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Determination of the HLA-DM Interaction Site on HLA-DR Molecules

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Summary

HLA-DM removes CLIP and other loosely bound peptides from MHC class II molecules. The crystal structures of class II molecules and of HLA-DM have not permitted identification of their interaction sites. Here, we describe mutations in class II that impair interactions with DM. Libraries of randomly mutagenized DR3 α and β chains were screened for their ability to cause cell surface accumulation of CLIP/DR3 complexes in EBV-B cells. Seven mutations were associated with impaired peptide loading in vivo, as detected by SDS stability assays. In vitro, these mutant DR3 molecules were resistant to DM-catalyzed CLIP release and showed reduced binding to DM. All mutations localize to a single lateral face of HLA-DR, which we propose interacts with DM during peptide exchange.

Introduction

HLA-DR molecules are synthesized in the endoplasmic reticulum, where they associate with invariant chain (Ii). A segment of Ii occupies the peptide binding groove of HLA-DR molecules (Ghosh et al., 1995), preventing other ligands from binding until delivery to endosomes (Roche and Cresswell, 1990; Busch et al., 1995). Upon entry into endosomes, Ii is degraded (Lotteau et al., 1990), leaving a nested set of peptides termed CLIP (class II–associated invariant chain peptides) in the groove. HLA-DM, an endosomal resident, nonclassical MHC class II molecule (Sanderson et al., 1994), is required for efficient removal of CLIP from most HLA-DR molecules. Therefore, CLIP/DR complexes accumulate at the surface of cells lacking expression of functional DM (Riberdy et al., 1992; Fling et al., 1994; Morris et al., 1994; Guerra et al., 1998) and of cells in which DM/DR interaction is disrupted (Mellins et al., 1994). Additionally, DM edits the peptide repertoire (Sloan et al., 1995; Katz et al., 1996; Kropshofer et al., 1996; van Ham et al., 1996) and stabilizes empty class II molecules (Denzin et al., 1996; Kropshofer et al., 1997; Vogt et al., 1997).

The kinetics of DM-catalyzed peptide release from purified MHC class II molecules in vitro (Sloan et al., 1995), as well as coprecipitation experiments (Sanderson et al., 1996), provide strong evidence for a direct association between DM and MHC class II molecules during peptide exchange. However, clues as to the physical nature of these interactions remain sparse. The acidic pH optimum of the interactions indicates a role for titratable functional groups (Sloan et al., 1995; Sanderson et al., 1996), and experiments using hydrophobic fluorescent probes suggest a role for hydrophobic interactions at the DM/DR interface (Ullrich et al., 1997). The recently solved crystal structures of HLA-DM (Mosyak et al., 1998) and its murine homolog, H-2M (Fremont et al., 1998a), overall resemble those of classical MHC class II molecules (e.g., Fremont et al., 1998b), except for the lack of a peptide binding groove. However, inspection of these structures yields no obvious clues as to how DM and DR interact.

In this study, we set out to identify point mutations in DR α or β chains that lead to a loss of interaction with HLA-DM. We predicted that this should result in surface accumulation of CLIP/DR complexes, as seen for cells that lack DM altogether. An efficient screen for this phenotype among cells with mutant DR3 molecules, followed by careful biochemical characterization of informative mutants, allowed us to map a face of DR3 that is critical for functional interactions with HLA-DM.

Results

Mapping of DM/DR Contacts

Random mutagenesis of HLA-DR3 (DRA*0101/DRB1*0301) was employed to identify mutations that decrease functional interaction with DM. Libraries of mutant DR α and β chain cDNAs were introduced into EBV-B cell lines lacking endogenous expression of the appropriate DR chain. These cells, carrying mutated DR molecules, were screened for accumulation of CLIP, a hallmark of reduced DM function.

The recipient cell lines were two previously generated EBV-B cell lines, 9.22.3 (DRA null) and 9.4.3 (DRB1 null) (Figure 1A). Retroviral transduction of the missing wild-type DR α chain into 9.22.3 cells rescued surface HLA-DR expression, as measured by flow cytometry using the HLA-DR-specific mAbs, L243 (data not shown) and ISCR3 (Figure 1B). Note that expression was restored for both DR3 (DRA/DRB1*0301) and DRw52a (DRA/DRB3*0101) molecules, encoded by the two DR β chain loci of the DR3 haplotype (Figure 1A). Similar results were obtained when the missing wild-type DRB1*0301 was transduced into 9.4.3 cells, except that endogenous DRw52a (DRA/DRB3*0101) expression gave rise to significant staining of untransfected cells with anti-DR mAbs (Figure 1B). Total DR levels increased about 2-fold after retroviral transduction.

Untransduced 9.22.3 and 9.4.3 cells expressed low but detectable levels of CLIP (measured by flow cytometry using the CLIP-specific mAb, CerCLIP.1; Figure 1B), believed to be associated with endogenously expressed DQ (R. C. D., unpublished data). Only a slight increase in CLIP levels was noted after transfection of wild-type

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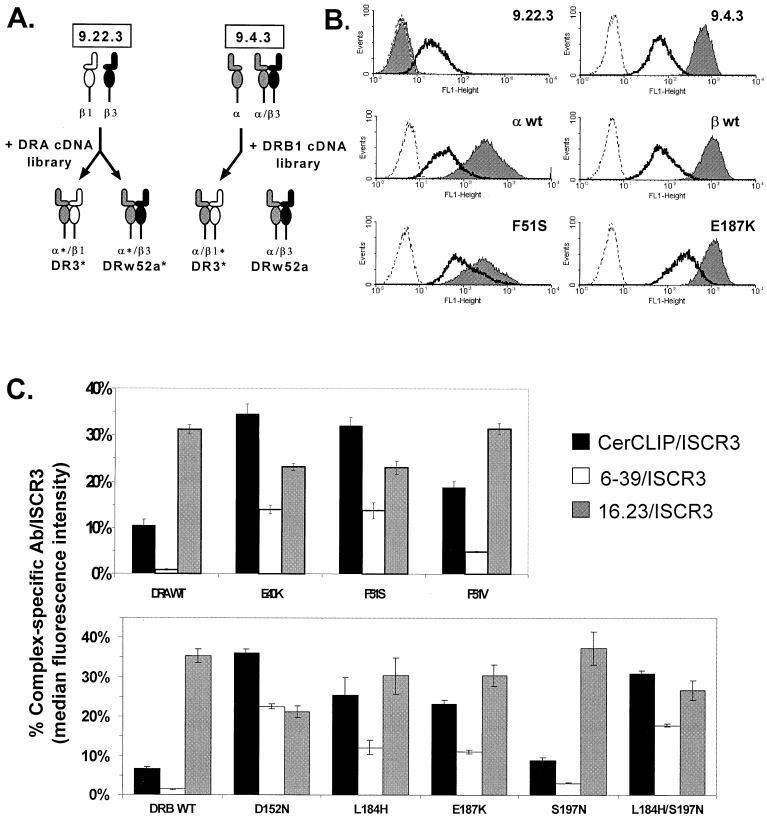

Figure 1. Cell Surface MHC Class II Expression and Peptide Loading of Mutant Cell Lines

(A) Experimental strategy. 9.22.3 lacks HLA-DRα expression; DRα transfection restores expression of HLA-DR3 and HLA-DRw52a, containing β chains encoded by DRB1*0301 and DRB3*0101, respectively. 9.4.3 expresses DRw52 but lacks DRB1*0301 and hence DR3 expression, which is reconstituted by transfection of DRB1*0301. An asterisk (*) denotes an HLA-DR molecule containing an introduced chain with or without random mutations.

(B) Cell surface expression of HLA-DR and associated CLIP on representative mutant cell lines and wild-type controls stained with anti-DR (ISCR3) or anti-CLIP (CerCLIP.1) mAbs. Cells were stained with anti-DR (ISCR3; shaded histogram) or anti-CLIP (CerCLIP.1; solid line) or without primary Ab (dotted line), incubated with FITC-conjugated goat anti-mouse IgG, and analyzed by flow cytometry.

(C) Cell surface binding of mAbs CerCLIP.1, 6–39 (anti-DR3/CLIP) and 16.23. Bar graphs represent ratios of median fluorescence intensity of CerCLIP, 6–39, and 16.23 to that of ISCR3, which corrects for slight variations in DR expression. Shown are averages from three independent experiments with standard deviation. Total surface expression of HLA-DR as measured by ISCR3 binding was similar for each set of cell lines (DRα mutants: ± 11%, DRβ mutants: ±8%). Upper panel, DRα mutants (and wild-type control). Lower panel, DRβ mutants (and wild-type control).

DR3, so DM expression in these cells was sufficient to allow release of CLIP from most wild-type DR molecules. While DR3 molecules accumulate CLIP in the absence of functional interaction with DM, DRw52a does not, due to its low affinity for CLIP (Sette et al., 1995; unpublished data]. Thus, these cell lines allow sensitive screening for high-CLIP mutants.

Mutations were introduced into DRA and DRB1 cDNAs by error-prone PCR, yielding a library with a frequency of point mutations that was high enough to contain every possible nucleotide substitution multiple times (see Experimental Procedures for details). To screen for mutations that disrupt DM-DR interaction, we generated retroviral particles carrying mutant DRA and DRB1 cDNAs and transduced them into 9.22.3 or 9.4.3 cells at a low multiplicity of infection (< 0.15) to avoid introduction of more than one cDNA per cell. HLA-DR levels in the culture containing mutants were similar to those found on cells reconstituted with wild-type DR chains (data not shown). To select mutants, we used FACS to clone the 0.1% cells displaying the highest levels of CLIP. Upon rescreening of surviving clones after expansion, many retained a high-CLIP phenotype (Table 1; data not shown).

To identify mutations associated with the high-CLIP phenotype, the mutant cDNAs were rescued from the clones by reverse transcription and high-fidelity PCR. DNA sequencing revealed the presence of single point mutations in most clones, and double mutations in a few (Table 1; data not shown). Mapping of the mutations on the published crystal structure of HLA-DR3 (Ghosh et al., 1995) revealed that one set of mutants clusters around the antigen binding groove; further analysis indicated that these mutations stabilize the DR3/CLIP complex (R. C. D. et al., unpublished data). However, a second group of six mutants clusters on the lateral face of DR3 that contains the N terminus of CLIP (Figure 2; Table 1).

Phenotypic Analysis

Although DM expression was observed in the mutant cells (data not shown), it was difficult to evaluate the con-

Table 1. HLA-DR Mutants Utilized in This Study

Chain	Clone	Mutation	Amino Acid Substitution
DR alpha	3C5	T-G 226	F51V
	3E11	G-A 193	E40K
	3G4	G-A 16	V (−20)[a]
		T-C 227	F51S
DR beta	2B10	G-A 541	D152N
	2E3	T-A 638	L184H
		G-A 677	S197N
	4C10	G-A 541	D152N
	4E10	G-A 646	E187K
	4F9	G-A 646	E187K

EBV-B cell clones with high CLIP at the cell surface were isolated following introduction of randomly mutagenized DRA or DRB cDNA libraries. cDNAs encoding the introduced DR chain were rescued by RT-PCR and sequenced. Nucleotides are numbered beginning with the first nucleotide of the cDNA. Clones 3G4 and 2E3 harbored two mutations each. Amino acids are designated by their one letter code. Amino acid numbering is from the first position in the mature protein unless otherwise noted.

[a] This mutation occurred in the segment encoding the signal sequence and is numbered by counting backwards from the first amino acid in the mature DRα chain.

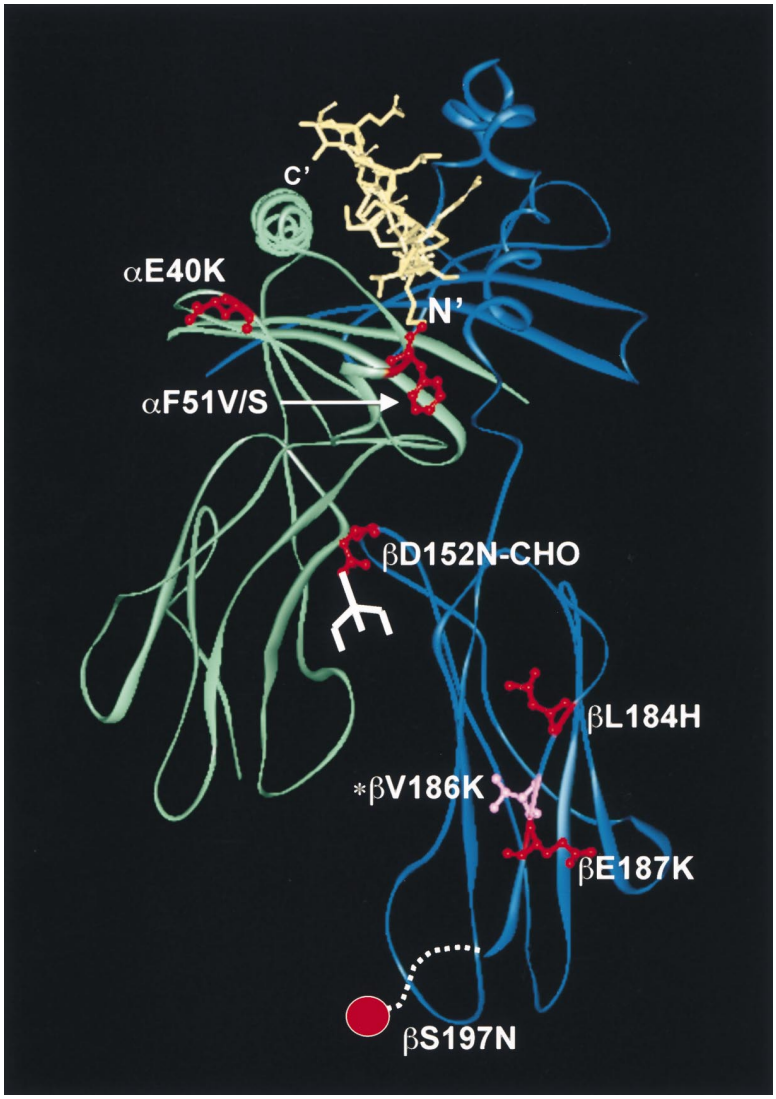

Figure 2. Mapping of Mutants onto the DR3/CLIP Crystal Structure

Ribbon diagram representation of the HLA-DR3/CLIP crystal structure. DRα chain is shown in green, DRβ in blue, and CLIP in yellow. The amino (N) and carboxy (C) termini of CLIP are labeled for orientation. Positions in HLA-DR3 that correspond to deleterious mutations described in this study are highlighted in red and labeled with the corresponding substitutions. The branched structure protruding from DRβ D152 represents the addition of a glycan onto this residue in the mutant βD152N. DRβ S197N is represented as a red dot extending off the last β chain residue (β Arg191) in the crystal structure. (*) Shown in purple is a mutant (β V186K) identified through targeted mutagenesis of several residues on the proposed interface. This mutant caused elevated surface DR3:CLIP levels (data not shown; see Discussion).

tribution of quantitative differences in DM and DR expression among the clones. Furthermore, other epigenetic changes, such as insertional inactivation of genes important for antigen processing, in the immunoselected clones could contribute to the observed phenotype. To overcome these difficulties, the rescued single and double mutants of DRA and DRB1 were reintroduced into 9.22.3 and 9.4.3 cells, respectively, generating polyclonal transduced lines in which any epigenetic variability should average out. The contributions of the individual mutations in the double mutants (2E3, 3G4) were analyzed by site-directed mutagenesis.

Flow cytometry was used to assess surface expression of mutant DR molecules and their associated peptide loading phenotype (Figures 1B and 1C). All mutant cDNAs reconstituted DR expression to a similar extent as wild-type, as measured using conformation-sensitive mAbs specific for DRβ (ISCR3; Figure 1B; data not shown) or DRα (L243; data not shown). Thus, the mutations did not impair HLA-DR assembly or surface expression. Staining with the CLIP-specific mAb, CerCLIP.1, revealed increased CLIP/DR ratios in all mutants over wild-type (Figures 1B and 1C).

CerCLIP.1 reactivity has been mapped to the CLIP N terminus (Avva and Cresswell, 1994), located close to some of the DR3 mutations described here (Figure 2). To rule out the possibility that the mutations, rather than causing CLIP accumulation, improved interactions between CerCLIP.1 and DR3, we measured binding with a second mAb, 6–39, which binds a different epitope that includes residues of DR3 and of the CLIP C terminus (R. C. D. et al., unpublished data). Overall, lower levels of staining were obtained using 6–39, but there was a good correlation between staining intensities obtained with the two antibodies. The antibody binding studies also revealed differences in CLIP accumulation, with αE40K, αF51S, βD152N, and the double mutant, βL184H/βS197N, being associated with stronger phenotypes. Of the two mutations found in clone 2E3, βS197N clearly made a smaller contribution when single mutants were analyzed. Note that comparisons between α and β mutants are difficult due to differences in the two recipient cell lines. Nonetheless, all of the mutations analyzed resulted in accumulation of varying amounts of CLIP.

We also analyzed reactivity with 16.23, a mAb that

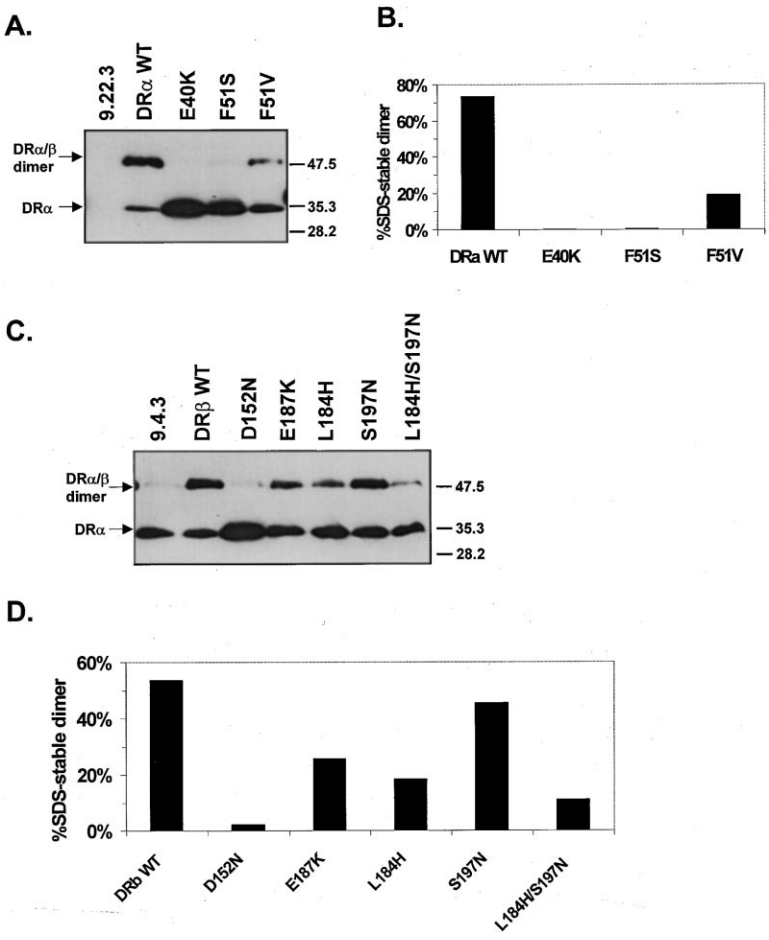

Figure 3. Mutant HLA-DR Molecules Fail to Acquire SDS Stability

(A and C) Unboiled, nonreduced whole cell lysates, prepared in 1% IGEPAL CA-630 detergent, were resolved by 10% SDS-PAGE, and Western blots were probed using DA6.147 (anti-DRα). The position of monomeric DRα and SDS stable DR α/β dimers are indicated by arrows. Molecular weight (kDa) standards are shown on the right. Data are representative of at least three experiments. No immunoreactive higher MW bands were detected (not shown). DRα and wild type control (A and B). DRβ mutants and wild type control (C and D). (B and D) Bar graph representing the ratio of SDS stable HLA-DR dimer to total HLA-DR [Dimer/(dimer + monomer)] in (A) and (C), respectively. Bands were quantified by densitometry. The absolute proportion of SDS stable dimers in wild type and mutant cells varied somewhat in different experiments, but the mutant molecules were less stable than wild type in all experiments (not shown).

binds to a subset of DR3 molecules that have undergone DM-catalyzed peptide exchange (Morris et al., 1994; Busch et al., 1998a). Although the differences in 16.23 staining were smaller than those found for anti-CLIP mAbs, higher CLIP accumulation correlated with lower 16.23 staining and vice versa.

The most profound CLIP exchange defect was produced by βD152N, which generated a consensus sequence for N-linked glycosylation. Reduced mobility of this mutant β chain on SDS-PAGE gels confirmed the presence of an additional glycan (cf. Figure 5B below).

Mutant HLA-DR Molecules Are SDS Unstable

HLA-DR3/peptide complexes isolated from wild-type cells are predominantly SDS stable, whereas the DR3/CLIP complexes of DM null cells are mostly SDS unstable (Mellins et al., 1990). SDS instability should be shared by mutant HLA-DR3 molecules that fail to interact productively with HLA-DM. To test this hypothesis, we compared the SDS stability of mutant HLA-DR molecules by Western blotting without sample boiling, followed by densitometry (Figure 3). All of the mutant HLA-DR molecules showed a reduction in the proportion of molecules that resist SDS-induced chain dissociation compared to the appropriate wild-type cell line. Thus, despite the presence of HLA-DM in these cells, these mutant molecules were unable to acquire a normal peptide repertoire that confers SDS stability.

DR3 Mutants Are Resistant to DM In Vitro

The phenotypes associated with the DR3 mutations are consistent with a block in DM interaction, but do not rule out other mechanisms, such as misrouting of HLA-DR molecules, altered interaction with other accesory molecules, or changes in CLIP affinity. To show a block in DM interaction directly, we examined the susceptibility of these mutant molecules to DM catalysis in vitro. Mutant HLA-DR molecules were affinity purified after transduction of the appropriate DRα and DRβ chains into the EBV-B cell line, 5.2.4, which lacks endogenous expression of DM and all DR isotypes (Mellins et al., 1991). The use of 5.2.4 transfectants allowed isolation of pure mutant HLA-DR3/CLIP complexes without co-purification of HLA-DRw52.

To examine the effect of the mutations on DM susceptibility in vitro, we measured dissociation of biotinylated CLIP (81–104) from purified mutant DR3 molecules in the presence and absence of purified, recombinant soluble DM (sDM; Figure 4). For these studies, synthetic CLIP was biotinylated at the C terminus, which is remote from the proposed DM interaction site (cf. Figure 2), to avoid any interference of the label with DM catalysis.

In the absence of sDM, the half-lives of CLIP release from all mutant HLA-DR molecules were comparable to wild- type, or slightly faster (Figure 4B). Thus, the CLIP accumulation seen in vivo cannot be explained by increased stability of the CLIP-DR complex. In contrast,

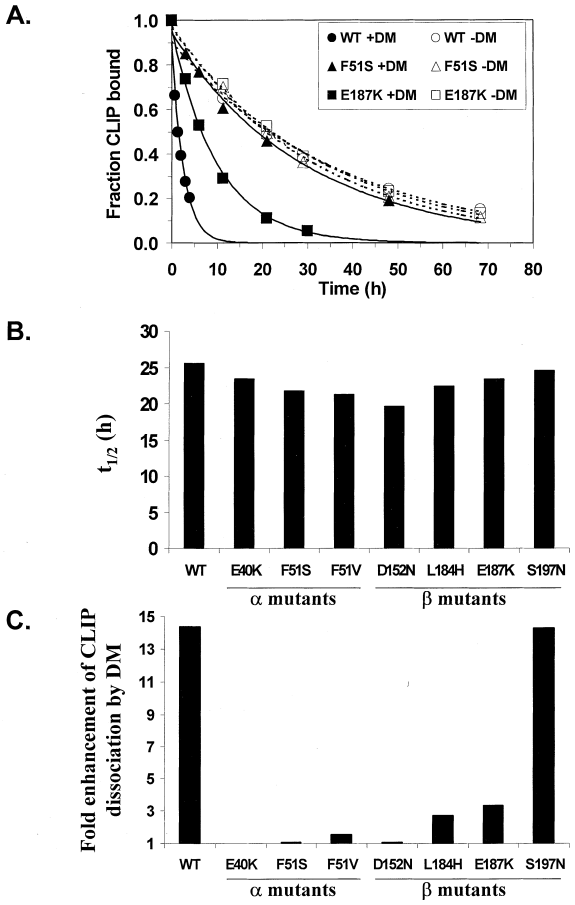

Figure 4. CLIP Dissociation from Mutant HLA-DR3 Molecules In Vitro

(A) Purified HLA-DR molecules (wild type and representative mutants) were loaded with C-terminally biotinylated CLIP [81–103] (20 μM), unbound peptide was removed, and dissociation of labeled CLIP was followed in the absence (open symbols) or presence (filled symbols) of 0.25 μM sDM at 37°C, pH 5.2. Starting counts for each molecule were normalized to a value of 1, and single-exponential decay curves were fitted.

(B) Dissociation half-lives ($t_{1/2}$) of DR3-CLIP complexes in the absence of sDM are shown for each mutant. Experimental error is ±5%.

(C) DM-enhancement of DR3-CLIP dissociation is equal to the $t_{1/2}$ in the absence of sDM divided by the $t_{1/2}$ in the presence of sDM. Data shown are representative of three or more experiments for each mutant.

dramatic differences were seen when sDM was added. Whereas CLIP dissociation from wild-type HLA-DR3 was accelerated by a factor of nearly 15-fold, most of the mutant HLA-DR molecules showed varying degrees of resistance to enhancement of CLIP release by sDM (Figure 4C). No enhancement was seen for αE40K, αF51S, and βD152N, whereas partial defects were observed for αF51V, βL184H, and βE187K. Only the weak mutant, βS197N, appeared indistinguishable from wild-type. The discrepancy for this mutant between the slight CLIP accumulation observed in vivo and normal sDM susceptibility in vitro likely is due to the position of the βS197N mutation on the connecting peptide between the β2 and transmembrane domains; this region is not likely to influence the in vitro dissociation assay, which is performed with soluble DM.

The DM resistance exhibited by each mutant in vitro correlated with CLIP accumulation and SDS instability in vivo. We concluded that the HLA-DR mutants presented in this study, except perhaps βS197N, diminish proper interaction with the ectodomains of DM.

Loss of DM Binding In Vitro

The simplest model to account for the diminished sDM susceptibility is a decrease in the affinity of HLA-DM for mutant HLA-DR molecules. Another possibility is that the DM binding site and site of catalytic action are topologically distinct, such that some mutations permit binding of the DM to DR but do not allow peptide release.

In order to examine these possibilities, we measured binding of mutant DR molecules to DM by coprecipitation. Purified mutant DR3/CLIP complexes were mixed with full-length recombinant DM, DM molecules were immunoprecipitated, and associated DR molecules were quantified by Western blotting (Figure 5). On the whole, the mutants bound less well to DM than wild-type DR3. Quantitative comparisons between the catalysis and binding assays are of limited value because of the semiquantitative nature of the coprecipitation assay, the use of different forms of recombinant DM in the two assays, and the use of different incubation conditions. Nonetheless, among the α chain mutants (Figure 5A), loss of DM binding correlated with loss of susceptibility to DM catalysis (cf. Figure 4): mutants αE40K and αF51S, which showed a greater loss of DM susceptibility than αF51V, also coprecipitated less well with DM. A similar trend was seen for the β chain mutants (Figure 5B). DRα- and DRβ-specific mAbs gave qualitatively similar results (Figure 5C), although the DRα-specific mAb was more sensitive to the effects of the mutations. Interestingly, densitometric analysis of DM binding to the βS197N mutant suggests some loss of binding, in contrast to the lack of a phenotype for this mutant in CLIP dissociation assays (cf. Figure 4C). Interaction with full-length DM (used in the coprecipitation assay) rather than soluble DM (used for CLIP release kinetics) may be required to reveal the defect caused by this mutation in the connecting peptide. Apart from this discrepancy, it was not possible within the accuracy of the assay to dissociate loss of DM binding of the DR3 mutants from loss of catalysis.

Discussion

We have described the isolation of several novel HLA-DR α and β chain mutants that accumulate CLIP due to a failure of the mutant molecules to interact productively with HLA-DM.

Most of the mutations do not appear to affect the overall folding of HLA-DR. All of the substitutions occur at relatively surface-accessible positions (αE40, αF51, and βE187 > 36%; βD152 and βL184 >15%; see Experimental Procedures) and are therefore unlikely to disrupt important inter- or intrachain contacts. No mutation disrupts surface expression, arguing that the mutant molecules are folded well enough to escape retention by the quality control apparatus in the ER (Ellgaard et al., 1999). Furthermore, conformation-specific antibodies recognize these molecules equally (Figures 1B and 1C; data

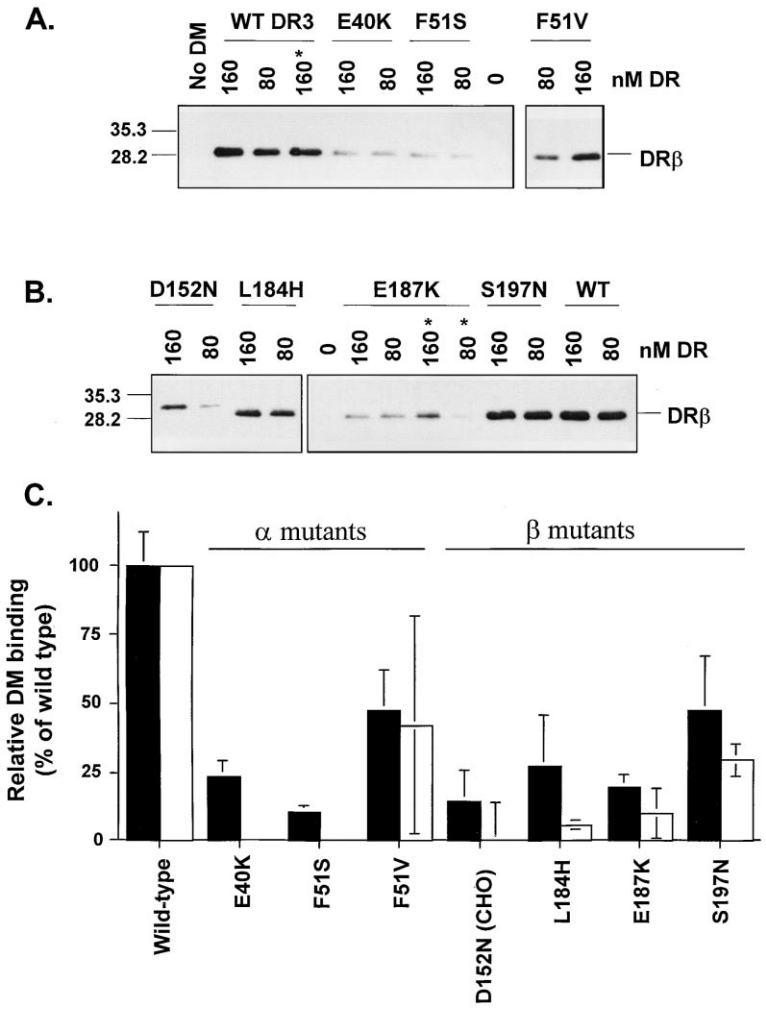

Figure 5. Mutant DR Molecules Are Impaired in Their Ability to Bind DM

DR molecules with mutations in the DR α (A) and β (B) chains were mixed with lysates of insect cells containing recombinant full-length DM. After incubation at 37°C (pH 5.0, 1% CHAPS), DM molecules were immunoprecipitated and associated DR molecules quantified by Western blotting. Levels of input DR and recovered DM were similar for all immunoprecipitations (data not shown). The asterisk (*) denotes the use of a second, independently purified DR preparation in some reactions. Note the decreased mobility of the βD152N mutant β chain, which carries an additional N-linked glycan.

(C) Densitometric quantitation of data pooled from two independent experiments like that shown in (A) and (B). Filled bars summarize results obtained using anti-DRβ (B10.a) and open bars those from anti-DRα (DA6.147) blots. Background-subtracted band intensities from film exposures in the linear range were normalized to wild type DR3 (= 100%) and corrected for variations in input DR and in recovered DM as described in Experimental Procedures. Qualitatively similar results were obtained in three to six additional experiments, although higher background seen in these experiments precluded precise densitometry.

not shown), and all mutants have near-normal rates of CLIP release in the absence of DM (Figure 4B). A possible exception is the glycosylation mutant, βD152N, which may well have a subtle conformational defect. This mutant releases CLIP slightly but consistently faster than the other mutants (Figure 4B), and the mobility of its α chain is somewhat reduced, suggesting alterations of remote α chain glycans (data not shown). In any case, the mapping information obtainable from this mutant is limited due to the large footprint of an N glycan.

As the mutations map to a common face on the DR crystal structure (Figure 2) and most do not detectably alter conformation, it is unlikely that the mutations influence DM interactions by changing the conformation of a distant DM contact site. Aberrant transport of mutant DR molecules to endosomes also is unlikely, because normal generation of CLIP (a prerequisite of isolation of high-CLIP mutants using CerCLIP.1) occurs only when endosomal transport is intact. Rather, the CLIP accumulation in mutant cells can be accounted for by the diminished interaction with DM, which was detectable by two independent in vitro assays. Thus, we propose that the mutations define the site at which DM binds DR during peptide exchange. Our mapping likely remains incomplete, because most mutations were isolated only once and because a previously isolated mutation that causes aberrant glycosylation of αN94 and poor interaction with DM (Mellins et al., 1994; Sloan et al., 1995; Denzin et al., 1996; Sanderson et al., 1996; R. B., unpublished data) was not found again in this study. However, extensive site-directed mutagenesis of adjacent charged and hydrophobic residues on this face of DR3 has revealed only one additional mutation (βV186K) that causes CLIP accumulation in vivo, so the map probably is not grossly incomplete (A. P, unpublished data). As our screen is biased against folding mutants, we may also have missed mutations that disrupt the DM binding site while simultaneously destabilizing the structure of the DR3 molecule.

Characteristics of the DM Binding Site on HLA-DR

Our data agree with previous results tentatively implicating this region of the molecule in interactions with DM: the mAb CerCLIP.1 prevents DM-mediated CLIP release by binding to the N terminus of CLIP (Denzin and Cresswell, 1995), and N-glycosylation of DRα residue 94 disrupts DM/DR interaction (see above). However, each of these results has inherent methodological limitations; at best, both results yield a large footprint with little information about the nature of the interaction.

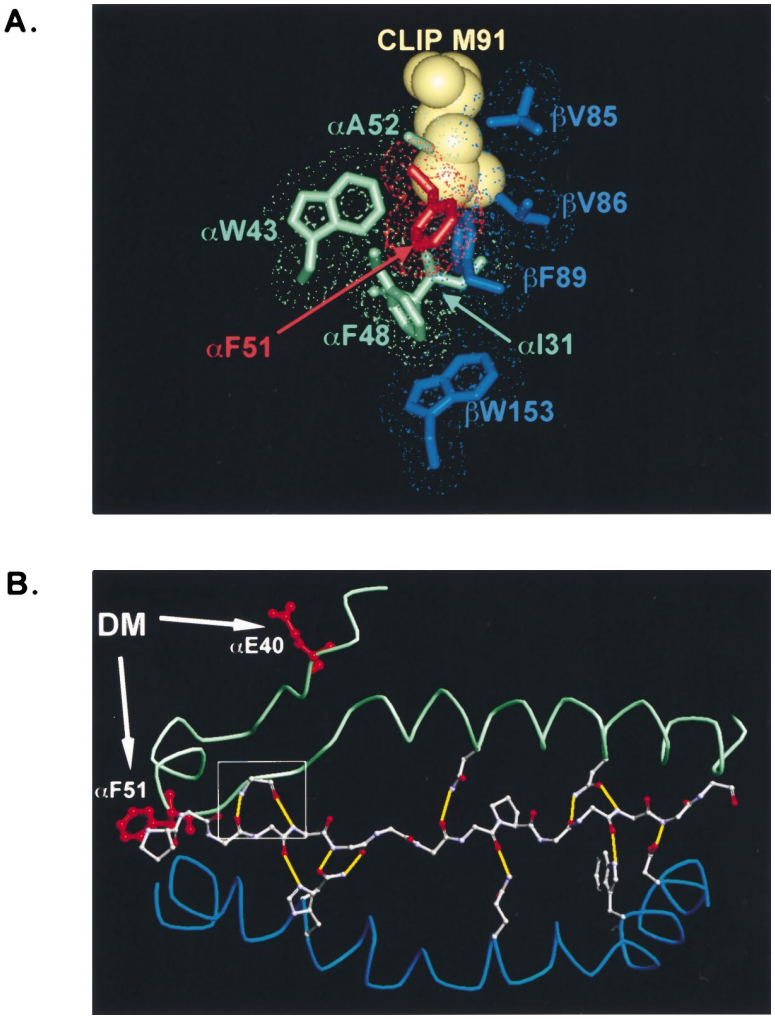

Figure 6. Proposed Mechanism of DM Action

(A) View of pocket 1 of the HLA-DR3-CLIP crystal structure and surrounding residues (same orientation as Figure 2), showing the cluster of hydrophobic amino acids connecting the P1 pocket of the groove to the surface exposed, DM-interacting residue, DRα F51. Engagement of this residue by DM may transmit a conformational change to the antigen binding groove. The P1 residue of CLIP (M91) is displayed in yellow, DRα residues are shown in green (except αF51, shown in red), and DRβ residues are shown in blue.

(B) TCR view of the peptide binding groove. The DM-interacting residues, DR αF51 and βE40, are positioned close enough to αS53 to allow the hydrogen bonds extending from this residue to the peptide backbone (boxed) to be broken.

The mutations presented here span the entire length of the HLA-DR ectodomains and are present on both chains. Binding is thus likely to involve a large surface area on HLA-DR (and HLA-DM) and may occur at an angle, across the β2 and α1 domains of DR. Additionally, a small contribution appears to be made by interactions between the connecting peptides of DM and DR. There was a conspicuous lack of disruptive mutations in the α2 domain, even after screening several additional site-directed mutants (charge reversal mutations at αGlu98, αArg100, and αGlu101), suggesting that this domain may not interact much with DM. Surface expression was normal for these DR mutants, arguing against the possibility that α2 domain mutations are unusually prone to causing folding defects. The previously described α2 domain mutant, αP96S, acts largely, if not entirely through addition of a bulky glycan at residue αN94, directly adjacent to the β chain (Mellins et al., 1994; Guerra et al., 1998).

The DRα chain is monomorphic, and the majority of polymorphisms occurring among HLA-DR β chain alleles localize to the peptide binding cleft. The homology among HLA-DR, -DP, and -DQ is also highest in regions outside of the peptide binding groove. DM itself is relatively nonpolymorphic, as is its murine homolog H-2M (Bodmer et al., 1995; Hermel et al., 1995). It is thus likely that DM interacts with the same surface on all MHC class II molecules. However, limited polymorphism does exist in DRβ on the surface proposed here to interact with HLA-DM. These polymorphisms could produce allelic differences in DM susceptibility. Additionally, there are several amino acid differences in the α and β chains of both HLA-DP and -DQ that have the potential to affect DM activity, based on their localization to the proposed DM interaction site. Further work with these other MHC class II molecules will be needed in order to determine whether these differences can alter peptide loading and presentation.

The putative DM binding site is distinct from the class II binding sites for SEB, TSST-1, and CD4. SEB binds solely to the α1 domain, primarily to the loops (turns) of the β-pleated sheet (Jardetzky et al., 1994). The closest residue in the DM binding site is αE40K, but this residue is well removed from SEB contact. The TSST-1 binding site overlaps that of SEB and does not involve the DM binding site (Kim et al., 1994). CD4 binds to the α2 and β2 domains of MHC class II molecules (Konig et al., 1992) but on the opposite face from the one proposed here. The DM/DR interface is also different from that between tapasin and MHC class I molecules (Suh et al., 1999).

Model for DM-Catalyzed Peptide Release

Our observations that exposed glutamic acid residues with expected pK_a values between 4.5 and 6 are part of the face of DR3 that interacts with HLA-DM (Figure 2) and are involved in DM interactions (Figures 4 and 5) may help explain the acid optimum of DM catalysis and binding (Sloan et al., 1995; Sanderson et al., 1996). A model involving direct interactions between charged residues of DM and DR is also compatible with the differential effects of salt on DM-catalyzed peptide binding to DR0401 molecules at neutral and endosomal pH (R. B., unpublished results). However, pH may also regulate DM/DR interactions indirectly through effects upon DM and DR conformation (Runnels et al., 1996; Ullrich et al., 1997).

Several lines of evidence have led to the notion that DM interacts best with open conformers of DR that are capable of rapid peptide release (Denzin et al., 1996; Kropshofer et al., 1997; McFarland et al., 1999). Stabilization of such a conformer by DM provides a simple mechanism that could explain the ability of DM to catalyze peptide exchange as well as to chaperone empty class II molecules. In this study, we have been unable to identify mutations that affect DM-catalyzed CLIP release from DR but not DM/DR binding. Although the number of mutations remains small, these results suggest that stable DM binding and catalysis of peptide release are highly concerted processes. In line with this view, we have recently observed that mutations in the antigen binding groove that stabilize CLIP/DR3 complexes also reduce binding to DM (R. C. D. et al., unpublished data).

Elucidation of the conformational changes induced by DM binding will require crystallographic studies of DM/class II complexes. However, the notion that small conformational changes may suffice to release the peptide is consistent with our observation that residues critical for DM/DR interaction were surface exposed in the crystal structure of DR3/CLIP and some were located near the groove, suggesting that major conformational adjustments are not required. Although breakage of hydrogen bonds may be a key step in DM-catalyzed peptide release (Weber et al., 1996), our results provide no evidence that DM interacts directly with DR residues that comprise the hydrogen-bonding network (Figures 2 and 6). Any weakening of hydrogen bonds by DM may instead involve indirect effects. One mechanism for this, previously suggested by Mosyak et al. (1998), is supported by our identification of two different mutations at position DR αF51 that impair DM interaction. This residue is exposed on the DM contact interface of HLA-DR at one end of a cluster of hydrophobic residues that communicate with the P1 sidechain pocket of the antigen binding groove (Figure 6A). Furthermore, this residue could act as a lever by which HLA-DM moves the extended strand including residues 51–53 such that the two-hydrogen bonds between Ser 53 and the peptide backbone are broken (Figure 6B). Residue αE40, on the adjacent strand, could also participate in this lever-like action. An open question is whether in addition to such local movements, the DM contact residues in the β2 domain are involved in more global conformational changes or whether they serve only to provide intermolecular contacts that contribute to affinity.

In conclusion, we have developed an efficient screen for mutant HLA-DR molecules with defective HLA-DM interaction. These mutants have allowed us to define a DM binding site on a lateral face of HLA-DR comprising exposed acidic and hydrophobic residues. Our results suggest a mechanism by which DM binding to MHC class II molecules translates into peptide release via disturbance of key interactions between the peptide and MHC class II molecule near the P1 pocket.

Experimental Procedures

Cell Lines and Antibodies

The following mutant EBV-B cell clones, all derived from a common progenitor, and their culture conditions, have been described previously. 9.22.3 lacks expression of DRα but expresses two DR β chains (DRB1*0301 and DRB3*0101) (Pious et al., 1985). 9.4.3 lacks DRB1*0301 expression but expresses DRα and the DRB3 gene product. The cell line 5.2.4 lacks expression of DR α, both β chains, as well as HLA-DM (Mellins et al., 1991). ϕNXA cells (kind gift of G.P. Nolan, Stanford University) were grown in DMEM supplemented with 10% heat-inactivated calf serum, 2 mM L-glutamine, and 1 U/ml Penicillin/Streptomycin (Life Technologies, Gaithersburg, MD).

The following antibodies have been described previously: L243 (Lampson and Levy, 1980), CerCLIP.1 (Avva and Cresswell, 1994), ISCR3 (Watanabe et al., 1983), 47G.S4 (Schafer et al., 1996), DA6.147 (Guy et al., 1982), 16.23 (Johnson et al., 1982), B10.a (Clark and Yakoshi, 1984), 5C1 (Sanderson et al., 1996), and 11323 (DM; gift of D. Zaller, Merck Research Laboratories; cf. Busch et al. (1998a). 6-39 is a murine IgG1 that recognizes DR3/CLIP complexes (E. D. M., M. Amaya, Z.-K. Pan, S. Lawrence, and Y. Paterson, unpublished data).

Cloning, PCR, and DNA Sequencing/DRA and DRB Constructs

Wild-type cDNAs encoding either DRA1*0101 (with 3 silent mutations) or DRB1*0301 were PCR-amplified using *Pfu* DNA polymerase under standard conditions, from pRC/CMV-DRA (kind gift of Ming-der Y. Chang, North Shore University Hospital-NYU School of Medicine) or pLNC5-DRB1*0301 (kind gift of William W. Kwok, Virginia Mason Research Center), respectively. The primers DRA-U1 (sense, 5′ CGA GAAGGATCCACTCCCAAAAAGAGCGCGCCCAA 3′) and DRA-L2 (anti-sense, CAGTGATCTGAATTCTAAGAAACACCATCACCTCC) were used to amplify the DRA*0101 cDNA; for DRB1*0301 we used DR3B-U1 (sense, CTGCTCGGATCCCTGGTCCTGTCCTGTTCTCC) and DR3B-L2 (anti-sense, CCTCTCGAATTCAAGAATAACAGCCAGGAG GGAAAGCTT) (all primers were made by Life Technologies). PCR products were cloned into the retroviral vector, pBMN-IRES-neo^r (kind gift of G. Nolan, Stanford University), using primer-encoded 5′ BamHI and 3′ EcoRI restriction sites.

cDNAs were mutagenized using error-prone PCR, using the following conditions (J. Caldwell, personal communication). Template cDNA (pBMN-DRA wt-IN or pBMN-DRB wt-IN; 10 fmol) was amplified using 10 pmol each of sense and antisense primers (pBMN-U1, AAGTAGACGGCATCGCAGCTT; and pBMN-L1, CCAGACCAACTG GTAATGGTA), and 2.5 U *Taq* polymerase (Life Technologies) in 7 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.01% gelatin (w/v), 5% DMSO (v/v), 0.2 mM dGTP, 0.2 mM dATP, 1 mM dTTP, and 1 mM dCTP. Amplification was carried out using 30 cycles of denaturation (94°C, 1 min.), annealing (50°C, 1 min.), and extension (72°C, 1.5 min.), followed by 10 min. at 72°C. Libraries were digested with BamHI and EcoRI and recloned into pBMN-IRES-neo^r. For both α and β chain libraries, about 150,000 colonies were obtained. Sequencing of ten randomly chosen clones each revealed mutation rates of about 4–5 $\times$ 10^{-4} mutations per nucleotide. The probability P that k mutations will occur in a sequence of length n at a given mutation rate, ϵ, was calculated as: $P = (n!/[(n-k)!k!)]\epsilon^k(1-\epsilon)^{n-k})$. By this estimate, 70% of the libraries should be wild-type, about 23% should have a single mutation, and a small minority should have two or more mutations. Each of the $\sim$2400 single point mutations should be represented an average of 16 times in the libraries.

We electroporated ligation reactions into Electromax DH10B cells (Life Technologies) using a BioRad Gene Pulser (0.1 cm cuvette, 2.5 kV, 100 Ω, 25 μF) and plated onto ten 15 cm LB agar plates containing 50 μg/ml ampicillin, yielding $\sim$1.5 $\times$ 10^5 colonies. Plasmid DNA was isolated without further expansion to prevent bias in the library.

cDNAs encoding the mutant HLA-DR chain of interest were rescued from cells by RT-PCR (Life Technologies) using the vector-derived primers, pBMN-U1 and pBMN-L1. They were cloned into the original pBMN-IRES-neo^r vector and sequenced at the Stanford PAN facility.

Site-directed mutagenesis was done by overlap extension PCR (Ho et al., 1989), using *Pfu* polymerase. Mutant cDNAs were cloned into pBMN-IRES-neo^r and verified by sequencing.

Molecular Modeling

DR3-CLIP images were generated from PDB coordinates (accession code 1A6A) using WebLab ViewerLite v.3.5 software (Molecular Simulations Inc., San Diego, CA). Surface accessibility was calculated using Swiss PDB viewer v.3.51 software (http://www.expasy.ch/spdbv/mainpage.html). Using this algorithm, only ~16% of all HLA-DR3 residues in the crystal structure have surface accessibility >36% and ~41% of all residues have >15% surface accessibility.

Retroviral Transduction

Production of amphotropic retrovirus using the pBMN vectors and ϕNXA packaging cell line was performed as described previously (Guerra et al., 1998) with minor modifications. In brief, 8 μg of plasmid DNA was transfected into 2.5×10^6 ϕNXA cells by calcium phosphate precipitation (Life Technologies). Twenty-four hours after transfection, cells were incubated in fresh media at 32°C. Retroviral supernatant was harvested at 48 hr posttransfection and centrifuged for 5 min at 500 $\times$ *g* to remove any cells. EBV-B cells were resuspended in the retroviral supernatant at a density of 5×10^5 cells/ml in the presence of 4 mg/ml polybrene (Sigma, St. Louis, MO). Following a 24 hr incubation at 32°C, transduced cells were washed and selected in media containing 1 mg/ml G418 (Life Technologies).

Flow Cytometry and FACSorting

Indirect staining of cells was performed as described (Busch et al., 1998a). Cells were analyzed on a FACScan flow cytometer using CellQuest v.3.1 software (Becton Dickinson, San Jose, CA).

For fluorescence-activated cell sorting, 10^7 cells were stained first with the mAb CerCLIP.1 and then a goat anti-mouse IgG-FITC (Caltag, Burlingame, CA) under sterile conditions and sorted on a Vantage FACSorter (Becton Dickinson) at the Stanford Shared FACS Facility. Cells with the highest levels of surface CLIP (arbitrarily set to the brightest 0.1%) were sorted into 96-well flat-bottom plates containing either fibroblast-conditioned RPMI or $\sim 10^5$ fibroblasts in RPMI.

Western Blotting

Western blotting of cell lysates was performed as described previously (Busch et al., 1998a). In brief, cells were washed, lysed in 1% IGEPAL CA-630 at of 10^7 cells/ml (1 hr, 4°C), and cleared of nuclei and debris. Nonreducing Laemmli SDS-PAGE sample buffer was added, and lysates were left unboiled (4°C) or boiled (10 min., 95°C) prior to loading on 10% SDS-PAGE gels. Separated proteins were transferred to PVDF (Immobilon P, Millipore, Bedford, MA). Blots were developed using goat anti-mouse IgG HRP conjugate and an enhanced chemiluminescence substrate (Renaissance; Dupont NEN, Boston, MA). Band intensities were quantified on light film exposures using a GS-710 densitomer and Quantity One software (BioRad, Hercules, CA).

Purification of HLA-DR Molecules and Soluble DM

HLA-DR molecules were purified from the EBV-B cell line, 5.2.4, transduced with the appropriate DRA and DRB cDNAs. Cells expressing high levels of surface HLA-DR were isolated by magnetic bead sorting (Dynal, Lake Success, NY) followed by FACS using the mAb L243. Purification was performed by affinity chromatography using an L243-CNBr-sepharose column as previously described (Mellins et al., 1994). 1–4 $\times$ 10^9 cells were lysed for purification of each mutant HLA-DR3 molecule. Purity and identity of protein samples were assessed by silver staining and Western blotting. The conformational integrity of DR $\alpha\beta$ heterodimers in each DR preparation was compared by a sandwich ELISA, using the anti-DR mAb, L243, to capture intact $\alpha\beta$ dimers (Stern and Wiley, 1992). Soluble DM molecules were purified from supernatants of transfected *Drosophila* cells as described (Sloan et al., 1995; Busch et al., 1998b).

In Vitro Peptide Dissociation Assays

Human CLIP with a C-terminal lysine [LPKPPKPVSKMRMATPLLM QALPK] was synthesized, derivatized with long-chain biotin, and purified by HPLC (Genemed Synthesis, Inc., South San Francisco, CA). Labeled CLIP was incubated with purified mutant DR3 in the presence of 1% n-octyl-glucoside (Boehringer Mannheim, Indianapolis, IN) for 24 hr at 37°C in citrate-phosphate buffer containing 0.5% IGEPAL CA-630, 1% BSA, and 0.05% NaN_3, and 1mM PMSF (pH 5.2). Peptide/DR complexes were separated from free peptide using a Sephadex G50-Superfine spin column and diluted to ~12 nM in citrate-phosphate buffer (as above without n-octyl-glucoside, pH 5.2). Unlabeled CLIP was added to a final concentration of 5 μM to prevent rebinding of dissociated biotinylated CLIP, and dissociation was allowed to proceed at 37°C with or without 0.25 μM soluble HLA-DM for various times. At each time point, reactions were stopped by adding 2 volumes of ice-cold 50 mM Tris-HCl (pH 8.2), 150 mM NaCl, 0.5% IGEPAL CA-630, 0.5% BSA, and 0.05% NaN_3. Neutralized reaction mixtures (100 μl) were transferred to L243-coated, blocked flat-bottom 96-well microtiter plates. After 2 hr of capture of DR complexes at 4°C, plates were washed in 200 μl PBS, 0.05% Tween-20, and 0.05% NaN_3. DR-bound biotinylated peptide was detected by addition of 0.1 μg/ml Eu^{3+}-conjugated streptavidin (EG&G Wallac, Gaithersburg, MD) in blocking buffer at 4°C for 1 hr. After further washes, enhancement solution (100 μl; EG&G Wallac) was added, and time-resolved fluorescence of Eu^{3+} was detected using a DELFIA 1234 fluorescence plate reader (EG&G Wallac).

DM/DR Binding Assay

Drosophila cells transfected with full-length DM cDNAs (DMA*0101/DMB*0101 (Busch et al., 1998b) under the control of the metallothionein promoter were induced with 1 mM $CuSO_4$ for 24 hr and lysed in PBS, 1% CHAPS, plus protease inhibitors, at a density of 5×10^7 cells/ml. DM concentration in the lysate (estimated by Western blotting using purified soluble recombinant DM as a standard) was ~90 ng/μl. DM-containing lysate (1–2 μl) was mixed with purified wild-type and mutant DR3 molecules (100–500 ng) in 50–60 μl reaction buffer [50 mM sodium acetate (pH 5.0), 150 mM NaCl, 1% CHAPS, 0.5% BSA, 0.05% NaN_3, and protease inhibitors]. After incubation at 37°C for 1–2 hr, reaction mixtures were immunoprecipitated with an anti-DM antiserum (11323; 2 μl serum bound to 15 μl protein A-sepharose) for 1 hr at 4°C. Immunoprecipitates were separated by 12% SDS-PAGE, and DM-associated DR3 molecules were detected by Western blotting using the DRα-specific mAb, DA6.147, or the DRβ-specific mAb, B10.a. Blots were reprobed with the DMβ-specific mAb, 47G.S4, or with the DMα-specific mAb, 5C1, to check for consistent recovery of DM. To ensure that comparable levels of all mutant DR molecules were used, the input DR molecules were also compared by Western blotting on the day of each assay. Densitometry was performed using a BioRad GS-710 densitometer. After subtraction of background, band intensities for the mutants were normalized to wild-type (= 1.0) and corrected for variation in recovered DM and input DR using the following formula:

$$\text{Corrected intensity} = 100 \times \frac{(\text{normalized DR band intensity from coIP})}{(\text{normalized input DR intensity}) \times (\text{normalized recovered DM intensity})}$$

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