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32. Crystal structures of Eco RV endonuclease bound to specific and nonspecific DNA fragments recently have been solved (*31*). The DNA in the specific complex has a 50° kink in the central TA step, and the overall conformation deviates significantly from that of B-form DNA; the DNA in the nonspecific complex, on the other hand, has a conformation similar to that of B-DNA. However, two fragments of DNA are bound to Eco RV in the nonspecific complex, with the terminal base pair of each duplex forming what would be the TA step in the specific complex. Because the DNA is discontinuous at the position where the greatest distortion is expected to occur, the conformation of DNA in such a complex might be significantly different from that of Eco RV bound to a continuous, nonspecific site.
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37. In view of the uncertainty about the exact number of base pair contacts involved in the specific and nonspecific Cro-DNA complexes, we have roughly estimated this number to be 20 bp (*14*).
38. It has been suggested that binding of a protein to a single side of DNA, as is the case for Cro, might favor DNA bending [J. B. Matthew and D. H. Ohlendorf, *J. Biol. Chem.* **260**, 5860 (1985)], thereby providing part of the driving force for protein-induced bending of nonspecific DNA.
39. This fragment is generated by first digesting the plasmid pDE13 [D. A. Erie, O. Hajiseyedjavadi, M. C. Young, P. H. von Hippel, *Science* **262**, 867 (1993)] with Sph I and then with Hind III. It is then separated by agarose gel electrophoresis, cut out of the gel, and purified with glass powder [B. Vogelstein and D. Gillespie, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 615 (1979)], followed by ethanol precipitation.
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43. We thank B. W. Matthews, E. Baldwin, W. A. Rees, B. Shoicet, L. Jen-Jacobson, P. H. von Hippel, and many of our laboratory colleagues for helpful discussions and comments on the manuscript. We are grateful to R. Albright and B. Matthews for providing us with purified λ Cro protein. Supported by U.S. Public Health Service (USPHS) research grants GM-32543 (C.B.), GM-15792 and GM-29158 (P. H. von Hippel), GM-20066 (B. W. Matthews), National Science Foundation grants MCB-9118482 (C.B.) and MCB-18945 (C.B. and D.A.E.), USPHS Postdoctoral Fellowship GM-12915 (D.A.E.), and a grant from the Lucille P. Markey Charitable Trust.

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Accumulation of HLA-DM, a Regulator of Antigen Presentation, in MHC Class II Compartments

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The *HLA-DM* genes encode an unconventional HLA (human leukocyte antigen) class II molecule that is required for appropriate binding of peptide to classical HLA class II products. In the absence of DM, other class II molecules are unstable upon electrophoresis in sodium dodecyl sulfate and are largely associated with a nested set of peptides derived from the invariant chain called CLIP, for class II–associated invariant chain peptides. DMA and DMB associated and accumulated in multilaminar, intracellular compartments with classical class II molecules, but were found infrequently, if at all, at the cell surface. Thus, DM may facilitate peptide binding to class II molecules within these intracellular compartments.

Major histocompatibility complex (MHC) class II molecules consist of heterodimers of a ~34-kD α and a ~28-kD β chain that interact with a third glycoprotein, the invariant chain (Ii) in the endoplasmic reticulum (ER). This complex is transported out of the ER through the Golgi stacks as a nonamer $(\alpha\beta Ii)_3$ (*1*). Invariant chain is then proteolytically removed and class II molecules accumulate in an acidic, lysosome-like compartment, termed MIIC in B lymphocytes (*2*). MIIC is placed late in the endocytic route and is a potential site for peptides derived from exogenous antigens to associate with class II molecules. Similar compartments have since been characterized in other cell types (*3*–*6*).

Insight into how peptides bind (that is, get loaded onto) class II molecules was provided by a series of cell lines with mutations in genes in the class II region of the MHC (*7*, *8*). These cell lines have three interesting properties: (i) class II molecules on the cell surface are not recognized by certain conformation-specific antibodies, such as the DR3 antibody 16.23; (ii) upon polyacrylamide gel electrophoresis (PAGE) in SDS, class II heterodimers are less stable than those of normal cells and dissociate into their constituent polypeptide chains; and (iii) the mutant cells are defective in the presentation of protein antigens to T cells (*9*). All three phenotypes of the mutant cells are the result of mutations in either of the linked *DMA* and *DMB* genes (*7*, *8*). In addition, another mutant cell line with a large deletion encompassing the *DM* locus (T2-DR3) develops abnormal MIIC and has class II molecules predominantly associated with the class II invariant chain–associated peptides (CLIP) (*10*, *11*).

To investigate further the function of DM, we made rabbit antisera; FS2 recognizes recombinant DM α chain protein and AK3 recognizes the derived COOH-terminal peptide of the DM β chain. To demonstrate the specificities of the antisera, we used glycoprotein preparations from the B lymphoblastoid cell line LCL721, the deletion mutant 721.174, and the cell line 7.9.6, which bears a point mutation in *DMB* (*7*, *9*, *12*). Both antisera recognized specific bands on protein immunoblots of 33 to 35 kD and 30 to 31 kD, for antibody to DMA (anti-DMA) and anti-DMB, respectively (Fig. 1, A and B). These values are consistent with the predicted molecular sizes of 26 kD given that the DMA sequence contains two putative N-linked glycosylation sites and DMB, one (*13*). As additional specificity controls, we demonstrated that anti-DMA did not cross-react with conventional class II molecules expressed in L cell transfectants and identified unique spots on two-dimensional SDS-PAGE (*14*).

The sequence similarities between DM and the classical class II genes, their locations adjacent to one another in the class II region, and the identical phenotypes of *DMA* and *DMB* mutants all suggest that the DM α and β chains associate to form a heterodimer. To confirm this, cells from the Burkitt's lymphoma cell line Raji were metabolically labeled for 20 min and lysed. Extracts were precipitated with the antiserum to DMA, FS2 (Fig. 2A). Immunoprecipitated protein was eluted from protein A–Sepharose and reprecipitated with either anti-DMA or anti-DMB. Reprecipitation with anti-DMA revealed bands at 30 and 33 kD. These were specific for DMA, and both were seen after metabolic labeling with ^{35}S for 5 min (*15*). The lower band is not present in appreciable quantities in the

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steady state (Fig. 1A) (*16*) and may represent an incompletely glycosylated form of DMA that is degraded in the ER. Reprecipitation with anti-DMB produced a band at 30 kD. No protein was isolated by AK3, when incubated with the peptide used for immunization. Thus, we showed that DMA and DMB associate and that this interaction takes place, as it does for classical class II molecules, early during biosynthesis. The association is not necessarily direct, and DMA and DMB could form a complex with other proteins.

Next we looked for expression of DM at the cell surface (Fig. 2B). Whereas DM in glycoprotein isolates was successfully biotinylated and could be immunoprecipitated with AK3, little if any was detected with the same antibody after biotinylating only cell surface proteins. DR was precipitated in large quantities from both preparations. These data suggest that unlike the classical class II molecules, DM is found mainly intracellularly.

To further define where in the antigen-processing pathway DM operates, we decided to localize it within the cell. Subcellular fractionation has recently been used as a means of analyzing class II antigen presentation (*3–6*). We used this technique to examine the steady-state distribution of DM in the melanoma cell line MelJuSo. Microsomes were separated in an electric field, and the positions of lysosomes (β-hexosaminidase activity) and of the total protein peak were determined (Fig. 3A). The fractions isolated from MelJuSo were then analyzed by protein immunoblotting and stained with antibodies to class I molecules (to determine the position of the

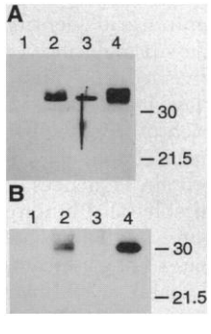

Fig. 1. Specificity of DMA and DMB antisera. Glycoproteins extracted on lentil lectin Sepharose (Pharmacia) were prepared, separated by 12% SDS-PAGE, and immunoblotted onto polyvinylidene difluoride membranes. Protein immunoblots were probed with (**A**) FS2 (anti-DMA) and (**B**) affinity-purified AK3 (anti-DMB). Lane 1, mutant 721.174 with a deletion encompassing much of the class II region of the MHC; lane 2, LCL721 parent cells; lane 3, mutant 7.9.6 with a point mutation in *DMB*, resulting in loss of *DMB* mRNA expression and 16.23 antibody reactivity (*7*); lane 4, Raji, Burkitt's lymphoma. The tight doublet bands may represent ER-confined forms (lower band) and post-ER forms with more mature carbohydrates (upper band) (*17*). Lanes 1 to 3 were loaded with glycoprotein extracted from 7×10^6 cells, lane 4 with glycoprotein from 2×10^6 cells (*26*). Molecular sizes are indicated on the right (in kilodaltons).

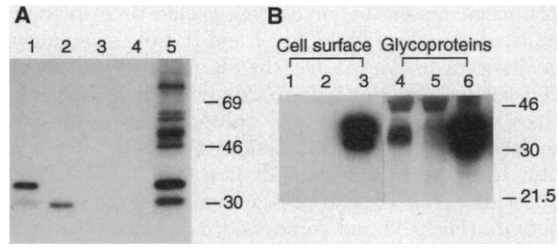

Fig. 2. (**A**) DMA-DMB association. Proteins from metabolically labeled Raji cells were immunoprecipitated with FS2 and then eluted from the protein A–Sepharose (*27*). One-tenth of the eluate was removed for loading directly onto the gel (lane 5), and the remainder was divided into four portions and reprecipitated with FS2 (anti-DMA) (lane 1), AK3 (anti-DMB) (lane 2), AK3 in the presence of competing DMB COOH-terminal peptide (lane 3), and an irrelevant antiserum (lane 4) (*28*). (**B**) Cell surface biotinylation. Whole Raji cells (lanes 1 to 3) and glycoproteins extracted from Raji lysates on lentil lectin Sepharose (lanes 4 to 6) were biotinylated with sulfosuccinimidyl-6-(biotinamido)hexanoate (Pharmacia). The cells were washed and lysed in NP-40 lysis buffer and thereafter the two samples were treated in an identical fashion by two rounds of preclearing followed by immunoprecipitation with AK3 (lanes 1 and 4), or AK3 preincubated with competing COOH-terminal peptide (lanes 2 and 5) or the DRα mAb 1B5 (lanes 3 and 6). The immunoprecipitates were separated by 12% SDS-PAGE, immunoblotted, and biotinylated proteins detected with avidin–horseradish peroxidase and enhanced chemiluminescence (*29*). Molecular sizes are indicated on the right (in kilodaltons).

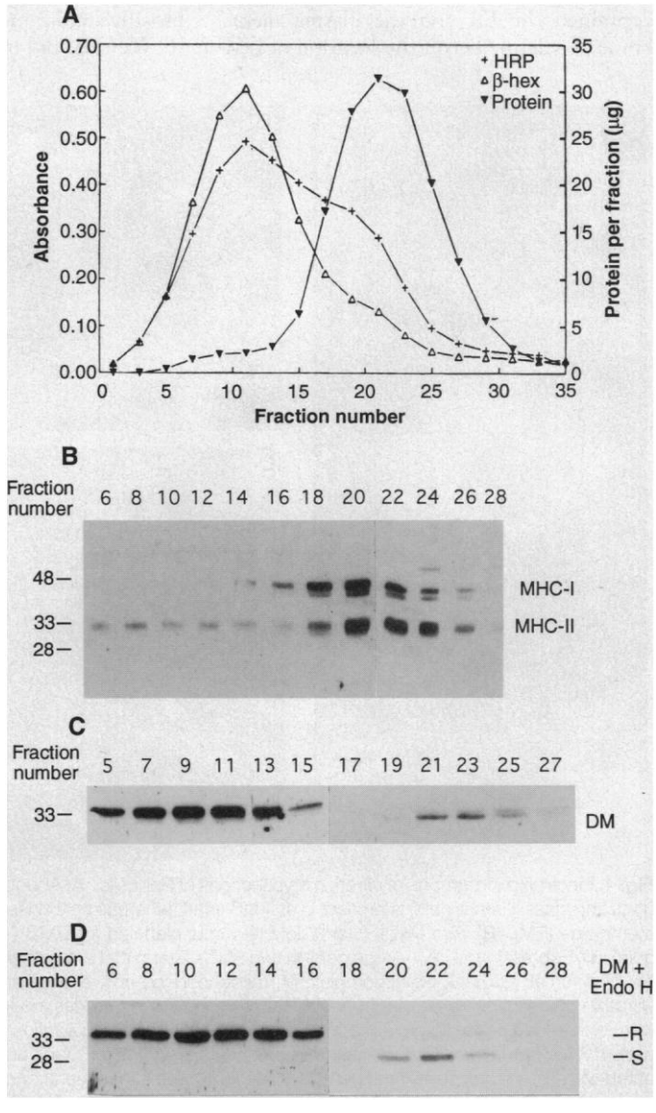

Fig. 3. Steady-state localization of DM in melanoma cells. The melanoma cell line MelJuSo was loaded with fluid phase HRP for 4 min and then chased for 10 min in HRP-free medium before fractionation. Postnuclear supernatant was generated, and the vesicles were separated in an electric field by density gradient electrophoresis (*5*, *19*). Before analysis by SDS-PAGE and protein immunoblotting, proteins were concentrated with trichloroacetic acid (TCA). (**A**) The enzymatic activity of β-hexosaminidase and HRP (left vertical axis), as well as the protein content in each fraction (right vertical axis), were determined. (**B**) The presence of HLA class I and II molecules in the fractions was determined by probing protein immunoblots with the class I mAb HC-10 (*30*) and an antiserum to class II β chain (*31*). The positions of HLA class I (MHC-I) and HLA class II (MHC-II) are indicated. Molecular size standards are indicated on the left (in kilodaltons). (**C**) The presence of DM in the fractions was determined with anti-DMA (FS2). (**D**) The position of DM molecules containing mature carbohydrates. TCA-precipitated proteins were digested overnight with Endo H before immunoblotting and detection with FS2. The positions of Endo H–resistant (R) and Endo H–sensitive (S) forms are indicated on the right. Samples for Endo H digestion were taken from the same fractionation as those analyzed in (C).

plasma membrane and of the ER), to class II molecules, and to DM. Class I and II molecules were distributed over the ER and the cell surface (fractions 16 to 26), whereas fractions 6 to 14 contained only class II molecules (Fig. 3B) (5). Fractions 6 to 14 also contained endocytosed horseradish peroxidase (HRP) and β-hexosaminidase activity (Fig. 3A) and corresponded to the position in the electric field of the recently defined class II compartment.

DMA was observed as a doublet in fractions 19 to 27, whereas in fractions 5 to 15 it mostly consisted of a slower migrating species (Fig. 3C). A similar distribution was seen for DMB (*17*). The peak of activity in these anodally migrating fractions mirrored that found for class II, as might be expected if the two molecules colocalized to one compartment. Fractions 18 to 28, where most of the class II molecules were found, contained the ER and the plasma membrane. To define better the location of DM in these fractions, they were digested with endoglycosidase H (Endo H), which removes immature (high-mannose) *N*-linked glycans (Fig. 3D). Whereas DMA in fractions 6 to 16 was Endo H–resistant, the lower and major component of the DMA doublet in the other fractions (18 to 26) was sensitive to Endo H, indicating residence in the ER. The fainter upper band was unchanged in position after Endo H treatment and could represent a minor proportion of the total protein present at the cell surface or possibly the Golgi, although the Golgi, as defined by galactosyl transferase activity, has been shown in this system to migrate slightly further toward the anode (*18*). Thus, the bulk of DM in MelJuSo was divided between the ER and a compartment that cofractionates with class II–containing vesicles. Percoll density gradient fractionation of Raji cells gave a broadly similar picture (*19*).

We then did immunoelectron microscopy on ultrathin cryosections of Raji cells. This confirmed that both DMA and DMB were located in typical multilaminar class II compartments or MIICs together with the majority of intracellular HLA class II molecules (Fig. 4, A and B). The plasma membrane had no detectable labeling for either DMA (Fig. 4A) or DMB. The ER labeling was weak, probably because of the large ER surface area and consequent lower density of DM. Ultrathin cryosections of 721.174 cells, which lack *DMA* and *DMB* genes, had no labeling with anti-DMA or anti-DMB. Morphologically, the DMA- and DMB-positive MIIC in Raji resembled those described in other human B cells (*2*, *10*). MIICs contain internal membrane vesicles and sheets and express lysosomal enzymes and membrane proteins (*2*, *10*). The DM-positive MIIC in Raji cells were positive for cathepsin D and the lysosomal membrane proteins CD63 and LAMP-1 (Fig. 4C), but were negative for the cation-independent mannose 6–phosphate receptor (CI-MPR), which is present on late endosomes but absent from lysosomes. The localization of DM to MIIC-like structures was also observed in the Epstein-Barr virus (EBV)–transformed B cell line LCL721 and in human dendritic cells isolated from peripheral blood (*20*).

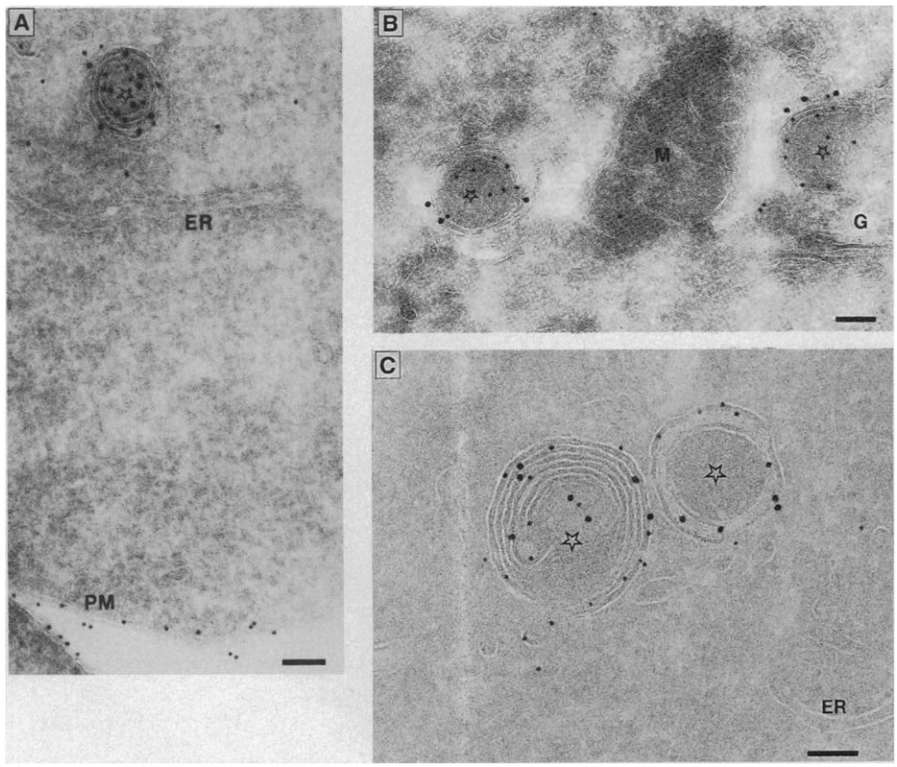

Fig. 4. Electron micrographs of ultrathin cryosections of Raji cells. (**A**) Abundant expression of DMA (large gold) and class II (small gold) is present in an MIIC (star), whereas only class II can be seen on the plasma membrane (PM). (**B**) Two MIICs (stars) double-immunolabeled for DMB (large gold) and class II (small gold). (**C**) Expression of DMA (large gold) in two MIICs (stars) that are further characterized by labeling for LAMP-1 (small gold). G, Golgi complex; M, mitochondrion; ER, endoplasmic reticulum, Bars, 0.1 μm. Ultrathin cryosectioning and immunogold labeling were done as described (*32*). Ultrathin cryosections were embedded according to a procedure that allowed better visualization of membranes (*33*). Sections were double-immunolabeled with either rabbit anti-DMA (FS2) or rabbit anti-DMB (AK3) and then incubated with 15-nm protein A–gold particles. Sections were subsequently incubated with rabbit antiserum to class II α-chain (*31*) or rabbit antiserum to LAMP-1 (*34*), and then with 10-nm protein A–gold particles. The location of the signals for the COOH-terminal part of DMB was consistent with this portion of the molecule being on the cytoplasmic face of the MIIC limiting membrane (Fig. 4B). Ultrathin cryosections incubated with irrelevant control antibody under the same conditions produced negligible background labeling.

The DM genes were first described, several features indicated that they were unconventional class II molecules that have a unique function. DM expression paralleled that of other class II genes—that is, it is constitutive in B cells and inducible with interferon γ in other cells (*13*). Comparison of the membrane-proximal immunoglobulin-related domains of DM with those of other MHC sequences shows that they share almost as much amino acid identity with class I as with class II molecules (~30%) and must have diverged at around the same time that class I and class II sequences split from each other, long before duplication of the main class II loci, DP, DQ, and DR. DM protein sequences do not contain a recognizable CD4 binding site. However, computer modeling based on the DR coordinates (*21*) indicates that the α1 and β1 domains of DM may fold in a similar way to the classical class II molecules (*22*).

Data from mutant cell lines implicating HLA-DM in class II antigen processing suggests a number of possible models for its function. DM might act as a CLIP sink, efficiently binding CLIP so as to leave conventional class II molecules available to seek other ligands. Alternatively, DM could act as a shuttle to deliver peptides into a compartment for interaction with class II. A third model invokes the concept of DM as a chaperone, tethering other class II molecules in the MIIC until antigenic peptide

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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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 (7×10^7) 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 (5×10^6/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 ($H\text{-}2A^k$) 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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