became exposed to the biotinylation reagent and therefore had arrived at the cell surface (Figure 4c and d). Biotinylated stable complexes of A^k and intact *HEL increased steadily during the chase, whereas biotinylated stable peptide–A^k complexes only became prominent at late chase times. A fraction of *HEL-containing stable complexes was already accessible to biotinylation after the 20 min pulse, suggesting a rapid recycling from early endocytic compartments.

Differential effects of chloroquine and leupeptin on SDS-stable processing products

The requirement for internalization to produce SDS-stable complexes containing full-length *HEL or peptides prompted us to investigate the involvement of low pH endocytic organelles. Classical inhibitors of the function of acidic organelles are lysosomotropic drugs like chloroquine, primaquine and ammonium salts (reviewed by Mellman *et al.*, 1986) or membrane-impermeant protease inhibitors like leupeptin (Blum and Cresswell, 1988). We pre-treated C3F6 cells with leupeptin (0.5 mM) or chloroquine (0.5 mg/ml) and then performed a standard pulse–chase experiment with *HEL in the presence of the inhibitors (Figure 5a–d). The amount of SDS-stable complexes was reduced by ~90% in chloroquine-treated cells and by ~50% in leupeptin-treated cells. By contrast, the amount of SDS-unstable bound *HEL was comparable in all three sets (Figure 5a).

A second dimension analysis of the stable complexes revealed that the formation of peptides was abolished completely by the chloroquine treatment and reduced by ~70% in leupeptin-treated cells. The decrease in the stable

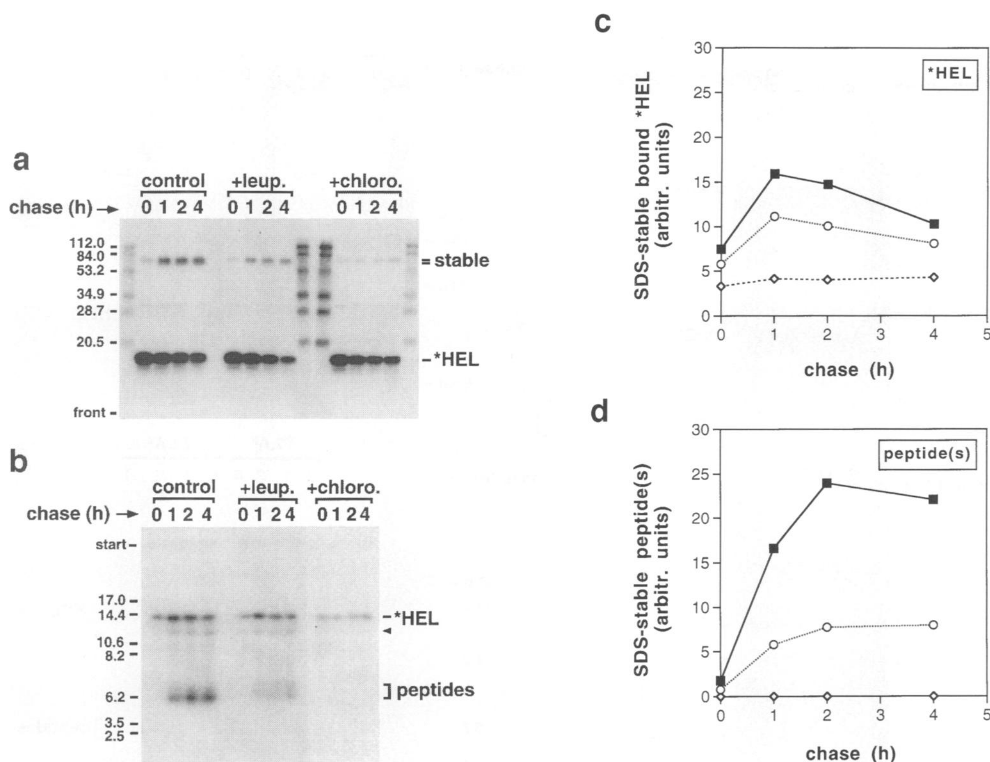


Fig. 5. Effect of chloroquine and leupeptin on A^k-bound *HEL processing products. C3F6 cells were pre-incubated with leupeptin (0.5 mg/ml) or chloroquine (0.5 mM) or without any addition (control) for 1 h at 37°C. They were then pulsed with *HEL for 10 min and chased for the times indicated with or without (control) the respective inhibitor. (**a**) A^k and the associated processing products were analyzed by Tris-glycine SDS–PAGE. (**b**) Material bound in SDS-stable complexes was dissociated and separated by Tris-tricine SDS–PAGE. Arrowhead: ~11 kDa degradation product of *HEL. (**c** and **d**) Material contained in SDS-stable complexes (see b) was quantified by densitometry. Note that the formation of SDS-stable bound peptide was completely abrogated in the presence of chloroquine, whereas a small amount of stable bound *HEL was still detectable. Control (■), leupeptin-treated cells (○), chloroquine-treated cells (◇).

bound peptides in leupeptin-treated cells was due mainly to a reduction of the lowermost band of the peptide doublet (Figure 5b). Both peptides also appear to migrate with slightly lower mobility relative to the control. The latter observations might indicate that leupeptin interferes with the proteolytic processing of *HEL and reduces the availability of peptides to loading-competent A^k molecules. Leupeptin had a minimal effect on the formation of stable bound *HEL, yet these complexes were reduced ~75% by chloroquine treatment. Thus, the formation of the large majority of stable *HEL–A^k complexes depended on endocytic organelles with a low luminal pH.

*Stable A^k association of *HEL on an alternative loading pathway*

To examine the intracellular routes involved in the formation of SDS-stable A^k complexes containing intact *HEL or peptide(s), we inhibited the established MHC class II loading pathway which required newly synthesized MHC class II and an HLA-DM type of molecule (Davidson *et al.*, 1991; Pinet *et al.*, 1994). For depletion of newly synthesized A^k, C3F6 cells were pre-incubated with cycloheximide for 0–6.5 h, then pulsed briefly with *HEL and chased for 2 h in the presence of the drug (Figure 6a and b). A pre-incubation with cycloheximide for >2.5 h abolished the formation of SDS-stable peptide–A^k complexes, but not of SDS-stable complexes containing *HEL. Minor SDS-stable bound products migrating at 11 kDa were also unaffected by the depletion procedure, supporting the notion that these were formed in the same pathway.

A similar result was obtained when the processing and loading of *HEL by the HLA-DM-negative cell line T2.A^k (Riberdy and Cresswell, 1992) was compared with its parent line T1.A^k (Figure 6c and d): both cell lines expressed similar numbers of A^k molecules at their cell surface (data not shown) and bound amounts of *HEL comparable with SDS-unstable complexes (Figure 6c). However, they differed in their production of SDS-stable products, with T2.A^k reaching only 30% of the level of T1.A^k. A second dimension analysis revealed that *HEL peptides were only formed efficiently in T1.A^k (at levels comparable with C3F6, see Figure 5b, control), whereas T2.A^k was almost entirely defective in this process (only trace amounts of peptides were visible at later chase times, see Figure 6d). All three cell lines formed SDS-stable A^k complexes containing *HEL and the 11 kDa product (Figures 6d and 5b, control). In T1.A^k, the 11 kDa form was more prominent than in the other two cell lines, which might indicate a slightly higher proteolytic capability in the binding or trafficking compartments for stable bound *HEL. In summary, the formation of SDS-stable complexes of *HEL and A^k was not dependent on the presence of newly synthesized A^k or HLA-DM, which were, however, required for the SDS-stable association of *HEL-derived peptides. This suggested that the two loading processes were distinct from each other and that they involved two separate cellular pathways.

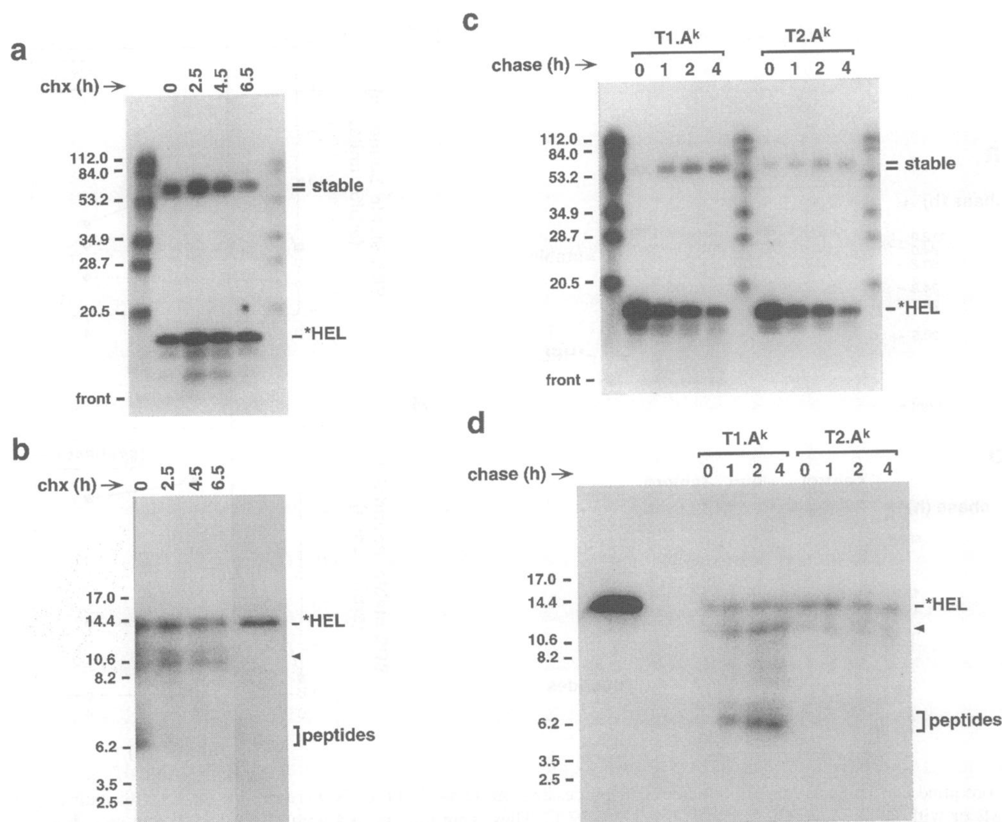


Fig. 6. Two distinct pathways give rise to the SDS-stable loading of peptide or full-length *HEL. (**a**) C3F6 cells were depleted of newly synthesized MHC class II by pre-incubation with cycloheximide (chx) for 0–6.5 h. Subsequently they were pulsed with *HEL and then chased for 2 h in the presence of the drug. A^k-bound products were analyzed in 15% Tris-glycine SDS gels. (**b**) Material from SDS-stable A^k complexes was isolated and electrophoresed on Tris-tricine SDS gels. *HEL was run in a separate lane of the same gel (right). Arrowhead: ~11 kDa degradation product of *HEL. (**c**) T1.A^k and T2.A^k cells were pulsed with *HEL for 10 min and then chased from 0 to 4 h. A^k-bound material was electrophoresed on 15% Tris-glycine SDS gels and visualized by autoradiography. (**d**) Analysis of *HEL-derived products in SDS-stable A^k complexes from T1.A^k and T2.A^k in Tris-tricine SDS gels. *HEL was run in a separate lane on the same gel (left). Arrowhead: ~11 kDa degradation product of *HEL.

*Free *HEL as the precursor for SDS-stable *HEL–A^k complexes*

We examined if SDS-stable *HEL–A^k complexes were formed by conversion of SDS-unstable *HEL–A^k derived from the plasma membrane or from internalized, free *HEL molecules. Cycloheximide-treated C3F6 cells were pulsed briefly with *HEL (2 min) to allow the formation of SDS-unstable *HEL–A^k complexes at the cell surface along with some fluid phase endocytosis of free *HEL. During the brief pulse, only a minimal amount of SDS-stable *HEL–A^k complexes was formed, which increased dramatically after a 2 h chase (Figure 7a and b, control). Addition of HEL 46–61 peptide or ciHEL to this pulse and a consecutive 2 h chase resulted in a competition of SDS-unstable and SDS-stable *HEL–A^k complexes by 70 and 100%, respectively (Figure 7a). A control peptide which does not bind to A^k (HEL 1–18) did not have any effect. Having established specificity and efficiency, we then added the competitors only during the chase, i.e. after the formation of SDS-unstable *HEL–A^k complexes. Under this condition, the formation of SDS-stable *HEL–A^k complexes was competed by the HEL 46–61 peptide and ciHEL but not by HEL 1–18 (Figure 7b). Note that the degree of competition with HEL 46–61 was again slightly lower than with ciHEL. These results show that SDS-stable *HEL–A^k complexes were formed from free *HEL molecules in a process competed by molecules endocytosed shortly after. The results from the cellular studies are summarized in a model depicted in Figure 8.

Discussion

Three main findings emerge from our studies tracing the fate of partially folded, iodinated lysozyme (*HEL) in an APC. First, our results demonstrate that a partially folded protein can spontaneously form SDS-unstable complexes with MHC class II molecules; however, these complexes are cleared rapidly by the APC. Second, we have identified an endosomal process that affects *HEL and results in the formation of SDS-stable, long-lived *HEL–A^k complexes. Lastly, we contrast this novel way of antigen processing with the catabolism of *HEL to peptides that bind to A^k in a different cellular event.

Partially folded *HEL bound to A^k like a typical MHC class II binding peptide: its affinity was identical to that of the HEL 48–61 peptide and also the kinetics of its interaction were comparable (Roof *et al.*, 1990). This is in contrast to the poor binding of native HEL. Other studies have demonstrated indirectly an interaction between denatured proteins or proteins with conformational flexible regions and MHC class II molecules by T-cell assays or by competitive binding experiments (Allen and Unanue, 1984; Lee *et al.*, 1988; Sette *et al.*, 1989). However, our studies present for the first time quantitative

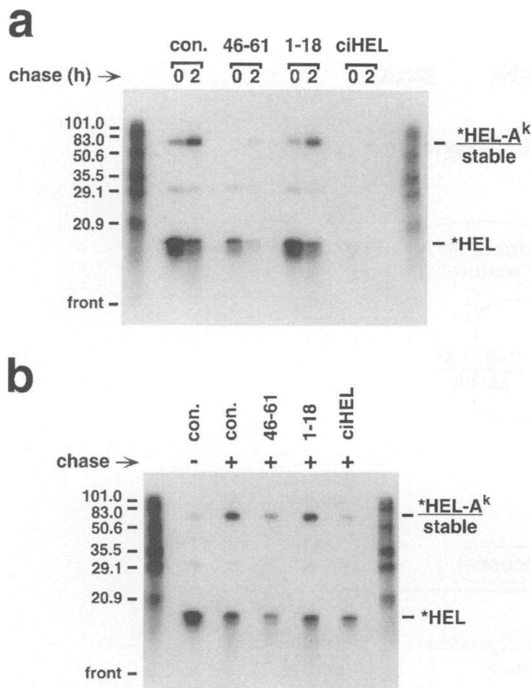


Fig. 7. The formation of SDS-stable *HEL–A^k complexes can be competed by HEL 46–61 peptide and ciHEL. (**a**) C3F6 cells were pre-treated with cycloheximide (10 μg/ml) for 3.5 h, pulsed with *HEL (1 μM) for 2 min, washed, and chased in the absence of *HEL for 1 h. Both pulse and chase incubations contained either no competitor (con), HEL 46–61, HEL 1–18 or ciHEL at 100 μM, respectively. Note that HEL 46–61 peptide competed the association of *HEL with cellular A^k by ~70%, whereas ciHEL was 100% effective. (**b**) C3F6 cells were pre-treated as in (a), then pulsed with *HEL for 2 min in the absence of any competitor, washed, and chased in the presence or absence of competitors as in (a). Note that only a very small amount of SDS-stable *HEL–A^k complexes was formed during the 2 min pulse. In the presence of HEL 46–61 or ciHEL in the chase, the SDS-stable *HEL–A^k complexes did not increase substantially over the initial amount.

data obtained in direct binding experiments. It is very likely that *HEL binds to A^k via the core segment of HEL 52–61, since its binding affinity was identical to HEL 48–61 (the strongest A^k binding peptide in HEL) and the *HEL–A^k complexes were recognized by 3A9 T cells.

We observed two differences in the interaction of *HEL and HEL 48–61 peptide with A^k: (i) the pH optimum for *HEL binding to A^k was 6.0–6.5, whereas it has been reported to be 5.5–6.0 for HEL 48–61 (Harding *et al.*, 1991); (ii) under all conditions tested so far, *HEL only formed SDS-unstable complexes with purified A^k, whereas HEL 48–61 formed exclusively stable complexes (Nelson *et al.*, 1993). The interaction of *HEL with A^k is therefore different from that of the HEL 48–61 peptide. SDS-stable complexes of A^k molecules and endoplasmic reticulum polypeptides have been detected recently in transfected HeLa cells (Busch *et al.*, 1996). These complexes were only formed in the absence of invariant chain and most likely involved polypeptides with a high degree of unfolding. This situation may not be the case in *HEL, which showed a Stokes' radius similar to the native protein. In a recent paper, evidence for an SDS-unstable kinetic intermediate in the SDS-stable binding of the influenza hemagglutinin peptide HA 306–318 to HLA-DR1 was provided (Sadegh-Nasseri *et al.*, 1994). The SDS-unstable complex of *HEL with A^k might be a 'frozen' kinetic intermediate which requires a much higher activation energy than the HA peptide in order to be converted to an SDS-stable state.

In an APC, the SDS-unstable *HEL–A^k complexes were formed mainly at the plasma membrane and cleared rapidly after their endocytosis. At present, we do not know the cellular mechanism that caused the disappearance of plasma membrane-derived, SDS-unstable *HEL–A^k complexes. This process seems to be independent of a low pH endocytic compartment or HLA-DM, since chloroquine-treated C3F6 cells and HLA-DM-negative T2.A^k cells showed no difference in the handling of unstable bound *HEL relative to controls. We also ruled out the possibility that there is a direct conversion of SDS-unstable *HEL–A^k complexes to SDS-stable ones, with *HEL staying bound to the same A^k molecule all the time. However, it is possible that SDS-unstable A^k complexes contribute their cargo to one or the other loading process after dissociation or degradation.

Our second finding provides evidence for a novel strategy of antigen processing which gives rise to SDS-stable, long-lived complexes of full-length *HEL and A^k. The formation of SDS-stable *HEL–A^k complexes required internalization of *HEL, mature (endosomal) A^k molecules and a low pH environment. It proceeded in the absence of HLA-DM or proteolysis and resulted in the surface expression of long-lived complexes. Thus SDS-stable *HEL–A^k complexes are not formed in the established antigen processing pathway (reviewed by Germain and Margulies, 1993; Cresswell, 1994; Wolf and Ploegh, 1995), but on an alternative route which, in some aspects, resembles the pathway recently described by Long's laboratory (Pinet *et al.*, 1994, 1995). In those experiments, the presentation of influenza hemagglutinin and myelin basic protein on HLA-DR1 was dependent on recycling, mature HLA-DR1 and did not require invariant chain or HLA-DM. A similar pathway may also be followed by exogenous protein antigens that are presented in the absence of invariant chain (Nadimi *et al.*, 1991) or HLA-DM (Brooks *et al.*, 1994).

It is likely that the formation of SDS-stable *HEL–A^k complexes took place in early endosomes or recycling endosomes (Gruenberg and Maxfield, 1995), since it occurred early after a pulse of *HEL, and the complexes, once formed, were transported rapidly to the cell surface. We also observed slow and only partial proteolysis of the SDS-stable *HEL–A^k complexes (to an 11 kDa product), in agreement with the low proteolytic potential of early endosomes or recycling endosomes. Interestingly, targeting of immune complexes to early endosomes has been shown recently to result in a rapid, cycloheximide-resistant, MHC class II-restricted presentation, although the bulk of the antigen was proteolysed only slowly (Bonnerot *et al.*, 1995). The alternative processing pathway may thus function independently of proteolytic fragmentation and may generally give rise to extended antigens bound to MHC class II. Polypeptides in the range of 2–10 kDa have been isolated occasionally from MHC class II molecules (Buus *et al.*, 1988; Davidson *et al.*, 1991; Srinivasan *et al.*, 1991), but their relationship to well characterized peptides <3 kDa (reviewed in Engelhard, 1994) remains unclear.

What process allows the APC to generate SDS-stable

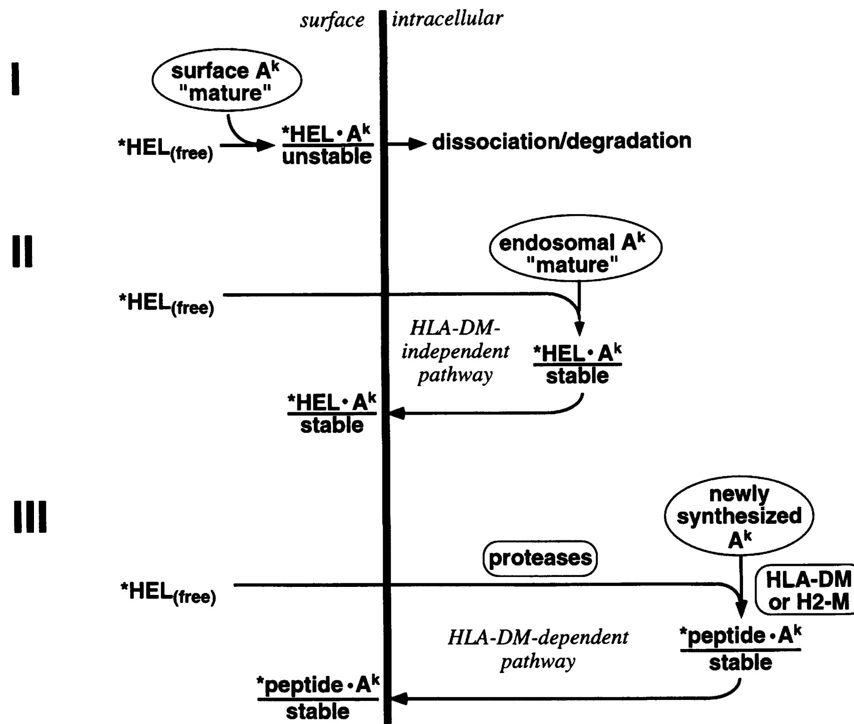


Fig. 8. Model for the cellular handling of *HEL.

*HEL–A^k complexes in endosomes? Our *in vitro* experiments showed that neither conditions like low pH nor the presence of reducing agents were sufficient for the formation of SDS-stable complexes. Likewise, the binding of *HEL to plasma membrane A^k did not result in the formation of SDS-stable complexes. We suggest that free *HEL molecules internalized into endosomes were stabilized in a more unfolded state that allows for an SDS-stable interaction with resident (mature) A^k. Such a change may be supported by molecular chaperones, like heat shock proteins (hsps), disulfide isomerases or prolylisomerases, which are known to bind partially folded or misfolded proteins (Gething and Sambrook, 1992). Hsp70 proteins were proposed to be involved in the processing of exogenous antigens and their MHC class II-restricted presentation (VanBuskirk *et al.*, 1989; Manara *et al.*, 1993), but no member of this protein family with a role in these events has been identified (Pierce, 1994). Our approach opens up the possibility of reconstituting *HEL processing and A^k loading *in vitro* and thus may enable us to define relevant cofactors.

Finally, our studies identified SDS-stable bound peptides as the other major radioiodine-containing processing products of *HEL. Most probably these were a nested set of peptides centered around HEL 48–61 (Nelson *et al.*, 1992), which is the immunodominant epitope of HEL presented on A^k (Allen *et al.*, 1984). Their formation followed an established pathway (reviewed by Germain and Margulies, 1993; Cresswell, 1994; Roche, 1995; Wolf and Ploegh, 1995), requiring internalization of *HEL into acidified, proteolytic compartments where the degradation of *HEL to peptides and their HLA-DM-facilitated association with newly synthesized A^k molecules took place. The almost complete lack of SDS-stable *HEL peptides in the HLA-DM-negative cell line T2-A^k indicates a strict dependence of peptide loading on HLA-DM even in those cases in which the invariant chain peptide CLIP interacts only weakly with the MHC class II molecule (Bangia and Watts, 1995; Liang *et al.*, 1995).

Materials and methods

Chemical reagents, monoclonal antibodies and cell lines

Guanidinium hydrochloride and urea (both electrophoresis grade) were purchased from Fisher Scientific (Pittsburgh, PA). Carrier-free ^{125}I was bought from Amersham (Arlington Heights, IL). *N*-hydroxysulfosuccinimidyl-LC-biotin (NHSS-LC-biotin) was obtained from Pierce (Rockford, IL). Dulbecco's modified Eagle's medium (DMEM) was from GIBCO BRL (Gaithersburg, MD), fetal calf serum (FCS) was from Hyclone (Logan, UT), Hank's buffered salt solution (HBSS) was from BioWhittaker (Walkersville, MD). All other reagents were analytical grade and were obtained from Sigma (St Louis, MO). The monoclonal antibodies (mAbs) 40F (anti-A^k, Pierres *et al.*, 1981) and P4H5 (anti-li, Mehringer *et al.*, 1991) were affinity purified on protein A–Sepharose according to standard procedures. mAb 30-5-7 (anti-L^d, Hansen *et al.*, 1988) was used as ascites. The cell lines M12.C3.F6 (C3F6; Nabavi *et al.*, 1989), 3A9 (Allen *et al.*, 1984), T1.A^k (Riberdy and Cresswell, 1992) and T2.A^k (Riberdy and Cresswell, 1992) were cultured in DMEM supplemented with 5% FCS (D5F). The interleukin-2 (IL-2)-dependent line CTLL-2 was propagated in D5F supplemented with 20 U/ml of recombinant IL-2.

*Preparation of *HEL*

*HEL was prepared by denaturing HEL (Sigma) in 6 M guanidinium hydrochloride (dissolved in 0.5 M Na/PO$_4$, pH 7) without reduction. Unfolded HEL was iodinated according to the chloramine-T procedure (McConahey and Dixon, 1980). Typically, 1 mCi of ^{125}I was used per 6 nmol of HEL. The reaction was stopped by *p*-hydroxyphenylacetic acid (10 mg/ml final) and NaI (10 mM final). Refolding was induced by rapid 10-fold dilution (Radford *et al.*, 1992) followed by desalting on a PD-10 column (Pharmacia) equilibrated with phosphate-buffered saline (PBS). *HEL was stored at 4°C for up to 1 week without loss of its properties.

*Assay for binding of *HEL to purified A^k*

A^k was purified from T2.A^k cells by affinity chromatography (Luescher and Unanue, 1990b) and by gel filtration on an HPLC-coupled Bio-Sil

SEC 250-5 column (Bio-Rad, Melville, USA). Binding assays consisted of 10 pmol of purified A^k and 15 pmol of *HEL in 33 µl of 0.5% Triton X-100, 200 mM 2-[*N*-morpholino]ethanesulfonic acid (MES), pH 5.5–6.5, acetate, pH 4.0–5.0 or *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), pH 7.0. No reducing agents were present unless specified. We also used mixtures of the buffers to rule out pH-independent effects. Standard incubations were 2 h at 37°C. For stop and neutralization, 300 µl of ice-cold 0.2 M $NaPO_4$, 0.5% Triton X-100, pH 7.0 was added. Binding or dissociation of *HEL at 4°C was negligible. Complexes were immunoprecipitated with 20 µg of the anti-A^k mAb 40F and eluted from the immunomatrix as published previously (Nelson *et al.*, 1994). Quantification was either by γ-counting, or by densitometry of autoradiographs after electrophoresis in 15% Tris-glycine SDS gels. Control precipitations of samples incubated without A^k or using irrelevant antibody gave <2% of the signal of the specific precipitation of an A^k-containing sample.

Pulse–chase *HEL uptake protocol

A total of 5×10^7 APCs (C3F6, T1.A^k, T2.A^k) were pulsed for up to 1 h at 37°C with 3–6 nmol of *HEL in 2 ml of D5F. After the pulse, the cells were chilled in cold HBSS, washed and then chased in D5F at 37°C or kept on ice (pulse only). The cells were washed at 4°C again and lysed on ice in 1 ml of TBST (50 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100, pH 7.5) supplemented with 5.1 µg/ml of leupeptin, 1 µg/ml of E-64, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM benzamidine and 2 mM iodoacetamide ($TBST^+$). A^k complexes were immunoprecipitated from the lysates using 10 µg/ml of mAb as described (Nelson *et al.*, 1994). Irrelevant antibodies precipitated <5% of the radioactivity precipitated by 40F. A^k and bound ligands were eluted from the immunomatrices by incubation with SDS sample buffer for 1 h at 37°C. The eluted material was first analyzed by SDS–PAGE on standard 15% Tris-glycine SDS gels. Radioactive material contained in SDS-stable complexes was analyzed further by second dimension electrophoresis in Tris-tricine SDS gels or by gel filtration on Superose 12 (see below).

Cellular assays with inhibitors

For endocytosis inhibition, 5×10^7 C3F6 cells were pre-treated for 10 min at 37°C with 5 mM NaN_3, 2 mM NaF and 5 mM 2-deoxyglucose (Schmid and Carter, 1990) in incubation buffer (PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.2% FCS). Subsequently, the cells were pulsed with 3 nmol of *HEL in 2 ml of incubation buffer plus inhibitors for 10 min at 37°C. After three washes with cold PBS plus inhibitors, they were chased in incubation buffer with inhibitors at 37°C or left on ice (pulse only). The cells were washed with cold PBS plus inhibitors and processed further as described above. The effect of leupeptin and chloroquine was investigated by pre-treating 5×10^7 C3F6 cells for 1 h at 37°C with 0.5 mg/ml leupeptin or 0.5 mM chloroquine in D5F. Subsequently, a standard pulse–chase experiment was performed with the inhibitors present in all the incubations. Chloroquine was included in all the washes as well. Newly synthesized A^k was depleted by pre-incubating 5×10^7 C3F6 cells with 10 µg/ml cycloheximide in D5F for 2.5–6.5 h at 37°C. Then the cells were pulsed for 10 min with 3 nmol of *HEL, washed and chased for 2 h at 37°C, all in the presence of the inhibitor. Further processing was as described in the uptake protocol.

Surface delivery assay

C3F6 cells (3×10^7) were pulsed with 6 nmol of *HEL in 2 ml of D5F for 20 min at 37°C, washed and chased in D5F for 0–4 h. Subsequently, the cells were washed with HBSS at 4°C, resuspended in 400 µl of 0.1 M $NaPO_4$, pH 7.4 (buffer B), and kept on ice. To this suspension, 20 µl aliquots of NHSS-LC-biotin (dissolved in buffer B at 5 mg/ml) were added three times over a period of 30 min. The reaction was stopped by washing the cells in ice-cold D5F. After another wash in ice-cold HBSS, the cells were lysed as specified in the *HEL uptake protocol. The pre-cleared lysate (1 ml) was applied onto a 1.3 ml column of monomeric avidin–Sepharose (Sigma), which had been recycled according to the manufacturer's instructions and equilibrated in $TBST^+$. The column was washed with 5 ml of $TBST^+$ and the flow-through of the lysate and the wash were combined. Then the column was eluted with 6 ml of $TBST^+$ containing 1 mg/ml D-biotin. All steps were performed at 4°C. From both the flow-through and the eluate, A^k was immunoprecipitated using 5 µg/ml of 40F. Further processing was as described in the uptake protocol.

Analysis of SDS-stable *HEL processing products

SDS-stable complexes of A^k were isolated after SDS–PAGE from the corresponding region of the gel (defined by pre-stained electrophoresis markers). For second dimension electrophoretic analysis, the gel slices were equilibrated in Tris-tricine sample buffer with reduced SDS (0.1%, Davidson *et al.*, 1991). After the removal of the buffer, the gel slices were heated to 100°C for 5 min to dissociate the A^k complexes, sealed on top of a Tris-tricine SDS gel with 1% agarose (in sample buffer with 0.1% SDS) and electrophoresed as described (Schägger and von Jagow, 1987). For gel filtration analysis of material contained in SDS-stable complexes, the corresponding gel pieces were heated to 100°C for 5 min without prior equilibration. The dissociated products were eluted for several hours at 4°C by incubation with 250 µl of 0.5 M Tris, pH 7.0, 8 M urea, 0.1% SDS, 10 mM dithiothreitol (buffer E) supplemented with 5.1 µg/ml of leupeptin, 1 µg/ml E-64, 0.1 mM PMSF, 0.5 mM benzamidine and 2 mM iodoacetamide. The sample was then ultracentrifuged (100 000 *g*, 15 min), the supernatant retrieved and the procedure repeated once. More than 90% of the radioactivity in the gel slice was recovered in the combined supernatant (500 µl). This sample was applied onto an FPLC-coupled Superose 12 column (HR 30/10, Pharmacia, Piscataway, NJ) equilibrated in buffer E. Fractions of 500 µl were collected and evaluated by γ-counting.

T-cell assay on chemically fixed cells

C3F6 cells in DMEM were fixed for 15 min at room temperature with 1% paraformaldehyde. After quenching (0.4 M lysine, 10 min at room temperature), the cells were washed with DMEM and resuspended in D5F. In a 96-well plate, 5×10^4 fixed C3F6 cells were titrated with HEL 48–61 peptide, native HEL or ciHEL. A total of 1×10^5 3A9 cells were added per well and the plate was incubated for 16 h at 37°C, 5% CO_2. The supernatants were harvested and assayed for IL-2 with the IL-2-dependent cell line CTLL-2.

Acknowledgements

We thank Peter Cresswell (Yale University) for generously providing T1.A^k and T2.A^k, Ted Hansen (Washington University) for the monoclonal antibody 30-5-7, Shirley Petzold and Brian Deck for technical assistance and Ernst Ungewickell, Ruth Knorr, Stuart Kornfeld, Paul Allen, Nick Viner and Chris Nelson for discussions and comments on the manuscript. This work was supported by grants from the NIH and a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft (R.L.).

References

Allen,P.M. and Unanue,E.R. (1984) Differential requirements for antigen processing by macrophages for lysozyme-specific T cell hybridomas. *J. Immunol.*, **132**, 1077–1079.

Allen,P.M., Strydom,D.J. and Unanue,E.R. (1984) Processing of lysozyme by macrophages: identification of the determinant recognized by two T-cell hybridomas. *Proc. Natl Acad. Sci. USA*, **81**, 2489–2493.

Amigorena,S., Webster,P., Drake,J., Newcomb,J.R., Cresswell,P. and Mellman,I. (1995) Invariant chain cleavage and peptide loading in major histocompatibility complex class II vesicles. *J. Exp. Med.*, **181**, 1729–1741.

Bangia,N. and Watts,C. (1995) Evidence for invariant chain 85–101 (CLIP) binding in the antigen binding site of MHC class II molecules. *Int. Immunol.*, **7**, 1585–1591.

Blum,J.S. and Cresswell,P. (1988) Role for intracellular proteases in the processing and transport of class II HLA antigens. *Proc. Natl Acad. Sci. USA*, **85**, 3975–3979.

Bonnerot,C., Lankar,D., Hanau,D., Spehner,D., Davoust,J., Salamero,J. and Fridman,W.H. (1995) Role of B cell receptor Igα and Igβ subunits in MHC class II-restricted antigen presentation. *Immunity*, **3**, 335–347.

Brooks,A.G., Campbell,P.L., Reynolds,P., Gautam,A.M. and McCluskey,J. (1994) Antigen presentation and assembly by mouse I-A^k class II molecules in human APC containing deleted or mutated HLA DM genes. *J. Immunol.*, **153**, 5382–5392.

Brown,J.H., Jardetzky,T.S., Gorga,J.C., Stern,L.J., Urban,R.G., Strominger,J.L. and Wiley,D.C. (1993) Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. *Nature*, **364**, 33–39.

Busch,R., Cloutier,I., Sékaly,R.-P. and Hämmerling,G.J. (1996) Invariant chain protects class II histocompatibility antigens from binding intact polypeptides in the endoplasmic reticulum. *EMBO J.*, **15**, 418–428.

Buus,S., Sette,A., Colon,S.M. and Grey,H.M. (1988) Autologous peptides constitutively occupy the antigen binding site on Ia. *Science*, **242**, 1045–1047.

Cresswell,P. (1994) Assembly, transport, and function of MHC class II molecules. *Annu. Rev. Immunol.*, **12**, 259–293.

Davidson,H.W., Reid,P.A., Lanzavecchia,A. and Watts,C. (1991) Processed antigen binds to newly synthesized MHC class II molecules in antigen-specific B lymphocytes. *Cell*, **67**, 105–116.

Denzin,L.K. and Cresswell,P. (1995) HLA-DM induces CLIP dissociation from MHC class II αβ dimers and facilitates peptide loading. *Cell*, **82**, 155–165.

Denzin,L.K., Robbins,N.F., Carboy-Newcomb,C. and Cresswell,P. (1994) Assembly and intracellular transport of HLA-DM and correction of the class II antigen-processing defect in T2 cells. *Immunity*, **1**, 595–606.

Engelhard,V.H. (1994) Structure of peptides associated with class I and class II MHC molecules. *Annu. Rev. Immunol.*, **12**, 181–207.

Fling,S.P., Arp,P. and Pious,D. (1994) HLA-DMA and -DMB genes are both required for MHC class II/peptide complex formation in antigen-presenting cells. *Nature*, **368**, 554–558.

Germain,R.N. and Margulies,D.H. (1993) The biochemistry and cell biology of antigen processing and presentation. *Annu. Rev. Immunol.*, **11**, 403–450.

Gething,M.J. and Sambrook,J. (1992) Protein folding in the cell. *Nature*, **355**, 33–45.

Gruenberg,J. and Maxfield,F.R. (1995) Membrane transport in the endocytic pathway. *Curr. Opin. Cell Biol.*, **7**, 552–563.

Hansen,T.H., Myers,N.B. and Lee,D.R. (1988) Studies of two antigenic forms of L^d with disparate β 2-microglobulin (β 2m) associations suggests that β 2m facilitates the folding of the α 1 and α 2 domains during the *de novo* synthesis. *J. Immunol.*, **140**, 3522–3527.

Harding,C.V., Roof,R.W., Allen,P.M. and Unanue,E.R. (1991) Effects of pH and polysaccharides on peptide binding to class II major histocompatibility complex molecules. *Proc. Natl Acad. Sci. USA*, **88**, 2740–2744.

Karlsson,L., Peleraux,A., Lindstedt,R., Liljedahl,M. and Peterson,P.A. (1994) Reconstitution of an operational MHC class II compartment in nonantigen-presenting cells. *Science*, **266**, 1569–1573.

Lee,P., Matsueda,G.R. and Allen,P.M. (1988) T cell recognition of fibrinogen. *J. Immunol.*, **140**, 1063–1068.

Liang,M.N., Beeson,C., Mason,C. and McConnell,H.M. (1995) Kinetics of the reactions between the invariant chain (85–99) peptide and proteins of the murine class II MHC. *Int. Immunol.*, **7**, 1397–1404.

Luescher,I.F. and Unanue,E.R. (1990a) The sites in the $I\text{-}A^k$ histocompatibility molecule photoaffinity-labeled by an immunogenic lysozyme peptide. *J. Biol. Chem.*, **265**, 11177–11184.

Luescher,I.F. and Unanue,E.R. (1990b) Purification and photoaffinity labeling of the $I\text{-}A^k$ histocompatibility molecule. *J. Immunol. Methods*, **135**, 233–245.

Manara,G.C. *et al.* (1993) New insights suggesting a possible role of heat shock protein 70-kD family-related protein in antigen processing/presentation phenomenon in humans. *Blood*, **82**, 2865–2871.

McConahey,P.J. and Dixon,F.J. (1980) Radioiodination of proteins by the use of the chloramine-T method. *Methods Enzymol.*, **70**, 210–213.

Mehringer,J.H., Harris,M.R., Kindle,C.S., McCourt,D.W. and Cullen,S.W. (1991) Characterization of fragments of the murine I-A-associated invariant chain. *J. Immunol.*, **146**, 920–927.

Mellman,I., Fuchs,R. and Helenius,A. (1986) Acidification of the endocytic and exocytic pathways. *Annu. Rev. Biochem.*, **55**, 663–700.

Morris,P., Shaman,J., Attaya,M., Amaya,M., Goodman,S., Bergman,C., Monaco,J.J. and Mellins,E. (1994) An essential role for HLA-DM in antigen presentation by class II major histocompatibility molecules. *Nature*, **368**, 551–554.

Nabavi,N., Ghogawala,Z., Myer,A., Griffith,I.J., Wade,W.F., Chen,Z.Z., McKean,D.J. and Glimcher,L.H. (1989) Antigen presentation abrogated in cells expressing truncated Ia molecules. *J. Immunol.*, **142**, 1444–1447.

Nadimi,F., Moreno,J., Momburg,F., Heuser,A., Fuchs,S., Adorini,L. and Hämmerling,G.J. (1991) Antigen presentation of hen egg-white lysozyme but not of ribonuclease A is augmented by the major histocompatibility complex class II-associated invariant chain. *Eur. J. Immunol.*, **21**, 1255–1263.

Nelson,C.A., Roof,R.W., McCourt,D.W. and Unanue,E.R. (1992) Identification of the naturally processed form of hen egg white lysozyme bound to the murine major histocompatibility complex class II molecule $I\text{-}A^k$. *Proc. Natl Acad. Sci. USA*, **89**, 7380–7383.

Nelson,C.A., Petzold,S.J. and Unanue,E.R. (1993) Identification of two distinct properties of class II major histocompatibility complex-associated peptides. *Proc. Natl Acad. Sci. USA*, **90**, 1227–1231.

Nelson,C.A., Petzold,S.J. and Unanue,E.R. (1994) Peptides determine the lifespan of MHC class II molecules in the antigen presenting cell. *Nature*, **371**, 250–252.

Pierce,S.K. (1994) Molecular chaperones in the processing and the presentation of antigen to helper T cells. *Experientia*, **50**, 1026–1030.

Pierre,P., Denzin,L.K., Hammond,C., Drake,J.R., Amigorena,S., Cresswell,P. and Mellman,I. (1996) HLA-DM is localized to conventional and unconventional MHC class II-containing endocytic compartments. *Immunity*, **4**, 229–239.

Pierres,M., Devaux,D., Dosseto,M. and Marchetto,S. (1981) Clonal analysis of B- and T-cell responses to Ia antigens. *Immunogenetics*, **14**, 481–485.

Pinet,V., Malnati,M.S. and Long,E.O. (1994) Two processing pathways for the MHC class II-restricted presentation of exogenous influenza virus antigen. *J. Immunol.*, **152**, 4852–4860.

Pinet,V., Vergelli,M., Martin,R., Bakke,O. and Long,E.O. (1995) Antigen presentation mediated by recycling of surface HLA-DR molecules. *Nature*, **375**, 603–606.

Radford,S.E., Dobson,C.M. and Evans,P.A. (1992) The folding of hen lysozyme involves partially structured intermediates and multiple pathways. *Nature*, **358**, 302–307.

Riberdy,J.M. and Cresswell,P. (1992) The antigen-processing mutant T2 suggests a role for MHC-linked genes in class II antigen presentation. *J. Immunol.*, **148**, 2586–2590.

Roche,P.A. (1995) HLA-DM: an *in vivo* facilitator of MHC class II peptide loading. *Immunity*, **3**, 259–262.

Roof,R.W., Luescher,I.F. and Unanue,E.R. (1990) Phospholipids enhance the binding of peptides to class II major histocompatibility molecules. *Proc. Natl Acad. Sci. USA*, **87**, 1735–1739.

Sadegh-Nasseri,S., Stern,L.J., Wiley,D.C. and Germain,R.N. (1994) MHC class II function preserved by low-affinity peptide interactions preceding stable binding. *Nature*, **370**, 647–650.

Sanderson,F., Kleijmeer,M.J., Kelly,A., Verwoerd,D., Tulp,A., Neefjes,J.J., Geuze,H.J. and Trowsdale,J. (1994) Accumulation of HLA-DM, a regulator of antigen presentation, in MHC class II compartments. *Science*, **266**, 1566–1569.

Schägger,H. and vonJagow,G. (1987) Tricine-SDS–polyacrylamide gel electrophoresis for the separation of proteins in the range of 1 to 100 kDA. *Anal. Biochem.*, **166**, 368–379.

Schmid,S.L. and Carter,L.L. (1990) ATP is required for receptor-mediated endocytosis in intact cells. *J. Cell Biol.*, **111**, 2307–2318.

Schmid,S.L. and Jackson,M.R. (1994) Making class II presentable. *Nature*, **369**, 103–104.

Sette,A., Adorini,L., Colon,S.M., Buus,S. and Grey,H.M. (1989) Capacity of intact proteins to bind to MHC class II molecules. *J. Immunol.*, **143**, 1265–1267.

Sherman,M.A., Weber,D.A. and Jensen,P.E. (1995) DM enhances peptide binding to class II MHC by release of invariant chain-derived peptide. *Immunity*, **3**, 197–205.

Sloan,V.S., Cameron,P., Porter,G., Gammon,M., Amaya,M., Mellins,E. and Zaller,D.M. (1995) Mediation by HLA-DM of dissociation of peptides from HLA-DR. *Nature*, **375**, 802–806.

Srinivasan,M., Marsh,E.W. and Pierce,S.K. (1991) Characterization of naturally processed antigen bound to major histocompatibility complex class II molecules. *Proc. Natl Acad. Sci. USA*, **88**, 7928–7932.

Stern,L.J., Brown,J.H., Jardetzky,T.S., Gorga,J.C., Urban,R.G., Strominger,J.L. and Wiley,D.C. (1994) Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. *Nature*, **368**, 215–221.

Tulp,A., Verwoerd,D., Dobberstein,B., Ploegh,H.L. and Pieters,J. (1994) Isolation and characterization of the intracellular MHC class II compartment. *Nature*, **369**, 120–126.

VanBuskirk,A.M., Crump,B.L., Margoliash,E. and Pierce,S.K. (1989) A peptide binding protein having a role in antigen presentation is a member of the HSP70 heat shock family. *J. Exp. Med.*, **170**, 1799–1809.

Wolf,P.R. and Ploegh,H.L. (1995) How MHC class II molecules acquire peptide cargo: biosynthesis and trafficking through the endocytic pathway. *Annu. Rev. Cell Dev. Biol.*, **11**, 267–306.

Ziegler,K.H. and Unanue,E.R. (1982) Decrease in macrophage antigen catabolism caused by ammonia and chloroquine is associated with inhibition of antigen presentation to T cells. *Proc. Natl Acad. Sci. USA*, **79**, 175–178.

Received on March 4, 1996; revised on September 9, 1996