In the endocytic pathway of antigen-presenting cells, HLA-DM catalyzes the exchange between class II-associated invariant chain peptide (CLIP) and antigenic peptides onto major histocompatibility complex class II molecules. At low pH of lysosomal compartments, both HLA-DM and HLA-DR undergo conformational changes, and it was recently postulated that two partially exposed tryptophans on HLA-DM might be involved in the interaction between the two molecules. To define contact regions on HLA-DM, we have conducted site-directed mutagenesis on those two hydrophobic residues. The HLA-DM αW62AβW120A (DMW62AW120A) double mutant was expressed in HLA-DR+ HeLa cells expressing invariant chain, and the activity of this DM molecule was assessed. Flow cytometry analysis of cell surface DR-CLIP complexes revealed that DMW62AW120A removes CLIP as efficiently as its wild-type counterpart.

DMW62AW120A was found in the endocytic pathway by immunofluorescence, and DM-DR complexes were immunoprecipitated from these cells at pH 5. Finally, mutations αW62A and βW120A on HLA-DM did not affect the association with HLA-DO. The complex egresses the endoplasmic reticulum and accumulates in endocytic vesicles. Moreover, DO and DMW62AW120A were co-immunoprecipitated at pH 7. We conclude that the α62 and β120 tryptophan residues are not required for the activity of DM, nor are they directly implicated in the interaction with DR or DO.

Major histocompatibility complex class II molecules are primordial for activation of CD4+ T cells and for immunological response against pathogens (1). Three major histocompatibility complex II αβ heterodimers associate with a trimer of invariant chain (Ii)1 to form a nanomeric complex in the endoplasmic reticulum (ER) (2). This association allows the proper folding and trafficking of the major histocompatibility complex II molecules (3–5). Gradual proteolysis of Ii occurs in the endocytic compartments by the sequential action of proteases including cathepsins (6). A residual peptide of Ii (CLIP) remains in the peptide groove of the class II molecule and stabilizes the heterodimer by preventing collapse of the groove (7, 8). For most allotypes, CLIP must be actively removed to allow binding of antigenic peptides. HLA-DM, a nonclassical intracellular class II molecule, plays a central role in the efficiency of antigen presentation as it catalyzes CLIP release from HLA-DR and stabilizes the class II in a chaperone-like fashion (9–14). In fact, mice lacking the H2-Ma gene express a high level of surface class II-CLIP complexes (15–17).

The precise molecular mechanism of action of HLA-DM remains to be established. X-ray diffraction studies on HLA-DM crystals revealed a closed peptide-binding groove and an overall quaternary structure similar to classical class II molecules (18, 19). Time-resolved fluorescence anisotropy and far-UV circular dichroism spectrum analysis using soluble HLA-DM revealed that its structure is quite rigid and that it is not subjected to gross pH-dependent conformational change. However, many other experiments by the same groups suggest that protonation in the endocytic pathway results in minor, reversible structural changes exposing hydrophobic regions of the DM heterodimer (20, 21). For example, fluorescence spectroscopy studies revealed that hydrophobic tryptophan residues are buried in native DM and would become more solvent-exposed at endosomal pH (20). Accordingly, Ullrich et al. (21) used 8-anilino-1-naphthalenesulfonic acid (ANS), a fluorescent dye binding to hydrophobic protein patches, to demonstrate subtle pH-induced change in DM. Since the interaction of DM with DR reduces ANS binding to both molecules, it was postulated that the surface of contact comprises these pH-sensitive regions (21, 22).

On the basis of these results, Wiley and co-workers (19) proposed that two partially exposed tryptophan (α62, β120), located on the same lateral surface of DM, could be critical for DR binding. The model predicts a major interaction between tryptophan α62 of DM and the protruding phenylalanine α51 of DR, resulting in the breakage of multiple hydrogen bonds at the end of the groove and in the release of unstably associated peptides. At least part of this model was recently confirmed when mutation of DRαF51 was shown to abolish the interaction with DM (23).

To confirm the possible interaction between DRαF51 and DMαW62, we have mutated the exposed tryptophan on HLA-DM and tested the ability of this mutant to release CLIP from HLA-DR. Our results show that this mutation on DM does not affect DR contact, CLIP release, or the localization of the protein within the cell. Similar conclusions were drawn after mutating the second exposed tryptophan at position β120. Finally, those amino acids substitutions on DM did not affect its interaction with HLA-DO, an intracellular nonclassical class II molecule that binds to DM and regulates its activity.

**EXPERIMENTAL PROCEDURES**

Plasmids—DMα cDNA was digested by EcoRI-Clal from pMCFRPAC and subcloned into EcoRV-Clal of pBluescript (pBS) (24). The
DMβ cDNA has been described previously (25). DMα and DMβ cDNAs from pBluescript were subcloned successively on the same pBudCE4-A vector (as previously described) (25). The Ii p35 cDNA was obtained by mutagenesis of the second ATG codon of Ii (kindly provided by Rafick Sékaly) and subcloned in the BamHI site of SRIopuro. Details will be provided elsewhere.

DMα and DMβ Mutagenesis—Mutations into the DMα and DMβ cDNA sequences were introduced by PCR overlap extension (26). Briefly, 5’ PCR products were generated from pBS DMα and pBS DMβ using mutagenic primers (DMαW62A/EcoRI; 5’-CTG AGC GCG GTC AGC GAA TTC GGG-3’; DMW120A/Neol; 5’-GAA GCC CGC CAC ATA GCG TCG CAT-3’) as well as the universal (DMα) or reverse (DMβ) primers. The 3’ PCR product was generated using the complementary mutagenic primers. The two overlapping PCR products were mixed, and a final PCR was performed using the flanking primers. Fragments were subsequently subcloned into the PstI-SalI sites of pBSDMa and SalI-HindIII of pBDSβm, thereby replacing the wild-type fragment with the PCR product containing the appropriate mutation. The nucleotide sequence was confirmed by DNA sequencing using T7 polymerase (Amersham Biosciences). Mutant cDNAs and wild-type were introduced into pBudCE4-A as NotI-Xhol (pBDSMa) and SalI-Xhol (pBDSβm) fragments.

Antibodies—Monoclonal antibody (mAb) 5A1 (IgG1), is directed against the luminal portion of HLA-DM (PharMingen International, Oakland, CA, Canada). mAb L243 (IgG2a) binds to a specific DR-DQ heterodimer (25). Goat anti-mouse IgG (H+L) coupled to Alexa Fluor 488 were obtained from Molecular Probes, Inc. (Eugene, OR). Biotinylated goat anti-mouse was from BIOCAN scientific (Mississauga, Canada), and Texas Red-coupled streptavidin was from Amersham Biosciences. The anti-DOβ serum was produced in C3H mice (H-2k) by repeated intraperitoneal injections of DAP fibroblasts transfected with DRα and DRβ cDNAs (28). Rabbit antisera against the cytoplasmic tail of HLA-DOα or HLA-DMβ have been described previously (25). The CD107a-specific monoclonal antibody H4A3 (IgG1) reacts with the heavy glycosylated 100-kDa lysosome-associated membrane protein (LAMP-1) (Developmental Studies Hybridoma Bank, NICHD, University of Iowa, Iowa City, IA).

Cell Lines and Transfections—HeLa DR1 (DRα plus DRβ 0101) cells were kindly provided by Dr. R. P. Sékaly. HeLa DO cells have already been described (25). In order to enrich for DO-expressing cells, the population was treated with γ interferon (PharMingen International) and sorted on magnetic beads for CLIP expression (Dynal ASA, Olso, Norway). mAb L243 (IgG2a) binds to a specific DR-DQ heterodimer (25). Goat anti-mouse IgG (H+L) coupled to Alexa Fluor 488. Cells were then analyzed by flow cytometry by FACScan (Becton Dickinson, Mississauga, Canada).

Flow Cytometry Analysis—Intracellular staining was done as previously described (25). Briefly, saponin-treated cells were incubated with the appropriate primary antibody, and then they were incubated with goat anti-mouse IgG (H+L) coupled to Alexa Fluor 488. Cells were then analyzed by flow cytometry on a FACScan (Becton Dickinson, Mississauga, Canada).

Fluorescence Microscopy—104 cells were plated on coverslips in 24-well plates and cultured for 3 days before staining (25). Cells were analyzed by fluorescence microscopy on a Zeiss axioscope microscope (Carl Zeiss, Thornwood, NY). Photographs were taken with a Zeiss microscope camera MC 100 on Eastman Kodak Co. elite chrome 400 films.

Immunoprecipitations and Western Blotting—Cells (4 × 106) were trypsinized, washed in phosphate-buffered saline, and lysed in 1% Triton X-100 at pH 7 or in CHAPS 1% at pH 5 as described previously (29). After centrifugation, supernatants were harvested and incubated with protein G coupled to CNBr-activated Sepharose 4B (Amersham Biosciences) and then added to the appropriate antibody (1.25 μg/mg mouse serum) antibody (anti-DOβ). Following washes in lysis buffer, samples were resuspended in nonreducing buffers and subjected to SDS-PAGE. After transfer to nylon membranes (Amersham Biosciences), proteins were blotted with the rabbit anti-DOα or anti-DMβ sera. Secondary antibody (peroxidase-coupled goat anti-rabbit; BIO/CAN Scientific) was used at a 1:1000 dilution for 2 h, and the signal was detected using ECL (Amersham Biosciences).

RESULTS

The crystal structure of DM reveals two partially exposed tryptophan residues that are thought to interact with HLA-DR and to participate in CLIP removal (19). Tryptophans ε62 and β120 are located on the same lateral face of HLA-DM in the α1 and β2 domains, respectively (Fig. 1). These amino acids are highly conserved throughout evolution, emphasizing their potential importance.

In an effort to define the binding site for HLA-DR and to gain insights into the mechanism of action of HLA-DM, we mutated those two bulky aromatic tryptophans to small alamines and tested in vitro the ability of mutant proteins to remove CLIP from HLA-DR. Various combinations of mutated and wild-type DM αβ cDNAs were transfected in HeLa cells expressing DR1 and Ii. DR1 is known to be dependent on DM for the release of CLIP at acidic pH (30). We obtained four stable cell lines: DR1 Ii DM, DR1 Ii DM W62A, DR1 Ii DM W120A, and DR1 Ii DM W62A/W120A. Expression of DR was monitored at the cell surface, while DM and Ii were analyzed in permeabilized cells. As measured by flow cytometry, all cell lines express high levels of the various transfected molecules (Fig. 2). The proper folding and intracellular sorting of DM was assessed by immunofluorescence microscopy. Fig. 3 shows that wild-type HLA-DM (B) accumulates in intracellular vesicles that are also positive for the lysosomal marker Lamp-1 (G). A tyrosine-based motif on the cytoplasmic tail of DMβ is responsible for its accumulation in endocytic compartments (31–33). The three mutated forms of DM were also found in peripheral vesicles (Fig. 3, C–E) and co-localized with Lamp-1 (Fig. 3, H–J). Control cells lacking DM showed a weak background using the DM-specific antibody but definite Lamp-1-positive compartments (Fig. 3, A, P, and arrows). Together with the fact that the MaP.DM1 conformational antibody efficiently recognizes the DM mutants, these results show that the overall structure of DM is not affected by replacement of tryptophans ε62 and β120.

The activity of these mutant forms of DM was verified by monitoring the levels of DR-CLIP complexes at the cell surface.

### Fig. 1. Position of mutations on HLA-DM. Highlighted tryptophan residues 62 (W62) and 120 (W120), respectively, are located on the same lateral face of HLA-DM in the α1 and β2 domains, respectively (Fig. 1). The GenBank accession numbers are NM006120 for DMα (red) and NM002118 for DMβ (orange) (10). Molecular modeling was done with Protein Data Bank reference number 1HDM and analyzed with the Swiss PDB Viewer (19).
of transfected cells. As demonstrated by many groups, introduction of HLA-DM in deficient cell lines favors the intracellular exchange of CLIP for more stable peptides (24, 34, 35). Consequently, CLIP expression is low at the surface of DM/H11001 cells. The cell surface expression of CLIP was measured using the Cer-CLIP monoclonal antibody that is specific for the N terminus of CLIP bound to class II molecules and which does not recognize the intact invariant chain (24). As shown in Fig. 4A, control DM/H11002 cells (HeLa DR1 Ii) express high levels of DR-CLIP complexes at their surface. On the other hand, cells expressing wild-type or mutant forms of DM do not express significant levels of CLIP (Fig. 4, B–E). These results suggest that DR-Ii complexes are sorted to the endocytic pathway, where Ii is degraded until a last fragment, CLIP, is actively removed from the peptide binding groove by wild-type or mutant forms of HLA-DM. To confirm the proper interaction between the DM mutants and DR, co-immunoprecipitations were carried out at acidic pH in the CHAPS detergent (36). Fig. 5 shows that DM can be co-immunoprecipitated by the DR-specific antibody only in cells expressing both molecules (Fig. 5, left three lanes). Also, the three mutant forms of DM could all be efficiently co-immunoprecipitated with HLA-DR (Fig. 5). The stronger DMβ signal obtained by Western blotting on the HeLa DR1 Ii DM_W62A/W120A samples most probably reflects the higher level of HLA-DR expression on these cells (mean fluorescence value = 789) as compared with the other DM–transfectants (mean fluorescence values = 248–374) (Fig. 2). Taken together, these results show that the mutations αW62A and βW120A do not affect the folding, sorting, and activity of HLA-DM, nor its ability to interact strongly with HLA-DR.

Although these tryptophan residues are not directly involved in the binding to HLA-DR, they might very well be crucial for the binding to HLA-DO. The latter is mostly expressed in B cells and is a nonclassical class II molecule that modulates the activity of DM (37–39). The mode of action of DO is not determined, but it clearly affects the peptidic repertoire when present in physiological amounts in an APC (40–42).

To further characterize the DO-DM contact regions, wild-type and double mutant (DM_W62A/W120A) DM molecules were stably transfected in HeLa DO/H11001 cells. Expression of both proteins was confirmed by intracellular staining and flow cytometry (Fig. 6). Since HLA-DO is absolutely dependent on DM association to egress the ER (43) and gain access to the endocytic pathway, we first verified the ability of mutant HLA-DM to direct the transport of DO. Immunofluorescence microscopy showed that HLA-DO reaches the endocytic pathway in the
presence of either the wild-type or the DM_{W62A/W120A} molecule (Fig. 7). As opposed to the diffuse ER-like pattern of expression observed for DO in DM\_H11002 cells (Fig. 7b), defined HLA-DO-containing vesicles can be seen in DM\_H11001 cells (Fig. 7, d and f). The co-localization between DO and DM_{W62A/W120A} in the endocytic pathway (Fig. 7, e and f) strongly argues for an efficient interaction between the two molecules, allowing DO to egress the ER.

The interaction between DO and DM was confirmed by co-immunoprecipitation experiments. Cells were lysed in 1% Triton X-100, and DO was immunoprecipitated using a polyclonal antibody against the cytoplasmic tail of the \( \beta \) chain. Western blotting with antibodies specific for DM or DO showed that wild-type DM and the DM_{W62A/W120A} double mutant were both efficiently co-immunoprecipitated with DO (Fig. 7B). Altogether, these results suggest that W62 and Q120 tryptophans on HLA-DM are dispensable for the interaction with DR or DO.

**DISCUSSION**

Understanding the molecular mechanism by which HLA-DM and HLA-DM/DO catalyze peptide exchange will allow the...
development of new approaches for manipulating antigen loading and presentation. With this in mind, we have undertaken the mapping of amino acids involved in the interactions between these molecules. So far, the group of Mellins has characterized regions of HLA-DR that interact with HLA-DM (23, 44). Random mutagenesis on HLA-DR allowed the identification of critical residues on a lateral face encompassing both the α1 and β2 domains. From these experiments, it was proposed that DM releases unstable peptides through its “leaver effect” on amino acids DRα40 and α51, the last one being critical for stability around the P1 pocket of the groove (23). A role for DM in destabilizing those P1 anchors was also proposed from the crystal structure of HLA-DM. The existence of a three-molecule complex between DM, DR, and DO allowed the identification of critical residues on a lateral face encompassing both the α1 and β2 domains (21). The presence of tryptophan residues in these regions is located on distinct faces of the H2-M heterodimer. Site-directed mutagenesis in the corresponding regions of DM should help delimiting contact sites with DR.

Based on the crystal structure of HLA-DM, the group of Wiley had already proposed that the contact between DM Trp62 and DR Phe51 could destabilize the P1 pocket and liberate an unstable peptide. Much experimental evidence points to a critical role of such hydrophobic residues in the contact between DM and DR. For example, based on studies measuring the binding of ANS, it was concluded that DR-CLIP complexes display a larger hydrophobic surface than DR molecules associated with stable peptides. Also, there is a preferential association of the two molecules at the acidic pH of endocytic vesicles, where both molecules would expose hydrophobic residues (21). The presence of tryptophan residues in those contact regions was deduced by their preferential binding of ANS as well as from spectroscopy studies measuring variations in fluorescence emission between exposed and buried residues (21, 45).

Two of 11 tryptophans are partially exposed and located on the same lateral surface on the crystal structure of DM. These residues may be part of the hydrophobic patches that become accessible to ANS at acidic pH but that are buried in the DM-DR interface (19). While DR Phe51 could interact with DM Trp62, tryptophan DM β120 may contact those hydrophobic residues identified by Mellins and co-workers (23) in the β2 domain of DR and which may serve to increase the affinity for DM. However, our results presented here do not support such a model. Mutation of the two tryptophans on DM did not disturb the activity, the sorting, or the conformation of the protein. Still, the possibility remains that those mutations could finely tune the specificity of DM and influence the peptide repertoire of the class II molecules.

The fact that random mutagenesis on HLA-DR allowed the identification of Phe51, Leuβ184, and Valβ186 residues certainly suggests the involvement of hydrophobic residues on DM as well (23). However, replacement of residue Gluβ187 for a lysine in the β2 domain also decreased DM binding in these studies. The importance of this charged residue on DR prompted us to evaluate the potential role of positively charged DM Lys60, Lysa115, Argβ93, and Argβ85 residues. However, the simultaneous mutation of these residues did not inhibit CLIP release by DM in our system (data not shown).

 Altogether, our results suggest that the DM/DR interaction relies on other hydrophobic residues in the above-described region or that it may implicate another interface of DM. Indeed, Fremont et al. (18) identified two other hydrophobic regions that are located on distinct faces of the H2-M heterodimer. Site-directed mutagenesis in the corresponding regions of DM should help delimiting contact sites with DR.

Finally, our results from immunofluorescence and co-immunoprecipitation studies revealed that tryptophans α62 and β120 on DM are not necessary to make contact with HLA-DO. Although DO also undergoes conformational change following acidification of the environment (46), it first interacts with DM in the ER, and the strong association is resistant to lysis in 1% Triton X-100 (43). These observations suggest that the nature of the interactions is likely to differ between DR-DM and DO-DM. The existence of a three-molecule complex between DM, DO, and DR suggests the presence of two distinct functional interfaces on DM, and random mutagenesis is under way to delineate the contact regions with HLA-DR and DO (47).

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Functional Analysis of Tryptophans α62 and β120 on HLA-DM
Amélie Faubert, Angela Samaan and Jacques Thibodeau

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