The phenotype of H-2M-deficient mice is dependent on the MHC class II molecules expressed

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For a broader view of the role of H-2M as an accessory molecule in antigen presentation, we investigated the degree to which different MHC class II isotypes and alleles depend on H-2M to function *in vivo.* We generated H-2M-deficient animals expressing $E^{k/b}$ or A^k molecules in addition to the A^b molecules already present in the mutant strain, and compared the ability of the different MHC class II molecules to present antigen at the cell surface for recognition by T cells, and contribute to positive selection of $CD4^+$ T cells in the thymus. Biochemical analyses were performed to assess MHC class II maturation, and to determine the peptide content of the molecules. In the absence of H-2M, $E^{k/b}$ molecules containd a more heterogeneous set of class II-associated invariant chain peptides (CLIP) than A^b did, which, unlike A^b-CLIP complexes, were not SDS-stable. Unlike A^b molecules, both $E^{k/b}$ and A^k efficiently presented exogenously added peptides to T cells in the absence of H-2M. In addition, epitopes from some proteins, especially those known to be invariant chain independent, were presented by A^k molecules in the mutant animals. To our surprise, expression of $E^{k/b}$ overcame the positive selection defect observed in H-2M-deficient mice expressing A^b alone. In contrast, A^k expression did not augment positive selection of $CD4^+$ T cells in the mutant animals. Some of these findings *in vivo* contrast significantly with findings from *in vitro* studies on murine MHC class II molecules in human DM-deficient cell lines.

Key words: MHC class II / Antigen presentation / H-2M

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1 Introduction

To be capable of presenting antigens effectively during an immune response, class II molecules must intersect an endocytic compartment containing peptides generated from antigens delivered there by various means, demanding that class II molecules diverge from the normal constitutive secretory pathway en route to the cell surface [1, 2]. This task is managed by another glycoprotein, the invariant chain (Ii). Following translocation into the endoplasmic reticulum (ER), the class II α and β chains interact with Ii to form a nonameric structure consisting of three $\alpha\beta$ heterodimers and three Ii molecules $(\alpha\beta Ii)_3$. Critical to this interaction is the binding of a segment of the lumenal domain of Ii (residues 81–105), designated CLIP (class II-associated invariant chain peptide), to the antigen-binding groove of MHC class II [3]. The $(\alpha\beta Ii)_3$ complex is transported through the Golgi and the trans-Golgi network (TGN), where the complex is sorted directly, via signals predominantly in the cytoplasmic tail of Ii, to compartments bearing late endosomal/lysosomal characteristics [1, 2].

For class II molecules to bind peptides, they must first be relieved of Ii [4–6]. Upon arrival of class II-Ii complexes in the endocytic pathway, Ii is degraded by the proteases that reside there [7, 8], rendering $\alpha\beta$-CLIP complexes. Removal of CLIP is catalyzed by an MHC-encoded, nonclassical class II molecule, designated H-2M in the mouse [9], and HLA-DM in humans [10]. H-2M and HLA-DM localize independently to the same endocytic compartment that contains class II molecules [11].

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Once DM intersects a class II-containing endocytic compartment, a transient interaction occurs between the two molecules [12, 13], during which CLIP is removed [14–16]. In the absence of HLA-DM/H-2M, class II molecules are preferentially occupied by CLIP [17–19].

Three functions have been ascribed to DM: (1) to act as a catalyst, substoichiometric amounts inducing the removal of CLIP or an Ii-derived substrate and facilitating its exchange by heterogeneous peptides [14–16]; (2) to serve as a molecular chaperone, preventing nonspecific aggregation of the temporarily empty $\alpha\beta$ dimers following CLIP release [20]; and (3) to function as a peptide editor, positively selecting peptides that can bind stably to a particular class II allele [21].

The requirement for HLA-DM in generating stable $\alpha\beta$-peptide complexes is not absolute, but instead depends on the class II allele in question. Transfection of different human and mouse class II alleles into the HLA-DM mutant cell line T2 revealed both HLA-DM-dependent and -independent peptide loading of class II molecules. The human alleles DR3 and DR4 [22, 23], and mouse alleles A^d and A^k [23, 24], all exhibited HLA-DM-independent CLIP release and peptide loading. The apparent lack of requirement for HLA-DM to accomplish removal of CLIP was attributed to the low affinity of these class II alleles for CLIP [25]. However, the dependence on DM does not always correlate with the affinity of the class II molecule for CLIP [26].

Recently, strains of mice lacking H-2M have been characterized [19, 27, 28], allowing a detailed characterization of the dependency of I-A^b molecules on H-2M to function in antigen presentation. APC from the mutant animals were unable to present peptides generated from whole proteins, and were heavily compromised in their ability to present peptides added exogenously. The A^b molecules in $M^{o/o}$ animals (H-2^b haplotype) lacked the usual heterogeneous array of antigenic peptides and instead carried essentially a single CLIP species [19]. Nonetheless, positive selection of $CD4^+$ thymocytes by A^b-CLIP complexes expressed in the thymus was rather efficient (30–50 % of wild-type levels), yielding a broad TCR repertoire [19, 27, 28]. To explore whether the phenotype of $M^{o/o}$ mice varies when other class II alleles/isotypes in addition to A^b are present, we have now generated and characterized $A^b/M^{o/o}$ animals expressing $E^{k/b}$ or A^k.

2 Results

2.1 Generation of H-2M-deficient mice expressing $E^{k/b}$ or A^k class II molecules

H-2M-deficient ($M^{o/o}$) mice were generated on a H-2^b genetic background, in which only A^b but no E class II molecules are expressed [19]. As the Ma locus maps to the class II region of the MHC, it is difficult to introduce class II genes specifying other allotypes or isotypes onto the $M^{o/o}$ background except by transgenesis. $M^{o/o}$ mice expressing $E^{k/b}$ or A^k molecules were thus generated by crossing the $M^{o/o}(A^b)$ mice with either $E\alpha^k$ [29] or $A\alpha^k/A\beta^k$ [30] transgenic mice. These transgenic animals express $E^{k/b}$ ($E\alpha^k/E\beta^b$) and A^k complexes, respectively, with proper tissue and cell type specificity, and display normal E-restricted and A^k-restricted antigen presentation [29–31]. Backcrosses were set up to generate matched littermates expressing the desired transgenes on an $M^{+/o}$ or $M^{o/o}$ background, designated: $E\alpha^k M^{+/o}$, $E\alpha^k M^{o/o}$, $A^k M^{+/o}$, and $A^k M^{o/o}$ mice.

Cell surface levels of class II molecules in these mice were evaluated by staining splenic B cells with a panel of mAb recognizing epitopes on E (14.4.4s, H39.91.1 and

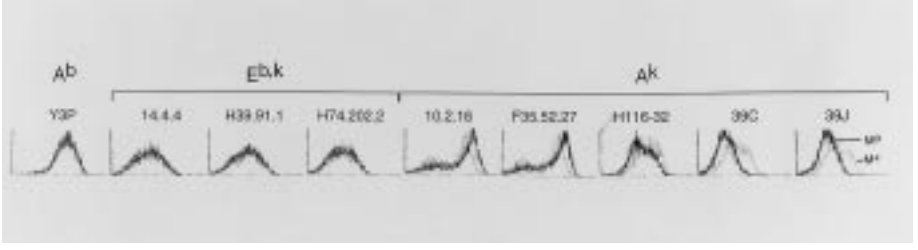


Figure 1. Cell surface expression of $E^{k,b}$ and A^k in H-2M deficient mice. Splenocytes from $M^{+/o}$ or $M^{o/o}$ mice expressing the transgenic class II molecules were stained appropriately with anti-E, or -A^k mAb followed by an anti-IgM counterstain, gated on IgM^+ B cells, and analyzed by cytofluorometry. A^b expressed on $M^{+/o}$ or $M^{o/o}$ splenocytes was stained with mAb Y-3P. The darker line represents the $M^{o/o}$ background, and the faint line represents $M^{+/o}$.

H74.202.2), $A\alpha^k$ (H116-32, 39J and 39C), or $A\beta^k$ (10-2-16 and F35.52.27) chains (Fig. 1). As described [19, 27, 28], A^b surface levels were normal in $M^{o/o}$ mice. The same held true for $E^{k/b}$ levels, as staining intensities with all mAb were superimposable in $M^{+/o}$ and $M^{o/o}$ mice (Fig. 1). In contrast to $E^{k/b}$, the level of A^k (haplotype-mixed or matched) detected varied with the anti-A^k reagent used. Slightly higher staining in the $M^{o/o}$ mutant compared with the wild-type control was obtained with anti-$A\beta^k$ mAb, while staining with anti-$A\alpha^k$ reagents was decreased: only slightly for H116-32, and more markedly for 39J and 39C (Fig. 1). This mAb-specific loss of staining may be explained by the presence of mixed-haplotype dimers in the $A^k M^{o/o}$ mice: A^b α and β chains and A^k α and β chains were shown to intermingle in wild-type cells [32, 33]. The presence of such mixed-haplotype I-A molecules on the cell surface may result in a change in the expression of key mAb epitopes. Specifically, $A\alpha^b A\beta^k$ dimers occupied by peptides such as CLIP in the absence of H-2M may be more stable than $A\alpha^b A\beta^k$ dimers containing other peptides, and may account for the slightly higher staining observed with the anti-$A\beta^k$ mAb for $A^k M^{o/o}$ cells. In summary, there are no gross abnormalities in expression of the transgene products in $M^{o/o}$ mice.

2.2 E- and A^k-restricted antigen presentation in $M^{o/o}$ mice

A^b class II molecules from H-2M-deficient mice were partially defective in their ability to present peptide antigens to T cells in an *in vitro* assay [19], which was evident as a 10- to 20-fold shift in dose-response curves. This was true with either fresh or fixed APC, indicating that loading of class II molecules with peptide at the cell surface was affected, rather than a mechanism dependent on internalization and further processing. Because the A^b molecules expressed in H-2M-deficient mice were predominantly occupied with a single CLIP species, this observation was interpreted as inefficient replacement of CLIP with antigenic peptides, reflecting high-affinity CLIP interactions with A^b [19]. We investigated peptide loading of $E^{k/b}$ and A^k molecules in the absence of H-2M by comparing the ability of splenic APC from the transgenic, H-2M-deficient animals, to stimulate IL-2 production by a panel of $E^{k/b}$- and A^k-specific T cell hybridomas (Fig. 2A). The $M^{o/o}$ mutation seemed to have no influence on the ability of $E^{k/b}$ to present peptides (Fig. 2A). Likewise, peptide presentation by A^k was also barely affected, although we did detect a degree of variation from one

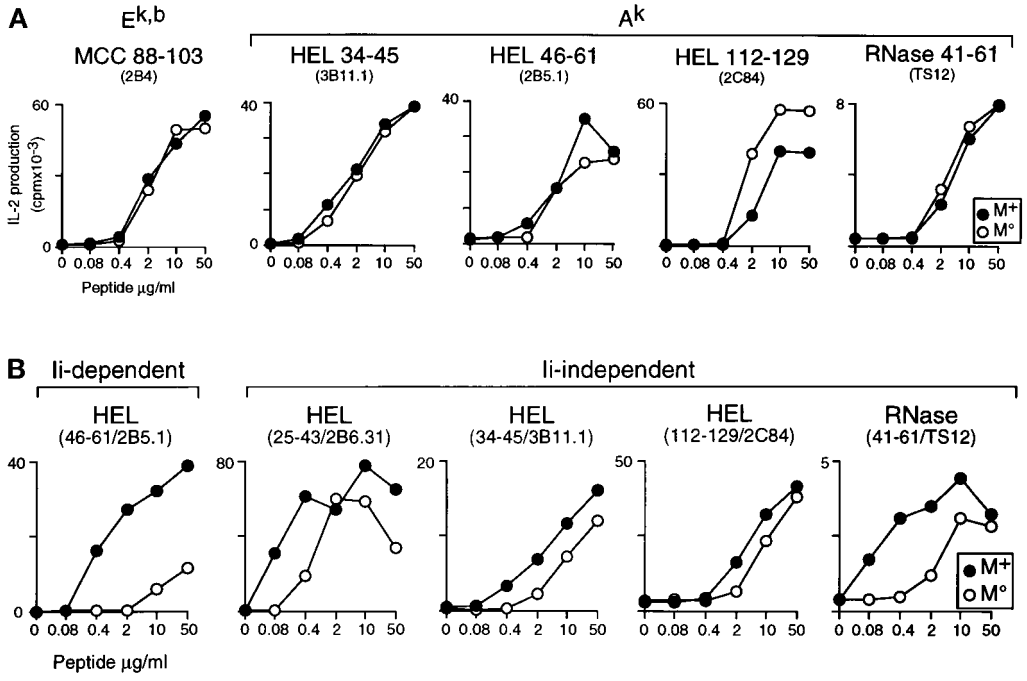


Figure 2. A^k and $E^{k,b}$ restricted antigen presentation in H-2M deficient mice. Spleen cells from $E\alpha^k M^{+/+}$, $E\alpha^k M^{o/o}$, $A^k M^{+/+}$, and $A^k M^{o/o}$ mice were used as APC for T hybridoma stimulation, measured as IL-2 production. The X-axis denotes [^{3}H]thymidine incorporation by the CTLL indicator cells. Antigens were provided in the form of (A) peptides: E-binding peptide MCC_{88-103}, and A^k-binding peptides HEL_{34-45}, HEL_{46-61}, $HEL_{112-129}$, and $RNase_{41-61}$, or the corresponding (B) whole proteins containing both Ii-dependent and -independent epitopes. The hybridoma used to recognize each class II-peptide complex is noted.

peptide to the next. Note that the T cell hybridomas used only recognize specific antigen in the context of haplotype-matched A^k dimers.

In the absence of H-2M, A^b molecules were unable to present peptides derived from whole protein antigens as well, reflecting the requirement for H-2M to remove CLIP from class II molecules intracellularly [19, 27, 28]. The ability of different class II alleles/isotypes to present whole protein antigens to T cells in the absence of H-2M was investigated. In particular, we wished to correlate the H-2M and Ii dependence of class II molecules for presentation of certain epitopes. For several proteins, the presentation of particular epitopes does not require that Ii target class II molecules to the compartments where they are processed. This behavior suggests that protein processing and peptide loading onto class II molecules can occur in compartments that class II molecules reach by an alternate route, such as by recycling from the cell surface [34–37]. It was not possible previously to ask whether H-2M would be required for the presentation of such epitopes, as Ii-independent presentation of peptides from exogenous proteins by A^b has yet to be found. However, this question could be readily asked with the $A^k M^{o/o}$ mice, as several Ii-dependent and -independent epitopes presented by A^k molecules are well defined [3]. Fig. 2B depicts representative antigen presentation experiments with splenocytes from $A^k M^{+/+}$ and $A^k M^{o/o}$ mice. Overall, there was some correlation with Ii-dependence: presentation of the hen egg lysozyme $(HEL)_{46–61}$ epitope to hybridoma 2B5.1 proved highly dependent on H-2M, in accordance with its strong dependence on Ii ([35] and our unpublished data). The activation of T cells specific for Ii-independent epitopes ($HEL_{34–45}$ and $HEL_{112–129}$) was, overall, less affected by the $M^{o/o}$ mutation. However, this correlation was not perfect: the presentation of $HEL_{25–43}$ or $RNase_{41–61}$ epitopes, which do not require Ii either *in vitro* or *in vivo* ([35] and our unpublished data), was clearly hampered by the absence of H-2M.

2.3 Positive selection of $CD4^+$ T cells in mice lacking H-2M but expressing additional class II alleles

The SDS-stable A^b-CLIP complexes of $M^{o/o}$ mice were able to select significant numbers of $CD4^+$ T cells, approximately 30–50 % of wild-type numbers [19]. How would the introduction of additional class II molecules ($E^{k/b}$ or A^k) into the $M^{o/o}$ mice affect positive selection? CD4/CD8 profiles were generated for thymocytes from $E\alpha^k M^{o/o}$ and $A^k M^{o/o}$ mice, gated on $CD3^{hi}$ cells to focus on those involved in positive selection (Fig. 3A). The additional expression of A^k in A^b-positive $M^{o/o}$ animals was not beneficial in selecting $CD4^+$ T cells in the thymus: the proportion of $CD4^+CD8^-$ cells in $A^k M^{o/o}$ mice was still reduced by roughly twofold as compared to $A^k M^{+/+}$ mice, with a large increase in the proportion of $CD4^-CD8^+$ cells, analogous to selection in $M^{o/o}$ mice expressing A^b alone. In marked contrast, the presence of the Ea^k transgene in $M^{o/o}$ mice restored selection to almost normal levels (Fig. 3A).

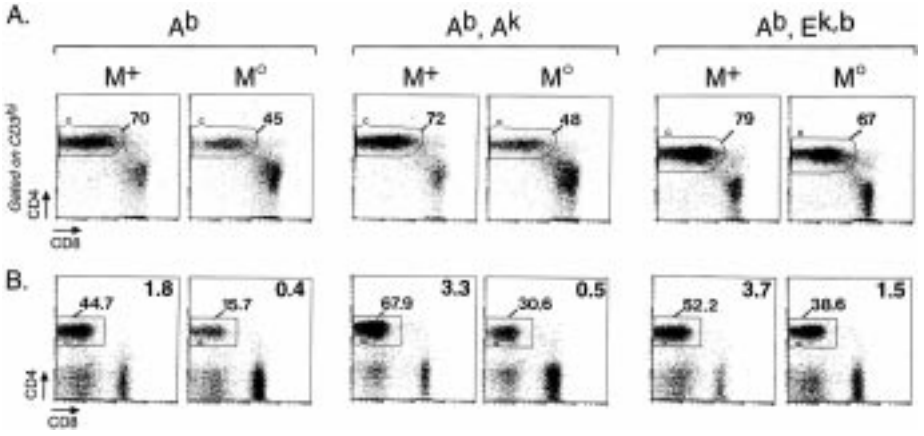


Figure 3. Selection of $CD4^+$ T cells in $M^{o/o}$ mice expressing additional class II alleles. (A) Thymocyte suspensions from the indicated mice were stained and analyzed by flow cytometry. Panels display the CD4/CD8 profiles gated on $CD3^{hi}$ cells. The values indicate the average percentages of $CD4^+CD8^-$ cells in these thymi of five different determinations. (B) Displays CD4/CD8 profiles of lymph node cells from the different mice. The percentage of $CD4^+$ cells (an average of four experiments) and the average CD4 to CD8 ratio are shown.

These trends also applied to T cell populations of peripheral lymphoid organs. CD4/CD8 staining profiles for lymph node populations revealed reduced numbers of $CD4^+$ T cells in the periphery of $M^{o/o}$ mutants expressing A^b alone (over several experiments, an $M^{o/o}/M^{+/+}$ ratio of $CD4^+$ cells of 0.41 ± 0.09, $n = 7$), with an inversion of the CD4/CD8 ratios (Fig. 3B). Such an inversion is also apparent with $M^{o/o}$ mice expressing A^k ($M^{o/o}/M^{+/+}$ ratio of $CD4^+$ cells averages 0.40 ± 0.07, $n = 4$). In contrast, these parameters were almost normal in $E\alpha^k M^{o/o}$ mice, the $E\alpha^k$ transgene restoring the preponderance of $CD4^+$ cells (CD4/CD8 ratio > 1.5; the $M^{o/o}/M^{+/+}$ ratio of $CD4^+$ cells averaged 0.75 ± 0.11, $n = 4$) (Fig. 3B).

2.4 Intracellular transport and peptide occupancy of $E^{k/b}$ and A^k molecules in $M^{o/o}$ mice

Could the restoration of $CD4^+$ T cell selection to that of wild-type levels by the mere addition of $E^{k/b}$ be indicative of a more diverse set of peptides occupying the groove of this class II molecule? Biochemical analyses were employed to determine whether the transgenic class II molecules synthesized in $M^{o/o}$ mice matured as normal into $\alpha\beta$-peptide complexes, or, analogous to A^b, remained occupied with CLIP. Pulse-chase and continuous labeling experiments were used to monitor intracellular transport and maturation of $E^{k/b}$ and A^k molecules in $M^{o/o}$ cells. Splenocytes from the appropriate mice were isolated, pulse-labeled for 1 h with [^{35}S]methionine, and chased for different times. Because the transgene-encoded class II molecules were expressed at lower levels than endogenously encoded A^b, splenocytes were also labeled continuously with [^{35}S]methionine for 5 h to improve recovery of labeled material. For both types of labeling experiments, cell lysates were immunoprecipitated first with a polyclonal rabbit anti-MHC class I serum to control for comparable levels of protein synthesis, and then immunoprecipitated with the anti-A^b mAb Y-3P, followed by the anti-E^k mAb 14-4-4s or a mixture of the anti-A^k specific mAb H116.32 and 10-2-16. Peptide loading of the transgene-encoded class II molecules was assessed by examining their stability in SDS [38].

The kinetics of glycan modification of $E^{k/b}$ in the presence and absence of H-2M was indistinguishable, and similar to that of A^b molecules (data not shown). As reported, A^b molecules in cells devoid of H-2M were essentially occupied with a single CLIP species [19, 27, 28], which was detected in anti-A^b immunoprecipitates as a low molecular mass species migrating just below the dye front on the gel (Fig. 4). Likewise, CLIP was clearly visible in the anti-$E^{k/b}$ immunoprecipitates from $E\alpha^k M^{o/o}$ lysates (Fig. 4). Nevertheless, whereas A^b-CLIP complexes in $M^{o/o}$ mice were SDS stable (although less so than A^b-peptide complexes in the presence of H-2M), $E^{k/b}$-CLIP complexes were unstable in SDS (Fig. 4).

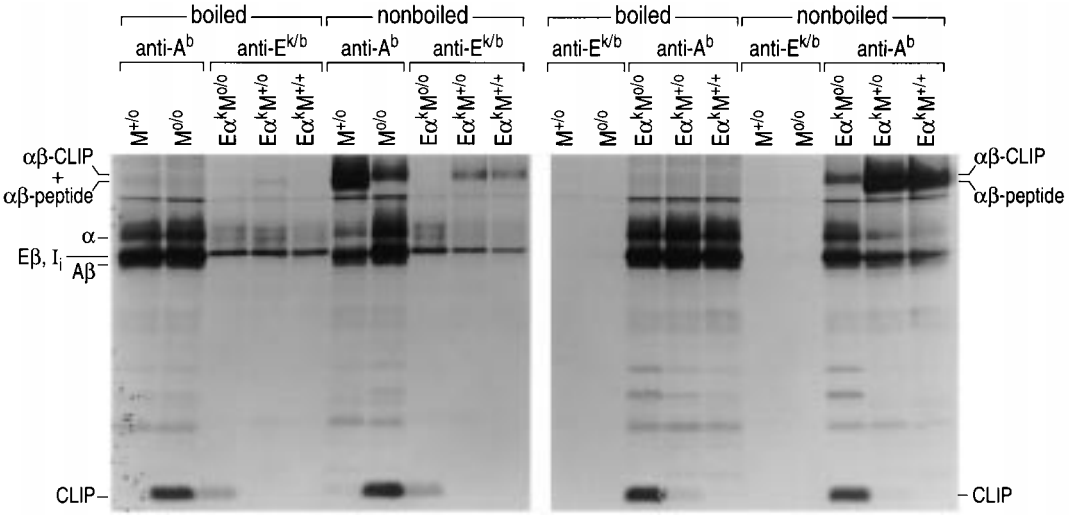
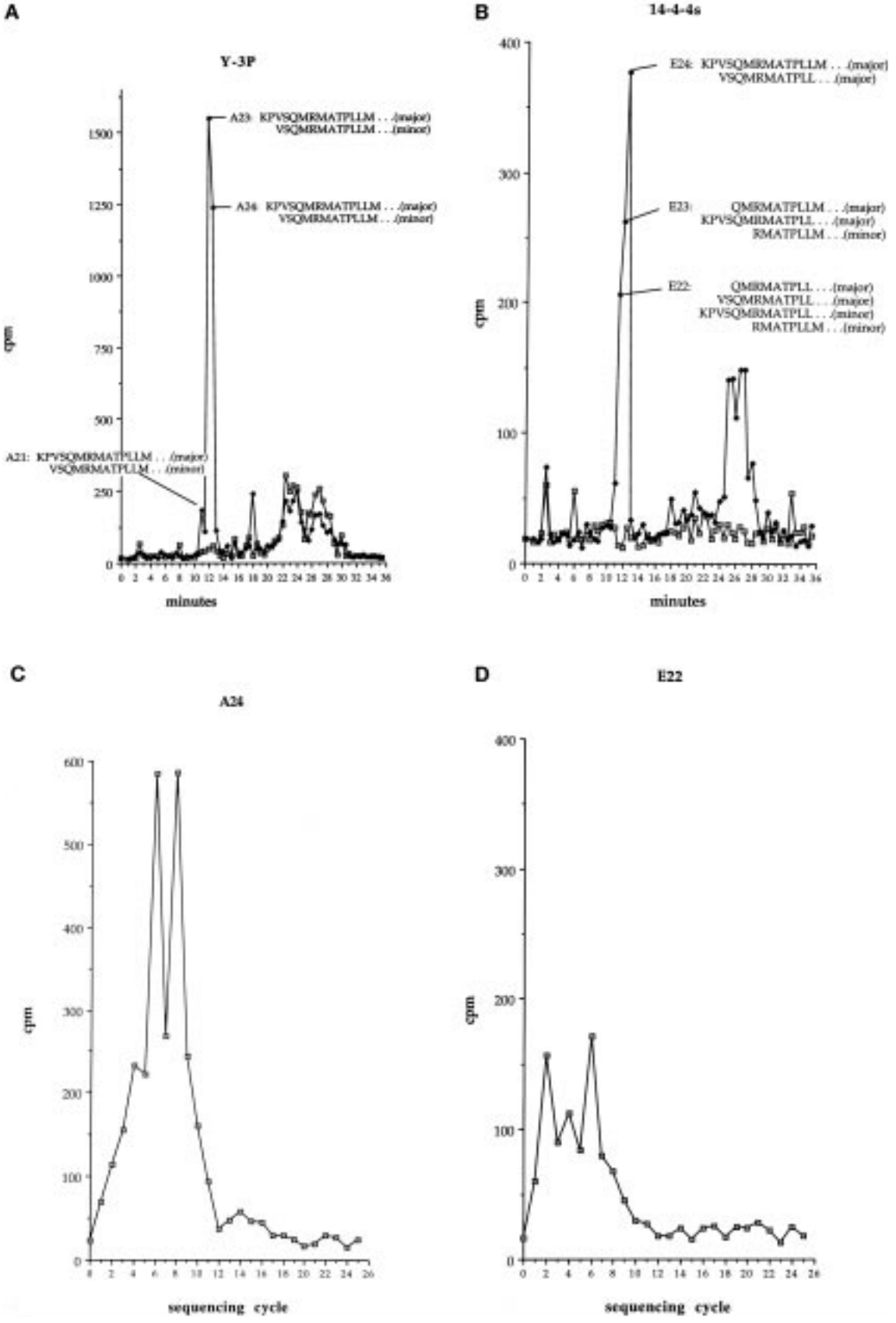


Figure 4. Acquisition of peptide by $E^{k,b}$ molecules. Splenocytes from $E\alpha^k M^{+/+}$, $E\alpha^k M^{+/o}$, or $E\alpha^k M^{o/o}$ mice were labeled continuously with [^{35}S]methionine for 5 h, and resulting lysates immunoprecipitated first with the anti-A^b mAb Y-3P, and then with the anti-E mAb 14-4-4s. The immune complexes were solubilized in SDS sample buffer either at 95 °C (boiled) or at room temperature (non-boiled), under reducing conditions, and analyzed on a 12.5 % gel. SDS-stable dimers, representing both $\alpha\beta$-peptide and $\alpha\beta$-CLIP complexes in the case of A^b, and only $\alpha\beta$-peptide complexes in the case of $E^{k/b}$, are denoted.


A
Y-3P
A23: KPVSQMRMATPLLM . . ./(major)
VSQMRMATPLLM . . ./(minor)
A24: KPVSQMRMATPLLM . . ./(major)
VSQMRMATPLLM . . ./(minor)
A21: KPVSQMRMATPLLM . . ./(major)
VSQMRMATPLLM . . ./(minor)
cpm
minutes
B
14-4-4s
E24: KPVSQMRMATPLLM . . ./(major)
VSQMRMATPLL . . ./(major)
E23: QMRMATPLLM . . ./(major)
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RMATPLLM . . ./(minor)
E22: QMRMATPLL . . ./(major)
VSQMRMATPLL . . ./(major)
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RMATPLLM . . ./(minor)
cpm
minutes
C
A24
cpm
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D
E22
cpm
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◀ *Figure 5.* Sequence analyses of CLIP peptides complexed with A^b and E^k in H-2M-deficient mice. Splenocytes from $E\alpha^k M^{+/+}$ and $E\alpha^k M^{o/o}$ mice were labeled continuously with [^{35}S]methionine for 5 h, and lysates immunoprecipitated with Y-3P (anti-A^b) and then 14-4-4s (anti-E). The immunoprecipitates were denatured in acid and run on an RP-HPLC column. The eluted peptides were detected by scintillation counting of the fractions. (A) Scintillation counts obtained for each HPLC fraction of Y-3P acid extracts from $M^{+/+}$ (open squares) and $M^{o/o}$ (closed diamonds) mice. Fractions A21, A23, and A24 were subjected to N-terminal sequencing (Edman Degradation), and the resulting CLIP peptides identified along with their relative amounts denoted. (B) Scintillation counts obtained for each HPLC fraction of 14-4-4s acid extracts from $M^{+/+}$ (open squares) and $M^{o/o}$ (closed diamonds) mice. Fractions E22, E23, and E24 were subjected to N-terminal sequencing, and the resulting CLIP peptides identified along with their relative abundance denoted. (C) Representative radioactive sequencing profile for one HPLC fraction obtained from Y-3P extract, fraction A24. (D) Representative radioactive sequencing profile for one HPLC fraction obtained from 14-4-4s extract, fraction E22.

Given the significant amounts of $E^{k/b}$-CLIP complexes recovered by immunoprecipitation from $M^{o/o}$ cells, we asked whether these CLIP species carried by $E^{k/b}$ molecules differed in sequence from those seen with A^b. Could allelic differences in H-2M dependence and/or affinities for CLIP be revealed by the types of CLIP species left in the class II peptide-binding groove after Ii breakdown? Longer CLIP species might be less dependent on H-2M for their displacement, or lower affinities for CLIP binding may yield a more heterogeneous set of CLIP fragments left in the groove. We chose to employ radioactive sequencing of HPLC fractions obtained from extracts of immunoprecipitated class II-CLIP complexes, since this method was successful in positioning the N terminus of the dominant CLIP species complexed to A^b in $M^{o/o}$ cells. The predominant CLIP species bound to A^b molecules in $M^{o/o}$ mice began at position 86, and extended at least 14 residues to position 99: KPVSQMRMATPLLM.... [19], based on sequencing of ^{35}S-labeled material. While the identification of the 14 N-terminal residues is unambiguous, we could not be certain of the C-terminal residue. Peptides eluted from A^b isolated from wild-type animals include CLIP 86–100, one residue beyond what we were able to sequence [39]. This CLIP fragment lacks region 81–89, reported to destabilize binding of CLIP 90–103 to HLA-DR1, even in the absence of HLA-DM [40, 41]. It should be noted that the radioactive sequencing described above and employed in this present study is merely qualitative and not quantitative: while it allows the determination of N-terminal cleavage sites of the most abundant CLIP species recovered in the immunoprecipitates, the relative amounts of each peptide sequences cannot be assessed accurately.

Splenocytes from $E\alpha^k M^{+/+}$ and $E\alpha^k M^{o/o}$ mice were labeled with [^{35}S]methionine for 5 h. A^b molecules were recovered first with the mAb Y-3P, followed by immunoprecipitation with 14-4-4s to recover $E^{k/b}$ molecules. Peptides were acid-extracted from the class II immunoprecipitates of $M^{+/+}$ and $M^{o/o}$ cell lysates, and subjected to reverse-phase (RP)-HPLC (Fig. 5). A sharp major peak of radioactivity with a similar retention time was observed in extracts from both Y-3P (Fig. 5A) and 14-4-4s precipitates (Fig. 5B) of $E\alpha^k M^{o/o}$ cells, which was absent from $E\alpha^k M^{+/+}$ samples.

Our previous analysis of Y-3P extracts from $M^{o/o}$ cells not carrying the transgenes yielded analogous results. We demonstrated that synthetic CLIP co-eluted with this major radioactive peak, and were able to identify a dominant sequence within the fraction by automated Edman degradation [19]. In the same manner, we took fractions A21, A23, and A24 of the Y-3P precipitate, and fractions E22, E23, and E24 of the 14-4-4s precipitate from $E\alpha^k M^{o/o}$ cells, and subjected all to 25 cycles of automated Edman degradation. For Y-3P fractions A21, A23, and A24, major spikes of radioactivity appeared at cycles 6, 8, and a minor peak at cycle 14, consistent with the predominant CLIP sequence beginning with residue 86, as reported previously for A^b [19] (sequence profile for A24 shown in Fig. 5C). Four CLIP sequences were identified in fraction E22: major spikes of radioactivity at cycles 2 and 6 at almost equivalent levels of radioactivity, with a less prevalent spike at position 4, suggested CLIP species beginning at position 90 (from cycles 2 and 4), and position 88 (from cycles 4 and 6) (Fig. 5D). Also present were fragments of CLIP with N termini at position 92 and 86 (Fig. 5D). Fraction E23 contained the largest variety of CLIP sequences, beginning at positions 92, 90 and 86 (data not shown). Two CLIP sequences were present in fraction E24, beginning at position 86 and position 88 (data not shown). In summary, although A^b was found complexed with a fairly homogeneous set of CLIP species in the absence of H-2M, a heterogeneous set of CLIP species were recovered from $E^{k/b}$. Thus, the CLIP species contained by $E^{k/b}$ in $E\alpha^k M^{o/o}$ mice arise from a variety of N-terminal cleavage sites, whereas the final N-terminal cleavage yielding A^b-CLIP complexes appears to prefer a single site.

A similar biochemical analysis of A^k molecules in $M^{o/o}$ mice was attempted, yet immunoprecipitations and their interpretation were complicated by the presence of mixed-haplotype dimers (*e.g.* $A\alpha^b A\beta^k$ and $A\alpha^k A\beta^b$) [32, 33]. Analysis of $M^{+/o}$ and $M^{o/o}$ mice expressing either the

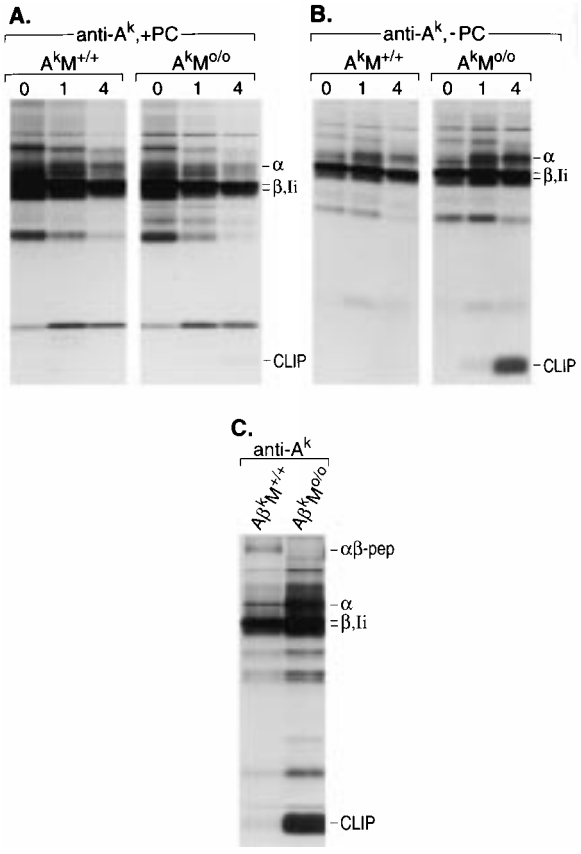

Figure 6. Biochemical analysis of A^k in the absence of H-2M. (A) Splenocytes from $A^kM^{+/+}$ mice and $A^kM^{o/o}$ mice were pulsed for 1 h with [^{35}S]methionine, then chased in cold methionine for 0, 1 and 4 h. Lysates were precleared with Y-3P to remove mixed-haplotype dimers, (+P/C), and then immunoprecipitated with a combination of anti-A^k mAb 10-2-16 and H11-632. One half of the samples were solubilized by boiling (shown), and the other half solubilized at room temperature (not shown), and analyzed under reducing conditions on a 12.5 % gel. The gel was autoradiographed for longer periods of time in an attempt to visualize equivalent amounts of A^k as seen in the –P/C samples (see B), so that the presence or absence of CLIP could be determined. B10.BR(H-2^k) served as a control for normal maturation of A^k molecules into SDS-stable A^k-peptide complexes (data not shown). (B) Cells were labeled as in (A), and the resulting lysates were directly immunoprecipitated with a combination of the anti-A^k mAb 10-2-16 and H11-632, without prior preclearing with Y-3P (–P/C). (C) Spleen cells from $A\beta^kM^{+/o}$ and $A\beta^kM^{o/o}$ mice, expressing the $A\beta^k$ transgene only, were labeled continuously for 5 h with [^{35}S]methionine, and resulting lysates immunoprecipitated with either a mixture of anti-A^k mAb 20-2-16 and H11-632 (not shown), or anti-A^b mAb Y-3P. Samples were solubilized at room temperature in SDS and analyzed under reducing conditions on a 12.5 % gel as above. Similar analyses of $M^{+/o}$ and $M^{o/o}$ mice expressing the $A\alpha^k$ transgene alone were also conducted (data not shown).

$A\alpha^k$ (data not shown) or $A\beta$ transgene alone (Fig. 6C) proved that mixed pairing with the endogenous A^b α and β chains occurred very readily. These mixed dimers were easily isolated with either anti-A^b or anti-A^k mAb, revealing $A\alpha^bA\beta^k$ dimers to be occupied predominantly with CLIP in $M^{o/o}$ animals (Fig. 6B, C). Unlike haplotype-matched A^b dimers, $A\alpha^bA\beta^k$-CLIP complexes were not SDS stable in $M^{o/o}$ mice (Fig. 6C). Preclearing with reagents (anti-A^b mAb Y3P, which binds both $A\alpha^bA\beta^k$ and $A\alpha^kA\beta^b$; [42]) to eliminate the mixed dimers prior to immunoprecipitation with the anti-A^k mAb resulted in very low recoveries of $A\alpha^kA\beta^k$ complexes (Fig. 6A), precluding a rigorous determination of the amount or sequence of CLIP peptides bound by these haplotype-matched A^k dimers in the presence or absence of H-2M. Yet, it was clear that the relative amount of A^k molecules occupied with CLIP in the absence of H-2M is significantly lower than for A^b, or for the mixed $A\alpha^bA\beta^k$ dimers.

2.5 Breakdown of Ii in $M^{o/o}$ cells expressing transgenic class II molecules

The expression of $E\alpha^k$ or the $A\alpha^k$ and $A\beta^k$ transgenes in either $M^{+/o}$ or $M^{o/o}$ mice did not affect the intracellular transport of endogenously encoded A^b molecules nor their maturation into SDS-stable complexes. However, a striking difference was observed in the breakdown pattern of Ii associated with class II molecules in $A^kM^{o/o}$ mice, as compared with $A^kM^{+/o}$, $M^{+/+}$, and $M^{o/o}$ mice (Fig. 7). Conversion of $\alpha\beta$Ii to $\alpha\beta$-CLIP was monitored via pulse-chase experiments. After a 1-h pulse with [^{35}S]methionine, intact Ii along with potential breakdown products migrating at approximately 24 kDa (p24) and 10 kDa (p10) were co-precipitated with A^b from $A^kM^{+/o}$ cells (Fig. 7A). As expected, the p24 species disappeared first, followed by p10, with a concomitant increase in CLIP (Fig. 7A). The identity of p10 and CLIP was confirmed by dissociating Y-3P immunoprecipitates via boiling in 1 % SDS, and reprecipitating with antisera specific for the N terminus of Ii and for CLIP, respectively (data not shown). Neither antiserum precipitated the 24-kDa species, suggesting that it is a lumenal fragment. Although the rate at which full-length Ii was converted to CLIP was indistinguishable for $A^kM^{+/0}$ and $A^kM^{o/o}$ cells, the intermediate p10 fragment was biochemically distinct in mice lacking H-2M. In $A^kM^{o/o}$ cells, a doublet was present at 10 kDa instead of the single polypeptide seen in $A^kM^{+/+}$ mice. Furthermore, the Ii breakdown products migrating at this position in $A^kM^{o/o}$ cells were not as predominant as their counterparts in $A^kM^{+/o}$ cells. The proteolytic destruction of full-length Ii molecules and conversion into CLIP did not always occur via a detectable p10 intermediate or precursor in $A^kM^{o/o}$ cells (Fig. 7A). In addition, a polypeptide migrating just above the dye

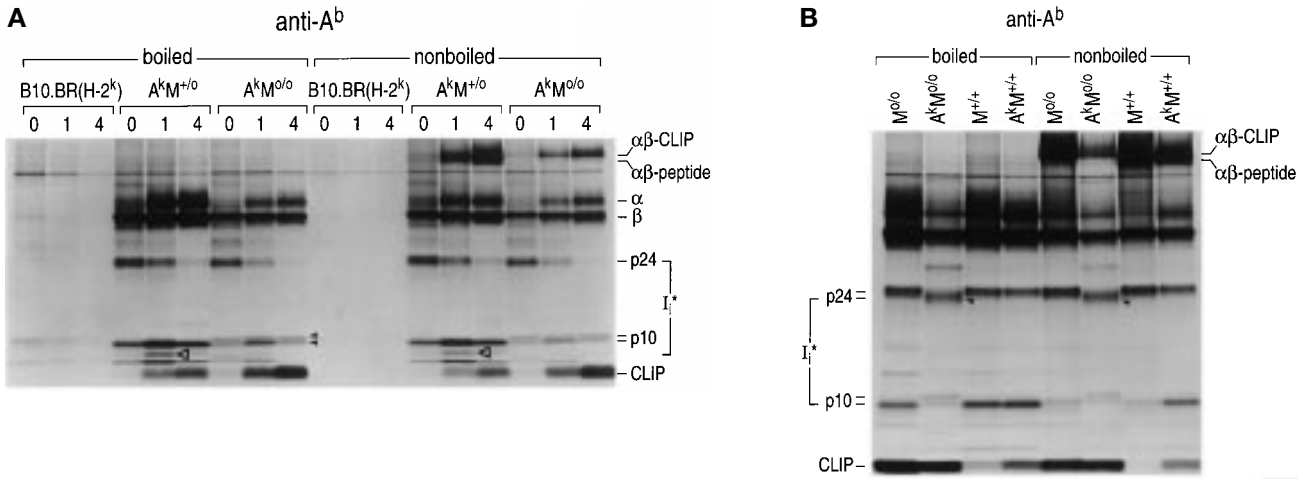

Figure 7. Breakdown of Ii associated with αβ dimers expressed in $A^kM^{o/o}$ mice. (A) Anti-A^b immunoprecipitates from pulsed-chased lysates described in Fig. 3 were solubilized by boiling or at room temperature, and analyzed under reducing conditions on a 12.5 % gel. Fragments representing potential Ii breakdown intermediates (p24 and p10) are bracketed and designated as Ii*. The p10 doublet recovered from $A^kM^{o/o}$ lysates is denoted with two filled-in arrowheads, and the intermediate fragment migrating just below p10 but above the dye front is marked with a single open arrowhead. (B) Splenocytes from $M^{o/o}$, $M^{+/+}$, $A^kM^{o/o}$ and $A^kM^{+/+}$ mice were continuously labeled for 5 h with [^{35}S]methionine, and the lysates immunoprecipitated with Y-3P. Samples were analyzed by either boiling in SDS, or at room temperature. Potential Ii breakdown intermediates are again designated as Ii*. The shift in migration of p24 recovered from $A^kM^{o/o}$ lysates as compared to p24 isolated from $M^{o/o}$ mice lacking the $A\alpha^k$ and $A\beta^k$ transgenes is highlighted with an arrowhead.

front, but below p10, in anti-A^b immunoprecipitates from $A^kM^{+/o}$ cells, was noticeably absent from $A^kM^{o/o}$ cells (Fig. 7A, 1-h chase). While in $A^kM^{o/o}$ cells the p10 intermediates appear less prevalent than in $A^kM^{+/o}$ cells, the final yield of CLIP is nonetheless significantly greater in $M^{o/o}$ mice.

Similar results were obtained when anti-A^b immunoprecipitates from continuously labeled cells were examined (Fig. 7B). In this experiment, the $M^{+/+}$ and $M^{o/o}$ mouse strains not expressing the transgenes were used for comparison. The altered pattern of Ii breakdown fragments was observed only when the $A\alpha^k$ and $A\beta^k$ transgenes were expressed together with the H-2M mutation (Fig. 7B). In contrast to the pulse-chase samples, the 24-kDa species co-precipitating with anti-A^b from continuously labeled $A^kM^{o/o}$ splenocytes migrated faster than the 24-kDa species present in the three other cell types (Fig. 7B). More CLIP was retrieved in precipitates from $M^{+/+}$ cells that also express the $A\alpha^k$ and $A\beta^k$ transgenes ($A^kM^{+/+}$), compared to $M^{+/+}$ cells without the transgenic class II molecules (Fig. 7B). Why would the proteolytic breakdown pattern of Ii be distinct only in mice lacking H-2M and expressing A^k? The distinct Ii breakdown pattern observed may possibly reflect the presence of mixed-haplotype dimers or nonamers which, when assembled with Ii, may exhibit an altered conformation (in the absence but not presence of H-2M). Interaction of these conformationally altered complexes with H-2M during Ii proteolysis, at a point prior to the end stage of αβ-CLIP, may be essential to achieve a "wild-type" pattern for Ii breakdown.

3 Discussion

The experiments presented here provide the first model in which the overall requirement for H-2M in antigen presentation can be assessed in a true *in vivo* system: an organism expressing multiple class II alleles. Mouse class II molecules were originally reported to be less dependent on HLA-DM for peptide loading and presentation than their human counterparts [19, 27, 28]. Analysis of $M^{o/o}$ mice proved otherwise for at least one allele: in the absence of H-2M, the A^b molecule was complexed predominantly with a single CLIP species and antigen presentation was compromised [23, 32, 33]. We have now generated $M^{o/o}$ mice which express not only A^b, but also the $E^{k/b}$ or A^k molecules, the latter claimed not to require HLA-DM for the removal of human CLIP and subsequent loading with heterogeneous peptides [23–25].

Biochemical analyses revealed $E^{k/b}$ in $M^{o/o}$ cells to be predominantly occupied with CLIP, analogous to A^b.

However, these $E^{k/b}$-CLIP complexes were not SDS stable, differing from A^b-CLIP. Furthermore, whereas A^b was occupied predominantly by a single CLIP sequence [19], $E^{k/b}$ bound a heterogeneous set of CLIP species including, but not limited to, the dominant peptide occupying the groove of A^b. The low levels of matched $A\alpha^k A\beta^k$ complexes remaining after preclearing precluded a reliable determination of their stability in SDS, but it appeared that less CLIP was bound by A^k than by A^b molecules in the absence of H-2M (although the mismatched $A\alpha^b A\beta^k$ heterodimers bound copious amounts of CLIP).

Could the distinctions in CLIP species bound to A^b and $E^{k/b}$ in the absence of H-2M reflect allelic differences in affinity for CLIP binding (and/or kinetic stability) and H-2M dependence? How the CLIP region of Ii fits in the groove of a particular class II molecule, as controlled by the affinity and/or stability of the interaction, may dictate the conformation of the complex, rendering different portions of Ii susceptible to proteolytic attack amongst different alleles. Thus, in the process of Ii removal, the CLIP species remaining could differ between class II molecules, and might already be preceded by discernible differences in the staged breakdown of Ii. The point at which class II-Ii complexes require interaction with H-2M *in vivo* (*i.e.* when class II-Ii complexes become substrates for H-2M), to ensure the end-stage of peptide loading, is unknown. The Ii breakdown pattern observed for $A\alpha^b A\beta^k$-Ii complexes in the absence of H-2M was indeed clearly altered. The data presented here, coupled with *in vitro* data reported previously [12, 14, 43], imply that H-2M interacts with class II-Ii complexes before Ii is completely destroyed, conferring a particular conformation of the class II peptide binding cleft.

The varying degrees of allelic/isotypic dependence on H-2M were best revealed through functional assays. In the absence of HLA-DM, most class II molecules are capable of presenting peptides added exogenously [44–46]. This, however, was not the case for A^b in $M^{o/o}$ mice [19], possibly owing to the unique resistance of the A^b-CLIP complex to dissociation in SDS. In contrast, both $E^{k/b}$ and A^k exhibited lower affinities for CLIP [25], and $E^{k/b}$-CLIP complexes were unstable in SDS (Fig. 4). This instability of $E^{k/b}$-CLIP molecules could possibly render them more susceptible to peptide exchange at the cell surface. As the affinities of these two class II molecules for CLIP predicted, the $M^{o/o}$ mutation had no effect on the ability of $E^{k/b}$ to present peptides, and barely affected A^k peptide binding and presentation.

Characterization of the original human DM mutant cell lines demonstrated that transfected HLA-DR1 and -DR3 molecules were incapable of forming intracellular complexes with peptides derived from intact protein antigens, whereas antigen presentation by transfected murine class II molecules showed little reduction in efficiency [23, 24, 26]. "DM-independent" antigen presentation was not restricted to mouse class II molecules expressed in a species-mismatched model, since DR4-restricted T cell responses to endogenous antigens were observed using the same mutant cell line as an APC. Published studies demonstrated that the requirement for HLA-DM in the presentation of antigen by A^k was both epitope and cell line dependent. A^k was defective in presenting KLH and $HEL_{46–61}$ determinants when expressed in the T2 mutant cell line, both of which were presented normally by A^k transfected into the 9.5.3 mutant cell line. Curiously, both KLH and $HEL_{46–61}$ epitopes are dependent on Ii for their presentation [47]. One might expect that epitopes not dependent on Ii for presentation by A^k may also be presented in an H-2M-independent fashion, assuming that in these cases CLIP would not be blocking the peptide binding groove. Indeed, H-2M was absolutely required for presentation of the Ii-dependent epitope $HEL_{46–61}$ by I-A^k, whereas presentation of Ii-independent epitopes $HEL_{34–45}$ and $HEL_{112–129}$ did not require H-2M. However, the correlation between Ii dependence and H-2M dependence was not perfect: presentation of two of the Ii-independent epitopes examined was somewhat impaired by the absence of H-2M. Characterization of $Ii^{o/o}/M^{o/o}$ animals expressing only A^b revealed that H-2M is required to a significant degree for the positive selection of T cells promoted independently of Ii [48].

The characterization of mice expressing more than one type of class II molecule is important to our understanding of class II-restricted antigen presentation in outbred populations. One could predict the introduction of additional class II alleles into the $M^{o/o}$ mice to either attenuate the severity of the mutation, or obliterate it altogether, depending on the particular class II molecule's requirement for H-2M. To our surprise, expression of $E^{k/b}$ overcame the selection defect observed in $M^{o/o}$ mice expressing A^b alone. In contrast, A^k expression was not beneficial to positive selection in the mutant mice. How could the introduction of $E^{k/b}$ rescue positive selection in $M^{o/o}$ animals? Clearly, $E^{k/b}$ is dependent on H-2M for peptide loading, as evidenced by the abundance of CLIP occupying its groove. This was not expected, since it has been demonstrated *in vitro* that CLIP exhibits a rapid-off rate when bound by $E^{k/b}$, not requiring (species-mismatched) DM for its release [23–25]. The increased diversity of CLIP peptides bound to $E^{k/b}$ identified by sequencing could have contributed to the observed selection of a broader repertoire of T cells, or, because CLIP is less strongly bound to $E^{k/b}$ than to A^b, exchange of CLIP for other peptides may have more readily

occurred. Indeed, a facile exchange of peptides in the $E^{k/b}$ groove might explain the response of T cells to the MCC peptide added exogenously. Whatever the mechanism, it clearly did not apply to A^k, which did not rescue positive selection in $M^{o/o}$ animals, even though the presentation of some determinants derived from intact protein antigens by A^k was not impaired by the absence of H-2M. It is possible that the $A\alpha^b A\beta^k$-CLIP complexes present in these animals more closely resemble A^b-CLIP, in that CLIP is very tightly bound within their groove.

In summary, the roles defined for H-2M as a catalyst and peptide editor apply, in some way or another, to all murine MHC class II molecules tested. Yet, the functional dependence on these described H-2M activities in the selection of T cells or in antigen presentation does exhibit subtle variability among different class II alleles or isotypes. Overall, whether or not H-2M is an essential co-factor in functional class II-restricted antigen presentation of a particular organism depends on the combined dependencies of the multiple class II alleles/isotypes expressed.

4 Materials and methods

4.1 Mice

Breeders used in these experiments included the following transgenic and mutant mice: $E\alpha^k$-transgenic mice (carrying the $E\alpha$16 transgene), at the 23rd backcross to C57BL/6 (B6) [29]; $A\alpha^k/\beta^k$-transgenic mice, generated by introducing the $A\alpha$46 and $A\beta$42 transgenes on the B6 background [30]; H-2Ma knockout ($M^{o/o}$) mice on a mixed B6 × 129 background (F2 equivalent) [19]; and H-2Ma wild-type ($M^{+/+}$ or $M^{+/o}$) mice on a mixed B6 × 129 background [19]. After a first cross of the transgenics to $M^{o/o}$ homozygotes, the resulting transgene-positive $M^{+/o}$ offspring were crossed again to $M^{o/o}$ homozygotes. This generated matched sets of transgene-positive and -negative littermates. In a similar fashion, mice carrying either the $A\alpha$46 or $A\beta$42 transgenes singularly were generated. The $E\alpha$ chain gene is invariant among haplotypes, and therefore any isotypic polymorphism observed between haplotypes for E molecules is provided by the β chain. We referred to the E molecules expressed in our mice as $E^{k/b}$ instead of E^b, to denote its transgenic origin.

4.2 Antibodies and flow cytometry

mAb reactive against A^b, $E^{k/b}$, or A^k class II molecules used in this study include Y-3P, 14-4-4s, H11-632 and 10-2-16 [42, 49–51]. Flow cytometric analysis of class II molecule and T cell marker expression was performed as described [49].

4.3 Immunoprecipitations and SDS-PAGE analysis

Spleen cells were labeled with [^{35}S]methionine as indicated in the figure legends, and lysed in NP40. Lysates were precleared four times, followed by immunoprecipitation with control or specific Ab, collected on protein A-agarose, and eluted in reducing SDS-sample buffer either at 95 °C or 20 °C to detect SDS-stable heterodimers [19]. All samples were analyzed by SDS-PAGE using 12.5 % polyacrylamide gels under reducing conditions.

4.4 Isolation and sequencing of CLIP peptides bound to class II complexes

Y-3P or 14-4-4s precipitates from lysates of continuously labeled spleen cells were extracted in 0.1 % trifluoroacetic acid (TFA) at 20 °C and applied to an RP-HPLC column (DeltaPak C18, 39 × 150 mm; Waters). Elution was as follows: 5 % acetonitrile in 0.1 % TFA for 5 min followed by a 5–95 % acetonitrile gradient for over 30 min at 1 ml/min. Fractions (0.5 ml) were collected, and aliquots were counted by liquid scintillation. Fractions generating the major radioactive HPLC peaks were subjected to automated Edman Degradation (25 cycles; performed by the Biopolymers Laboratory at the Massachusetts Institute of Technology), and radioactivity at each cycle counted to determine the N-terminal sequence of CLIP species present in a given fraction.

4.5 Antigen presentation assays

The A^k- or $E^{k,b}$-restricted T hybridomas used in these studies included 2B4 ([50, 52], and received from R. Schwartz), 3B11, 2B5.1, 2C84, 2B6.31 ([53], and received from L. Adorini and F. Momburg) and TS12 ([54], received from P. Allen). All were challenged with their cognate antigen, either as whole protein or as a synthetic peptide, in the presence of splenocytes from various mice (5×10^4 T cells with 3×10^5 whole splenocytes as APC in 250 μl RPMI/10 % FCS in 96-well plates). Twenty-four hours later, IL-2 secretion in the supernatants was assayed by using the IL-2-dependent CTLL cell line, measured as [^{3}H]thymidine incorporation by the indicator cells.

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