is bound. Our results show that DM accumulates in the intracellular class II–containing compartment implicated as the site of class II peptide loading. But whereas classical class II molecules reside principally at the cell surface, in the steady state little if any DM was present there. It is possible that DM does reach the cell surface and is then rapidly internalized. Consistent with this hypothesis is the presence of a Tyr-X-X-Leu (X, any amino acid) sequence in the cytoplasmic tail of DMB (23). This consensus sequence functions in a number of other proteins (including LAMP-1, which colocalizes with DM to the MIIC) as a signal for rapid internalization from the cell surface in clathrin-coated pits. The accumulation of DM in an intracellular compartment where loading of class II molecules probably occurs suggests that DM may be directly involved in this process.

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14. Lysates from Raji cells and from L cells expressing DQ, DP, or DR, as well as lysates from the untransfected cells, were analyzed by SDS-PAGE and protein immunoblotting. Anti-DRα and anti-DP reagents (mouse mAb 1B5 and a polyclonal rabbit antiserum raised against purified DP, respectively) recognized protein in Raji lysates and in the lysate from the relevant transfected cell line, whereas FS2 reacted only with Raji and not with any of the transfected cells. Glycoproteins were prepared from Raji cells and separated by nonequilibrium two-dimensional gel electrophoresis. The second dimension was immunoblotted and the membrane probed with FS2 and with 1B5. These two reagents produced different and unique patterns of reactivity, again indicating that the FS2 reagent does not cross-react with DRα.
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16. On pulse-chase analysis of Raji cells, FS2 precipitated a 30- to 33-kD doublet immediately after a 20-min labeling period. The 30-kD component gradually disappeared over the next 2 hours, leaving a doublet at 33 to 35 kD.
17. Identical protein immunoblots were probed with AK3.
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19. Membranes were prepared from Raji cells with a ball-bearing homogenizer and separated on a Percoll density gradient. Fractions were analyzed as for the MelJuSo cells, with similar results. Here we have presented only the data from MelJuSo, because this system has previously been characterized and described (5).
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26. Anti-DMA (FS2) was raised against a truncated DMA product (amino acids 4 to 104 of the predicted mature protein sequence) produced as inclusion bodies in *Escherichia coli*, by use of a T7 expression system (*24*). Rabbit anti-DMB (AK3) was raised against the COOH-terminal 15 amino acids of the molecule (*13*). AK3 was purified according to a standard protocol (*25*). Blots were developed through the use of enhanced chemiluminescence (Amersham).
27. Raji cells were starved for 30 min in cysteine- and methionine-free medium followed by metabolic labeling for 5 min with [$^{35}$S]methionine and [$^{35}$S]cysteine and lysed in 1% NP-40 lysis buffer. Immunoprecipitation with FS2 was carried out essentially as described (*15*) except that before addition of the antiserum the postnuclear supernatant (PNS) was denatured by boiling for 5 mins in 2% SDS and then diluted 10× in NP-40 lysis buffer. Analysis by SDS-PAGE revealed a doublet with bands at 30 and 33 kD.
28. Raji cells ($7 \times 10^7$) were metabolically labeled for 20 min as described (*15*). The cells were lysed in 0.5% NP-40 lysis buffer and immunoprecipitated with FS2. The immunoprecipitate was eluted off protein A–Sepharose beads by boiling in 2% SDS, 0.5 mM dithiothreitol, 20 mM tris (pH 7.4) for 3 min. Before reprecipitation, the eluate was diluted 10-fold in NP-40 lysis buffer.
29. Sulfosuccinimidyl-6-(biotinamido)hexanoate was used at a concentration of 0.1 mg/ml and incubated either with the glycoprotein preparation (0.1 mg/ml) or with whole cells in phosphate-buffered saline ($5 \times 10^6$/ml) for 1 hour at room temperature. The reaction was stopped by the addition of glycine to 10 mM. Biotin was removed from glycoproteins by spinning through a microfiltration device (Amicon). Immunoprecipitation was carried out as described (*15*) except that the antibodies were prebound to protein A–Sepharose.
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# Reconstitution of an Operational MHC Class II Compartment in Nonantigen-Presenting Cells


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Professional antigen-presenting cells (APCs) have a distinct compartment in which class II molecules are proposed to acquire antigenic peptides. Genetic evidence suggests that human leukocyte antigen (HLA)–DM, an unusual class II molecule, participates in this process. Peptide acquisition was reconstituted in nonprofessional APCs by transfection of class II, invariant chain (Ii), and H-2M, the murine equivalent of DM. The H-2M heterodimer appeared in an endosomal compartment, not at the cell surface, and the localization was independent of Ii. The data presented show that H-2M, class II, and Ii are the minimally required components for efficient formation of stable class II–peptide complexes, and thus for a functional class II compartment.


Recent reports have described the existence of a special major histocompatibility complex (MHC) class II–containing endosomal compartment in APCs where class II is thought to bind (that is, to be loaded with) antigenic peptides (*1*, *2*). This MHC class II compartment has been defined on the basis of subcellular fractionation and electron microscopy, but the biochemical requirements necessary for the function of this compartment and for peptide–class II association are not known. The observation that B cell lines with mutated HLA-DM genes are poor at antigen processing (*3*, *4*) prompted us to investigate the role of H-2M for the formation of a functional class II compartment.

The formation of compact, SDS-stable class II dimers has been correlated with a change in their association with peptides (*5*) and with their ability to present exogenous antigens (*6*, *7*). We asked whether H-2M could improve the formation of stable peptide–class II complexes in nonprofessional APCs transfected with human (HLA-DR3) or murine (H-2A$^k$) class II molecules together with combinations of Ii and H-2M (*8*). We found that only in the presence of both Ii and H-2M could SDS-stable DR dimers be detected (Fig. 1A).


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Neither Ii nor H-2M alone was sufficient to stabilize the DR molecules (9). In contrast, HeLa cells expressing H-$2A^k$ and Ii did give rise to some compact dimers, although in the presence of H-2M the amount of dimer was increased substantially (Fig. 1B). Expression of H-$2A^k$ alone gave rise to minor amounts of a high molecular weight smear but few or no compact dimers. Coexpression with H-2M, in the absence of Ii, increased the amount of SDS-stable smear, but did not lead to appearance of compact dimers.

To see whether stochiometric amounts of class II and H-2M were needed for stabilization, we titrated the amount of H-2M by dilution of the amount of transfected DNA. Omission of H-2M DNA rendered DR molecules SDS sensitive, but even small amounts of H-2M stabilized the DR molecules (Fig. 1C). Immunofluorescence staining showed that the amounts and patterns of DR and Ii expression were not affected by the dilution of H-2M (*10*).

Neither the HLA-DM nor the H-2M protein has been characterized. If H-2M is important for antigen processing, it should be present in normal APCs. To determine if this was the case, we briefly metabolically labeled (pulsed) B10.M splenocytes with $^{35}$S-methionine and $^{35}$S-cysteine. Radiolabeled material was immunoprecipitated from cell lysates either with 10-2-16, a monoclonal antibody reactive with H-$2A^f$ (*11*), or with K553, a rabbit antiserum to purified H-2M (*12*). SDS–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the 10-2-16 precipitate showed a series of bands 31 to 41 kD in size, as expected for H-$2A^f$ (*13*) (Fig. 2, A and B). The K553 antiserum precipitated bands of 27 to 32 kD immediately after labeling (Fig. 2C). One of the bands probably was Iip31, and similar analysis of pulse-labeled HeLa cells separately transfected with the H-2Ma (Ma), H-2Mb (Mb), or Iip31 complementary DNA (cDNA) clones allowed us to identify the major bands. Endoglycosidase H (Endo H) treatment of the precipitates reduced the size of Ii and Mα to the same extent (Fig. 2D), indicating that Mα, like Ii, contained two N-linked carbohydrates, as predicted from the Mα amino acid sequence (*14*). Addition of unlabeled medium to the prelabeled B10.M splenocytes for increasing times caused Ii to disappear and Mα to become more resistant to Endo H treatment. However, as with other class II α chains, only one of the two N-linked carbohydrates acquired resistance to Endo H (*15*). Mβ, in contrast, was not sensitive to Endo H, consistent with the lack of N-linked addition sites in the Mb2 sequence (see below) (*16*). Two-dimensional gel electrophoresis of K553-precipitated material (Fig. 2E) confirmed that the band seen in Fig. 2, A to D, was indeed Iip31. The Mα chain is acidic and barely migrated into the gel under the conditions used.

The recently described class II compartment is part of the endosomal system, and H-2M could therefore be expected to have an endosomal location if it is directly involved in peptide processing. We investigated the subcellular localization of H-2M using indirect immunofluorescence (*17*) (Fig. 3). Figure 3A shows the costaining of H-$2A^d$ and H-2M in a B cell line analyzed by confocal microscopy. The separate images (Fig. 3B) show H-$2A^d$ to be present both intracellularly and at the plasma membrane. The H-2M staining, in contrast, was almost completely located to intracellular vesicles. Virtually no staining of the plasma membrane could be seen. The lack of H-2M at the cell surface was confirmed by K553 staining of nonpermeabilized HeLa cells transiently transfected with the H-2M cDNAs, as well as with H-$2A^k$ and Ii (Fig. 3C). H-$2A^k$, in contrast, was abundant at the plasma membrane (Fig. 3D). Staining of permeabilized cells expressing the same molecules showed staining similar to that seen in the B cell line. Figure 3, E and G, show the distinct vesicular localization of H-2M in permeabilized HeLa cells cotransfected with only the Ma and Mb cDNAs. To a large extent the H-2M–containing vesicles costained with the lysosomal markers LAMP-1 (Fig. 3F) and CD63 (*18*), and

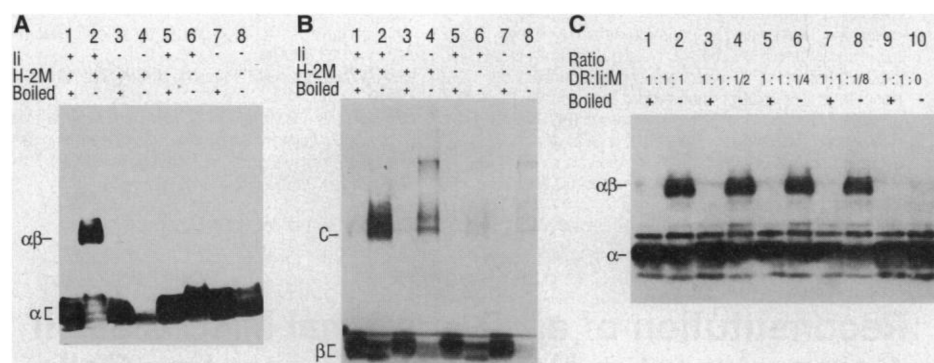


**Fig. 1.** Effect of H-2M expression on peptide loading of class II in transfected HeLa cells. (**A**) Formation of SDS-stable dimers of HLA-DR3 in cells transfected with different combinations of DR3, H-2M, and Ii (*6*). Lanes 1 and 2 represent transfections with all three components, lanes 3 and 4 transfections with only DR3 and H-2M, and lanes 5 and 6 transfections with only DR3 and Ii. Lanes 7 and 8 represent transfections with only DR3. (**B**) Transfections as in (A), except that DR3 was substituted for H-$2A^k$ and murine Ii was used instead of human Ii. (**C**) Formation of SDS-stable dimers of HLA-DR3 in cells where the amount of H-2M was titrated by substitution of Ma and Mb cDNAs with vector DNA. Lanes 9 and 10 represent transfections where no H-2M DNA was included. Whole-cell extracts in SDS sample buffer were either boiled or left at room temperature before being subjected to SDS-PAGE and transfer to protein immunoblots. Blots were probed with mAb DA6.147 (anti-DRα) (*34*) (A and C) and with monoclonal antibody 10-2-16 (anti–H-$2A\beta^k$) (B). In (A) and (C), α indicates DRα monomers, αβ indicates DR heterodimers; in (B), β indicates H-2Aβ monomers and C indicates compact H-2A dimers.

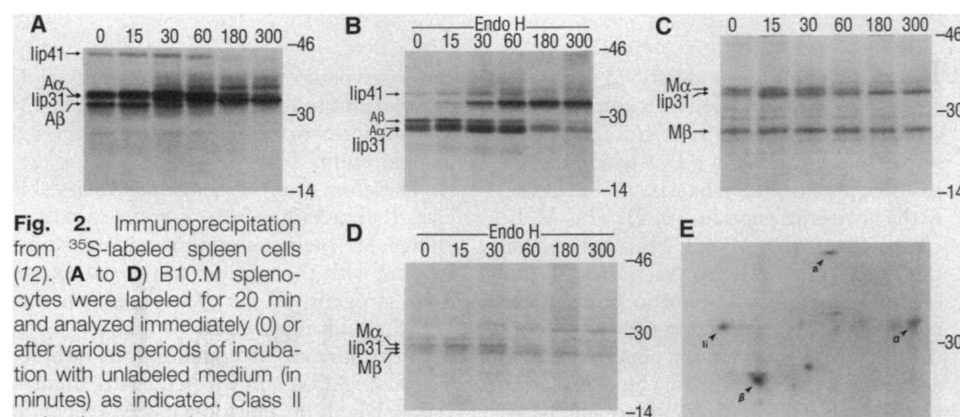


**Fig. 2.** Immunoprecipitation from $^{35}$S-labeled spleen cells (*12*). (**A** to **D**) B10.M splenocytes were labeled for 20 min and analyzed immediately (0) or after various periods of incubation with unlabeled medium (in minutes) as indicated. Class II molecules were immunoprecipitated with 10-2-16 (anti–H-$2A^{f,k}$) (A and B) or K553 (anti–H-2M) (C and D). Samples were analyzed by SDS-PAGE without (A and C) or with (B and D) Endo H treatment. (**E**) B10.M splenocytes were labeled for 4 hours. H-2M was immunoprecipitated from the cell lysates with K553 and analyzed by two-dimensional gel electrophoresis. a, actin; α, H-2Mα; β, H-2Mβ; Ii, invariant chain p31.

to some extent also with the cation-independent mannose-6-phosphate receptor (M6PR) (Fig. 3H), a marker for late endosomes (*19*); however, the vesicles did not stain for transferrin receptors or γ-adaptin, markers for early endosomes and the trans-Golgi network, respectively (*20*). H-2A$^k$ was not seen in vesicles in the absence of Ii, confirming the role of Ii in directing class II to the endosomal pathway (*21*). In contrast, the presence or absence of Ii did not affect the localization of H-2M, indicating that H-2M has its own targeting information. K553-staining of cells transfected with only Ma (Fig. 3I) or Mb (*10*) cDNAs showed endoplasmic reticulum (ER) staining. Therefore the two H-2M chains, like those of other class II molecules, must associate in order to be transported out of the ER.

To see whether the H-2M-containing compartment was accessible to fluid-phase markers, we allowed HeLa cells to internalize biotinylated dextran for increasing lengths of time. After 1 hour, vesicles costaining for H-2M and dextran could be seen, and after 2.5 hours a large number of vesicles stained for both markers (Fig. 3J).

The H-2M gene locus contains one Ma gene and two Mb genes (*22*). Sequence analysis of genomic DNA showed that the published Mb cDNA sequence corresponds to the Mb1 gene (*23*). The Mβ2 protein sequence was found to be quite different from the Mβ1 sequence, with the differences concentrated to the membrane-distal domain (Fig. 4). The site for N-linked glycosylation in Mβ1 is not present in Mβ2. Unexpectedly, when we analyzed splenocyte mRNA sequences from five different mouse strains, we could only detect Mb2 sequences. Further analysis indicated that less than 10% of spleen Mb RNA was derived from the Mb1 gene, a finding consistent with the apparent lack of glycosylated Mβ1 protein in the immunoprecipitation experiments. As with the human DM genes (*24*), we found the Ma and Mb2 sequences to be essentially nonpolymorphic.

Our data show that H-2M is expressed as a heterodimer in normal splenocytes. It is mainly localized to the endosomal pathway and only small amounts are present at the cell surface, confirming previous suggestions that DM would function intracellularly (*3*). H-2M is associated with Ii during synthesis but does not, in contrast to other class II molecules, appear to require Ii for targeting. Two other functions

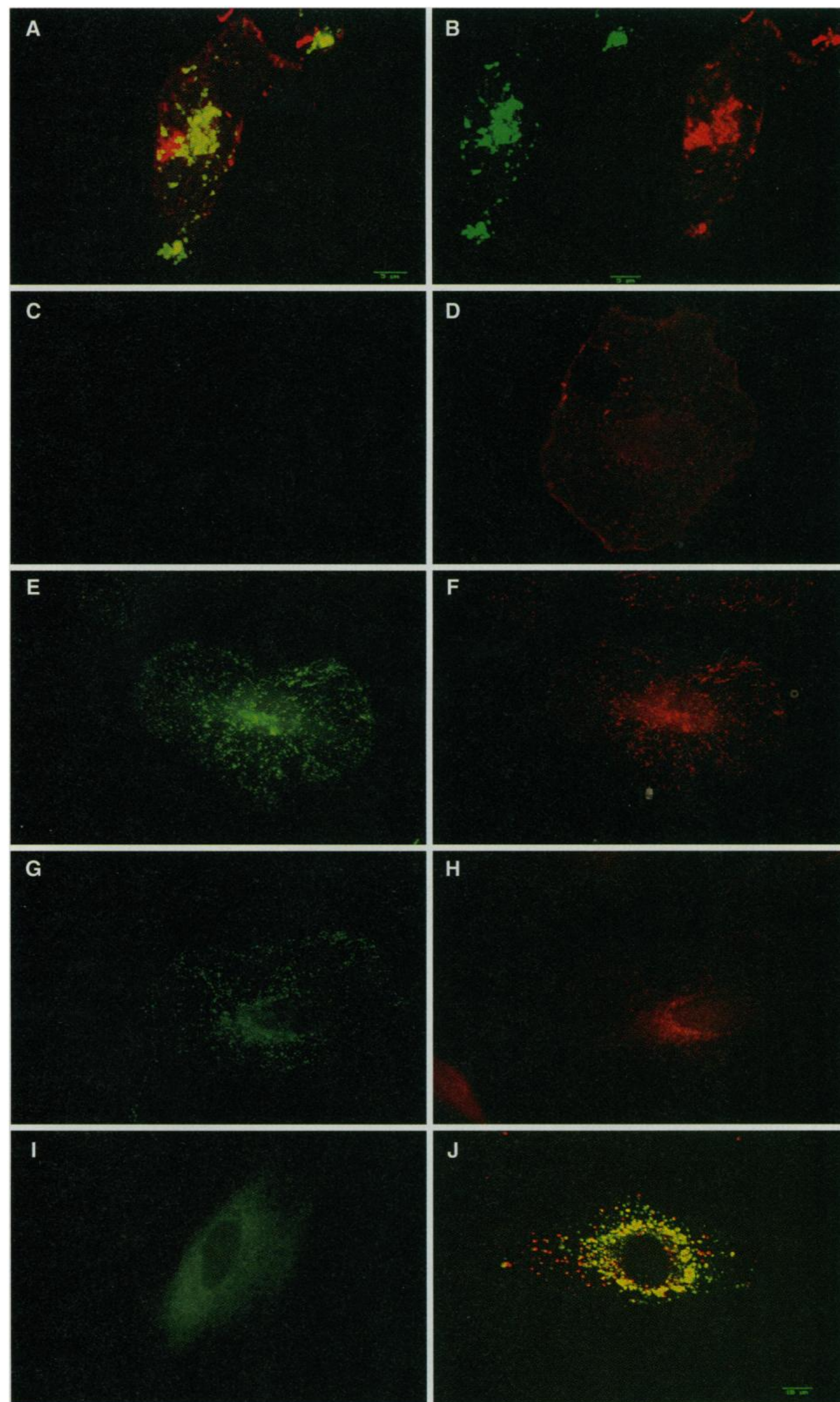


**Fig. 3.** H-2M has an endosomal location (*17*). (**A** and **B**) Confocal images of LK35.2 stained with K553 (anti–H-2M) (green) and BP107 (anti–H-2A$^d$) (red) (*35*). Yellow staining indicates colocalization (A). The separated images (B) show K553 staining to be confined to vesicular structures, whereas the BP107 staining is located both at the cell surface and intracellularly. (**C** and **D**) HeLa cells transfected with H-2M, H-2A$^k$, and Ii. In nonpermeabilized cells, K553 staining is absent from the plasma membrane (C), whereas 10-2-16 staining (anti–H-2A$^k$) is abundant at this location (D). (**E** to **H**) H-2M–transfected HeLa cells, in the absence of class II and Ii, costained with K553 (E) and LAMP-1 (F), or K553 (G) and CI-M6PR (H). (**I**) HeLa cells transfected with only Ma stained with K553 show the single chains to be localized in the ER. (**J**) Confocal image of HeLa cells transfected with H-2M, H-2A$^k$, and Ii. Cells were allowed to internalize biotinylated dextran for 2.5 hours before fixation and staining with K553 (green) and streptavidin (red). Yellow staining indicates colocalization.

```
         -18                β1→
Mβ2      MAALWLLLLVLSLHCMGAGGFVAHVESTCVLNDAGTPQDFTYCVSFNKDL 32
         -------------------------------*------------------
Mβ1      MAALWLLLLVLSLHCMGAGGFVAHVESTCVLDDAGTPQDFTYCVSFNKDL

         LACWDPDVGKIVPCEFGVLSRLAEIISNILNEQESLIHRLQNGLQDCATH 82
         ------*------------**---**-*---**---**------*---*-
         LACWDPIVGKIVPCEFGVLYPLAENFSRILNKEESLLQRLQNGLPDCASH

                      β2→
         TQPFWDVLTHRTRAPSVRVAQTTPFNTREPVMLACYVWGFYPADVTITWM 132
         -----**------*------------------------------------
         TQPFWNALTHRTRPPSVRVAQTTPFNTREPVMLACYVWGFYPADVTITWM

         KNGQLVPSHSNKEKTAQPNGDWTYQTVSYLALTPSYGDVYTCVVQHSGTS 182
         --------------------------------------------------
         KNGQLVPSHSNKEKTAQPNGDWTYQTVSYLALTPSYGDVYTCVVQHSGTS

                 cp→       tm→                   cyt→
         EPIRGDWTPGLSPIQTVKVSVSAATLGLGFIIFCVGFFRWRKSHSSSYTP 232
         --------------------------------------------------
         EPIRGDWTPGLSPIQTVKVSVSAATLGLGFIIFCVGFFRWRKSHSSSYTP

         LPGSTYPEGRH                                        243
         -*---------
         LSGSTYPEGRH
```

**Fig. 4.** Comparison of amino acid sequences (*23*) between H-2Mβ2 sequence derived from BALB/c (Mβ2[d]) and the previously published H-2Mβ1 sequence (Mβ1[d]). Dashes indicate identity between the sequences; asterisks indicates amino acid differences. The signal sequence is underlined and the N-linked carbohydrate addition site is double-underlined. Amino acids are numbered from the putative first amino acid in the mature protein. cp, connecting peptide; tm, transmembrane region; cyt, cytoplasmic tail.

have been attributed to Ii: It facilitates class II assembly and transport out of the ER, and it prevents premature antigen binding to class II molecules (*25*). Because H-2M assembly is not noticeably affected by the presence of Ii (*10*), we propose that Ii binding to H-2M serves to prevent H-2M from exercising its function before H-2M arrives in the endosomal system. The finding that H-2M increases the amount of SDS-stable H-$2A^k$ also in the absence of Ii, suggests that removal of Ii or Ii-derived CLIP (class II–associated invariant chain peptides) fragments from class II is not a direct function of H-2M, although H-2M may induce conformational changes in class II leading to decreased affinity for Ii and increased ability to bind other peptides or proteins. In the absence of Ii, only the latter effect would be seen. The present data also do not exclude a function of H-2M as a peptide carrier delivering peptides to class II.

Class II, Ii, and H-2M were sufficient to induce peptide loading onto class II in nonprofessional APCs, but it is not clear which structural compartments are necessary for antigen processing and whether these are specialized or general in nature. Morphologically distinct class II–rich endosomal compartments (MIICs) have been described in B cells (*1*), but the relation between the structural MIIC and functional class II loading remains unclear. Recent reports describe MIIC-like structures in transfected cells expressing only class II or, in a mutant cell line, expressing only Ii (*26*) (although transfection of class II, in the absence of DM or H-2M, into the mutant cell line disrupts the MIIC structure). One explanation for these findings is that expression of either class II, Ii, or possibly H-2M can induce MIIC-like structures. Alternatively, similar compartments may already exist in most cell types, assuming the role of "class II compartment" and becoming more pronounced when the cells express class II, Ii, or H-2M. Different types of professional APCs may also contain special compartments or molecules that may skew the repertoire of presented peptides. However, for formation of stable class II–peptide complexes, expression of class II, Ii, and H-2M appears to be both necessary and sufficient. The model system presented here is easily manipulated and will allow us to investigate the true nature of the class II compartment.

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7. In contrast to the human HLA-DR, murine H-2A has been described to migrate in SDS-PAGE gels in two different SDS-stable conformations, "floppy" and "compact," in addition to the single α and β chains (*25*, *27*). The compact form is thought to contain peptide, but also unstable and possibly floppy dimers can contain peptides and are not necessarily empty (*28*).
8. HeLa cells were transiently transfected (*29*) with constructs encoding DRA, DRB3, Ma, Mb, human Iip31-p33, and human Iip41 (Fig. 1, A and C) or $Aa^k$, $Ab^k$, Ma, Mb, murine Iip31, and murine Iip41 (Fig. 1B) in molar ratios 1:1:1:1:1.5:0.5. (Fig. 1, A and B). Vector DNA was used to substitute for omitted DNAs as indicated. Seventy-two hours after transfection, the cells were detached from the plates with 2 mM EDTA–phosphate-buffered saline (PBS) and lysed in nonreducing SDS–sample buffer containing 2% SDS. Samples were split into two portions, one of which was boiled for 5 min and one of which was left at room temperature for 20 min. Samples were separated on 10% polyacrylamide gels and blotted onto nitrocellulose. Horseradish peroxidase–labeled antibody to mouse immunoglobulin (Ig) was used together with enhanced chemiluminescence (ECL, Amersham).
9. The decreased level of DRα monomer in lane 4 is consistently seen, despite the lack of DR dimers. We do not see increased high molecular weight staining in this lane even with long exposures.
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12. Ma and Mb cDNAs (*15*) were modified so that the transmembrane and cytoplasmic tails of Ma and Mb were replaced by six histidines followed by a stop codon. These constructs were expressed in *Drosophila melanogaster*–derived Schneider cells (*30*). Soluble H-2M protein was purified from the culture medium by metal ion chromatography and gel filtration. The protein (90% pure) was used to immunize rabbits. The serum K553 reacted specifically with H-2M dimers and monomers (both α and β) in immunofluorescence and immunoprecipitation from transfected HeLa cells. No reactivity was seen with either dimers or monomers of H-2A or H-2O. Monoclonal antibodies 10-2-16 and BP107 do not recognize transfected cells expressing H-2M in immunofluorescence or immunoprecipitation. Cell labeling and immunoprecipitation were done as described (*13*). Immunoprecipitates were harvested with protein A–Sepharose, washed, and resuspended in SDS-PAGE sample buffer or isoelectric focusing (IEF) sample buffer. Samples were separated on 7.5 to 12.5% polyacrylamide gels directly or after NEPHGE (pH 3.5 to 10). Gels were fixed, dried, and autoradiographed.
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16. Note that the Mα chain decreases slightly in size with time. The blurriness of the Mβ band seen at early time points in the Endo H–treated samples is due to the superimposition of Ii and Mα.
17. LK35.2 cells were grown on cover slips coated with Cell-Tak (Collaborative Biomedical Research, Bedford, MA) before fixation with 4% formaldehyde–PBS. HeLa cells were transfected as in Fig. 1 with expression plasmids encoding the indicated cDNAs. After 48 hours, the cells were trypsinized and plated onto cover slips coated with Cell-Tak and grown for 12 hours before fixation with 4% formaldehyde–PBS, except for Fig. 3J where the cells were incubated in medium with biotinylated fixable dextran (2 mg/ml; Molecular Probes) for different times before fixation. After fixation, cells were washed with 50 mM $NH_4Cl$, PBS. Antibody incubations were made in PBS with 0.6% fish skin gelatin and 0.2% saponin for permeabilization, except in Fig. 3, C and D, where the saponin was excluded. Texas red–labeled streptavidin was used to detect biotinylated BP107 for staining H-$2A^d$ on LK35.2 and to detect biotinylated dextran in Fig. 3J. For all other stainings, fluorescein isothiocyanate–labeled antibody to rabbit Ig (Cappel) and Texas red–labeled antibody to mouse immunoglobulin (Molecular Probes) secondary reagents were used.
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CBA/J, and SJL mice were reverse-transcribed according to a standard protocol (32). Primers derived from the untranslated regions of the published Ma and Mb sequences (10) were used in polymerase chain reaction to amplify DNA from the first strands. This noncloned DNA was directly sequenced with the use of internal Ma or Mb primers (32) and fluorescent terminators. Genomic DNA derived from cosmid 5.22 (33) was subcloned and sequenced in the same way. For quantitation of the relative amounts of Mb1 and Mb2 transcripts, we used pairs of internal primers derived from sequences identical in both transcripts. Amplified material was divided into two portions and digested with Eae I (Mb1-specific) and Xba I (Mb2-specific), respectively. More than 90% of the material was cleaved with Xba I, less than 10% with Eae I. The amplified Ma and Mb cDNAs from B10.M were cloned. These clones were transferred to suitable expression vectors and used for all transfection experiments. Sequence analysis of spleen Mb cDNA showed that the Mβ2 amino acid sequences were identical in BALB/c (H-2$^{d}$), C57BL/6 (H-2$^{b}$), and B10.M (H-2$^{f}$). In SJL (H-2$^{s}$) and CBA/J (H-2$^{k}$), amino acid 70 was Q, not H, and amino acid 96 was P, not A. In CBA/J, amino acid 105 was A, not T. Compared to the published Mα sequence, we found the following differences: B10.M, C57BL/6, BALB/c, SJL, and CBA/J all had amino acid 75 D, not G; amino acid 101 P, not S; amino acid 194 Q, not H; and amino acid 212 G, not A. C57BL/6, SJL, and CBA/J had amino acid 220 M, not I. C57BL/6 also had amino acid 9 F, not L. SJL had amino acid 7 S, not P and amino acid 89 Q, not K. Amino acid numbering is as in Fig. 4. DNA sequences are available on request. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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# Reversion of the Mouse *pink-eyed unstable* Mutation Induced by Low Doses of X-rays

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Deletions and other genome rearrangements can be caused by radiation and are associated with carcinogenesis and inheritable diseases. The *pink-eyed unstable* ($p^{un}$) mutation in the mouse is caused by a gene duplication and reverts to wild type by deletion of one copy. Reversion events in the mouse embryo were detected as black spots on the fur of the animals or microscopically as partially black hair in a background of colorless hair. The frequency of partially black hair was increased by x-rays at very low doses. A linear dose-response relation was found between 1 and 100 centigray.

Sources of low-level radiation are almost ubiquitous in our environment and include nuclear explosions, radiation accidents, and medical diagnostic, therapeutic, and occupational exposure. Consequently, there is much interest in the occurrence of carcinogenesis and the genetic effects associated with exposure to low-dose radiation. The Oxford childhood survey that was started in the 1950s and other studies have shown about a twofold increase in the occurrence of cancer after diagnostic intrauterine x-ray exposure at doses of approximately 2 cGy (*1*). In most cases no increased risk has been detected after exposure to doses below 10 cGy among atomic bomb survivors and individuals exposed to therapeutic irradiation (*1*). This finding may indicate a higher susceptibility of fetal tissue to radiation. Currently, no genetic end points or biological markers in animals or humans are available to detect irradiation doses close to or below 2 cGy. Only one study suggests that doses below 10 cGy may be mutagenic in human lymphoblasts (*2*). However, this result was obtained by applying 30 daily doses of 1 to 10 cGy, and no effect was found with an acute x-ray exposure of 5 cGy.

Ionizing radiation is mutagenic and carcinogenic and preferentially induces deletions rather than point mutations (*3*). Genome rearrangements such as deletions are frequently associated with tumor cells (*4*). Because of this association, a system selecting for deletions by intrachromosomal recombination has been constructed in the yeast *Saccharomyces cerevisiae* (*5*) and has been termed deletion (DEL) assay. DEL recombination can be induced with a wide variety of carcinogens, including x-rays and carcinogens that have no effect in most other short-term tests (*6*). In addition, deletion of one copy of a duplication of part of the *hprt* gene in CHO cells can be induced by x-rays and by several mutagenic carcinogens (*7*).

To determine the effect of x-ray exposure on the frequency of deletion events between two alleles of a gene duplication in mammals in vivo, we used the *pink-eyed unstable* ($p^{un}$) mutation in the mouse. The $p^{un}$ mutation causes a reduction in the pigment in coat color and eye color. The $p^{un}$ mutation is caused by a disruption of the *pink-eyed* dilute locus, which results in a DNA sequence duplication of about 70 kb in a head to tail conformation (*8*). Spontaneous reversion of $p^{un}$ is caused by a deletion of one of the two copies of the duplicated sequence, which results in production of wild-type melanin in melanocytes. Reversion events are measured as black spots on the gray coat. The reversion frequency of $p^{un}$ is at least three to five orders of magnitude greater than that of other recessive mutations at other coat color loci (*9*). Approximately 1.8% (*8*) to 3.8% (*10*) (5.6% in our study) of the offspring of homozygous C57BL/6J $p^{un}/p^{un}$ mice have patches of wild-type color in their coats and are thus mosaic revertants.

Homozygous mice (C57BL/6J $p^{un}/p^{un}$) (*11*) were used in these experiments. An increase in reversion events would give rise to an increase in the number of animals that show dark patches. The protocol used for this test was similar to the "mouse spot test" (*12*). Matings were set up between mice homozygous for $p^{un}$ and pregnancy was timed. Female mice were irradiated with 100 cGy of x-rays at 8.5, 9.5, and 10.5 days after conception. Dark patches on the coats of the offspring were counted, and their size and distribution were recorded. Irradiation at 8.5 days after conception caused neonatal deaths in about 40% of the offspring (Table 1). With irradiation at later stages, the viability of offspring improved, and only about 1% of neonatal deaths occurred when irradiation was done 10.5 days after conception. Less than 20% of the offspring irra-

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