Enhanced Dissociation of HLA-DR–Bound Peptides in the Presence of HLA-DM

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Human leukocyte antigen (HLA)–DM is a critical participant in antigen presentation that catalyzes the release of class II–associated invariant chain–derived peptides (CLIP) from newly synthesized class II histocompatibility molecules, freeing the peptide-binding site for acquisition of antigenic peptides. The mechanism for the selective release of CLIP but not other peptides is unknown. DM was found to enhance the rate of peptide dissociation to an extent directly proportional to the intrinsic rate of peptide dissociation from HLA-DR, regardless of peptide sequence. Thus, CLIP is rapidly released in the presence of DM, because its intrinsic rate of dissociation is relatively high. In antigen presentation, DM has the potential to markedly enhance the rate of peptide exchange, favoring the presentation of peptides with slower intrinsic rates of dissociation.

Class II histocompatibility molecules such as HLA-DR bind antigenic peptides generated in antigen-presenting cells and display them at the cell surface for recognition by $CD4^+$ T lymphocytes (*1*). Newly synthesized class II αβ heterodimers associate with the invariant chain in the endoplasmic reticulum, and the molecules are transported to endosomal compartments where the invariant chain is partially removed by proteases (*2*). DM-deficient antigen-presenting cells are defective in presentation of protein antigens to T cells (*3*), and the class II molecules expressed in these cells are largely occupied by a nested set of CLIP peptides that share a core sequence (*4*) that interacts with the peptide-binding site in a defined manner (*5*). Thus, DM is required for the removal of the last fragments of the invariant chain, freeing the peptide-binding site for acquisition of antigenic peptides. The DM molecule directly induces the release of CLIP from class II molecules (*6–8*). With one exception (*6*), DM was not observed to release peptides other than CLIP (*6–8*). The structural features that make HLA-DR–CLIP complexes susceptible to DM have not been defined.

We investigated the sequence requirement for susceptibility to DM-induced release of peptides from HLA-DR molecules by using a series of synthetic peptides and affinity-purified DM isolated from human lymphoblastoid cells (*9*). In agreement with previous experiments (*6–8*), we found that low concentrations of DM but not control proteins markedly enhanced peptide binding to purified DR molecules and selectively induced release of the CLIP(81–104) fragment but not the antigenic peptide fragments HA(306–318) and HSP(3–13) from DR1 and DR3, respectively (Fig. 1). The crystal structure of DR3-CLIP complexes (*5*) indicates that the peptide complex is stabilized by hydrogen bonds between conserved residues in DR and peptide main-chain atoms. Peptide side chains interact in a series of pockets that accommodate residues P1 (Met^{91}), P3, P4, P6, P7, and P9 (Met^{99}). The potential requirement for NH_2-terminal residues that extend out of the binding site (*10*) was excluded with truncated peptides. CLIP(89–100) and longer CLIP peptides bound DR1 and DR3 with equal affinity and were equally released by DM. The possibility that DM interacts with core peptide side chains that extend out of the binding site also was excluded, because substitutions at nonanchor positions did not affect release (Fig. 1B).

The role of peptide anchor (A) residues was evaluated with chimeric CLIP(89–100) peptides containing anchors from the HA(306–318) peptide fragment at P1, P4, P6, and P9 (Fig. 2A). CLIP(89–100). A-HA was minimally released from DR1 during a 4-hour incubation with 9 nM DM, as was an analog singly substituted at P1, whereas

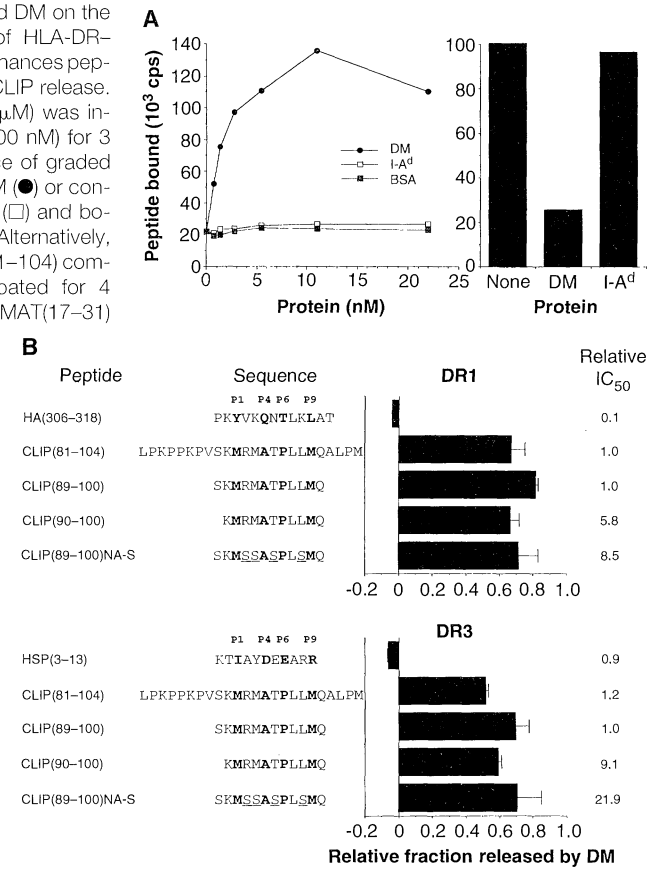

Fig. 1. Effect of affinity-purified DM on the formation and dissociation of HLA-DR–peptide complexes. (**A**) DM enhances peptide binding to HLA-DR and CLIP release. Biotinylated HA(306–318) (1 μM) was incubated with purified DR1 (100 nM) for 3 hours at 37°C in the presence of graded amounts of affinity-purified DM (●) or control proteins [murine H-2 I-A^d (□) and bovine serum albumin (■)] (*9*). Alternatively, preformed DR1-biotin-CLIP(81–104) complexes (100 nM) were incubated for 4 hours with 100 μM unlabeled MAT(17–31) and 8 nM DM or I-A^d. DR-biotin-peptide complexes were quantified in duplicate samples by a streptavidin-europium fluorescence immunoassay (*8*), and results are expressed as mean fluorescence counts per second (cps). (**B**) Release of biotinylated variants of CLIP from HLA-DR by DM. Preformed DR-biotin-peptide complexes (50 nM) were incubated with or without 9 nM affinity-purified DM in the presence of excess unlabeled specific peptide for 4 hours at 37°C. Biotin-peptide complexes from triplicate samples were quantified by the europium immunoassay. The relative fraction released by DM was determined by comparing the fraction of labeled peptide remaining bound to HLA-DR to the amount bound in the absence of DM. The affinity of each peptide is reported as the IC_{50} value (median inhibitory concentration) relative to CLIP(89–100) (*11*). Peptide anchor positions (*15*) (boldface under "sequence" column) correspond to pockets P1, P4, P6, and P9 of the DR binding site, according to the DR1-HA(306–318) (*17*) and DR3-CLIP (*5*) crystal structures. Anchor positions for HSP(3–13) were assigned on the basis of placement of aspartic acid in the P4 pocket (*18, 19*). In CLIP(89–100)NA-S, nonanchor residues in the core sequence were replaced by serine (underlined). Amino acid abbreviations are listed in (*15*).

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those substituted at P4, P6, or P9 were released to an extent similar to the parent peptide. The importance of the anchor residues was confirmed by the observation that chimeric HA(306–318) containing anchors (P1, P4, P6, P9) from CLIP is released by DM. An HA(306–318) analog containing methionine (M) instead of tyrosine (Y) at the dominant anchor position (P1) (HA.Y308M) was substantially released from DR1 in the presence of DM. Similar results were obtained with HSP(3–13) and DR3. Chimeric HSP(3–13) containing anchors from the CLIP sequence was released from DR3, whereas CLIP(89–100) with anchors from HSP was not. The residue at P4 (aspartic acid or alanine) was critical in determining the sensitivity to DM-induced release. Thus, the residues at dominant anchor positions (P1 for DR1 and P4 for DR3) in the absence of other substitutions can control sensitivity to DM. However, substitutions at nondominant anchor positions also can modulate the DM sensitivity of DR-peptide complexes (DRp). For example, DR1 complexes containing CLIP(89–100) with aspartic acid instead of alanine at P4 are relatively resistant to DM, which suggests that the collective identity of the residues at anchor positions rather than the identity of any single anchor residue determines whether a DRp will dissociate in the presence of low concentrations of DM.

The sensitivity of a given DRp complex to DM does not always correlate with apparent affinity measured in competitive inhibition assays (*11*). For example, CLIP(89–100) and HSP(3–13) are differentially sensitive to DM but bind DR3 with similar IC_{50} values. In contrast, sensitivity to DM correlates with intrinsic dissociation rates measured in the absence of DM (*12*). Peptides with intrinsic dissociation half-times of <20 hours were released by DM and those with half-times of >100 hours were not, under the experimental conditions used (Fig. 2A).

This result suggested the possibility that DM can accelerate the dissociation of any peptide: DM catalyzed the dissociation of all DRp tested, with first-order kinetics (Fig. 2B and Table 1). The rate constants observed, k_{obs}, were linear functions of the DM concentration and the intrinsic first-order peptide dissociation constants, k_{in}. Remarkably, the slope of k_{obs} versus the total concentration of DM ($[DM]_{total}$) was directly proportional to k_{in} for all DRp, including peptides with sequences unrelated to CLIP, so that

$$k_{obs} = k_{in} + jk_{in}[DM]_{total} \quad (1)$$

where the constant j is estimated to be $(1.2 \pm 0.7) \times 10^9$ M^{-1}. Thus, the DM-catalyzed rate is directly proportional to the intrinsic rate of peptide dissociation. This finding can be interpreted in terms of a simple model in which DM reversibly binds DRp and stabilizes a transition state of DRp, lowering the energy of activation for peptide release (Fig. 3A). Under conditions in which

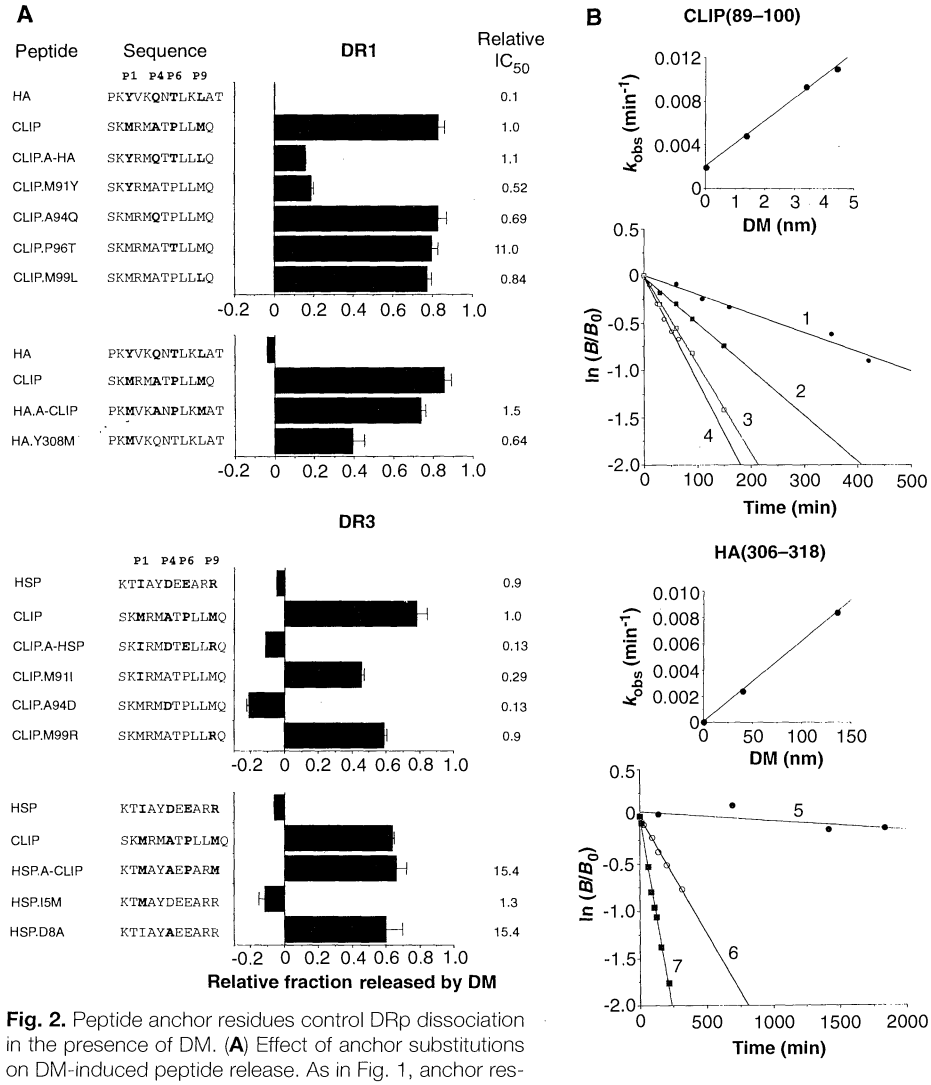

Fig. 2. Peptide anchor residues control DRp dissociation in the presence of DM. (**A**) Effect of anchor substitutions on DM-induced peptide release. As in Fig. 1, anchor residues for HA, CLIP, and HSP are in boldface. But for the mutant peptides, boldface residues indicate mutated anchor residues (*15*). Peptide release and relative affinities were determined as described in the legend to Fig. 1. CLIP refers to CLIP(89–100), HA to HA(306–318), and HSP to HSP(3–13). (**B**) The peptide dissociation rate is a linear function of DM concentration. The dissociation of fluorescein-labeled peptides from DR1 was measured in the presence of various concentrations of affinity-purified DM at pH 5 and 37°C by high-performance size-exclusion chromatography (HPSEC) (*20*). Observed first-order dissociation rate constants (k_{obs}) were calculated from the slopes of plots of $\ln(B/B_0)$ versus time, where B_0 represents the concentration of peptide complexes at time 0. Best-fit lines were calculated by the least squares method. Curve 1: 0 nM DM, half-time $t_{1/2}$ = 5.7 hours; curve 2: 1.5 nM, $t_{1/2}$ = 2.4 hours; curve 3: 3.4 nM, $t_{1/2}$ = 1.2 hours; curve 4: 4.4 nM, $t_{1/2}$ = 1.0 hours; curve 5: 0 nM DM, $t_{1/2}$ = 120 hours; curve 6: 39 nM, $t_{1/2}$ = 4.7 hours; curve 7: 135 nM, $t_{1/2}$ = 1.4 hours.

Table 1. The DM-catalyzed rate of peptide dissociation is directly proportional to the intrinsic rate of peptide dissociation. The dissociation of fluorescein-labeled peptides from DR1 was measured by HPSEC (*20*). Observed first-order dissociation rate constants (k_{obs}) were calculated for three concentrations of DM and slopes were derived from plots of k_{obs} versus $[DM]_{total}$. Best-fit lines were calculated by the least squares method. Intrinsic dissociation rate constants (k_{in}) represent y intercepts of the plots. The slopes of these plots were approximately proportional to k_{in}.

Peptide	k_{in} (min^{-1})	Slope/k_{in} (M^{-1})*
CLIP(89–100)	2.1×10^{-3}	1.0×10^9
CLIP-M91I	1.7×10^{-3}	1.0×10^9
CLIP-A94Q	4.1×10^{-3}	1.0×10^9
MBP(90–102)	1.5×10^{-3}	0.8×10^9
CLIP-M91Y	3.4×10^{-5}	2.8×10^9
MAT(17–31)	1.5×10^{-4}	1.2×10^9
HA(306–318)	7.3×10^{-5}	0.8×10^9

*Slopes were determined from plots of k_{obs} versus DM concentration.

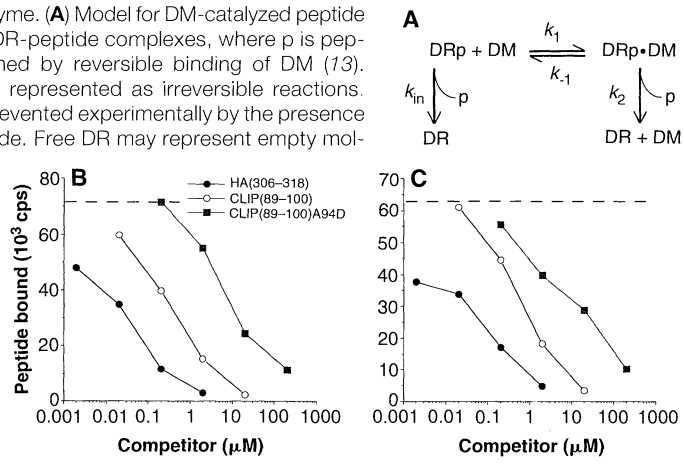

Fig. 3. DM acts like an enzyme. (**A**) Model for DM-catalyzed peptide release. DRp represents DR-peptide complexes, where p is peptide and DRp•DM is formed by reversible binding of DM (*13*). Peptide dissociations are represented as irreversible reactions. Reverse reactions were prevented experimentally by the presence of excess unlabeled peptide. Free DR may represent empty molecules (which are unstable at 37°C), inactivated DR, or DR molecules bearing unlabeled peptide. (**B** and **C**) Peptide competition in the absence (B) and presence (C) of DM. Purified DR1 (100 nM) was incubated at pH 5 with 0.1 μM biotin-HA(306–318) and various concentrations of the indicated unlabeled peptides for 18 hours (B) or for 4 hours in the presence of 17 nM affinity-purified DM (C). DR1-biotin-peptide complexes were quantified by the streptavidin-europium fluorescence immunoassay.

peptide release is irreversible and $[DM]_{total} \ll [DRp]$, one can apply the steady-state rate equation for enzyme-catalyzed reactions (*13*). If $[DRp] \ll K_m$ (Michaelis constant), the equation simplifies to

$$k_{obs} = (k_2/K_m)[DM]_{total} + k_{in} \quad (2)$$

Combining Eqs. 1 and 2 gives

$$k_2/k_{in} = jK_m \quad (3)$$

This equation provides a means of estimating the relative theoretical rate of peptide dissociation from DM-DRp complexes compared with the rate of peptide dissociation from DRp alone. Given preliminary data suggesting that K_m is $\geq 10^{-5}$ M, we estimate that peptide dissociation could be increased $\geq 10^4$-fold from DM-DRp complexes compared with dissociation from free DRp.

The observation that the rate of DM-catalyzed peptide dissociation is determined by the k_{in} value of a given peptide supports the idea that bonds involving peptide main-chain atoms rather than side chains are disrupted in the DM-stabilized transition state. This condition would reduce the activation energy for peptide release or exchange to the same degree regardless of peptide sequence. It is unlikely that a structural change in a particular pocket in DRp is responsible for enhanced peptide dissociation because substitutions at different positions in bound peptides can alter sensitivity to DM. The presence of DM should not alter the equilibrium binding of individual peptides. Indeed, no change is observed in the order of potency of peptides in competitive inhibition binding assays in the presence or absence of DM (Fig. 3, B and C), indicating that the rates are changed but not the equilibrium. However, it is unlikely that equilibrium is achieved during antigen processing, given the relatively rapid transport of newly synthesized and recycling DR molecules through different intracellular compartments with local environments that may differ in peptide composition, DM concentration, and pH. Thus, in addition to its critical role in removing CLIP from the peptide-binding site of newly synthesized class II molecules, DM also may modify the repertoire of peptides made available for recognition by $CD4^+$ T cells, as originally proposed by Sloan *et al.* (6), favoring peptides with slow intrinsic rates of dissociation.

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9. DR1 and DR3 molecules were purified from LG2 and T2-DR3 cells (*4*), respectively, with an L243 affinity column as described (*8*). DM was isolated from LG2 detergent lysates by affinity chromatography on Sepharose beads coupled to rabbit antibody to the COOH-terminus of DMβ (*8*); eluted in 0.5% *S*-octyl glucopyranoside, 100 mM glycine, and 0.15 M NaCl (pH 11.5); and neutralized with 1 M tris-HCl (pH 7.5). The eluted protein was ~25% DM as judged by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining. The DM concentration was determined by measuring absorbance at 280 nm relative to DR1 and assuming molar absorption coefficients of 94,680 and 64,710 for DM and DR1, respectively. The presence of contaminating protease activity was excluded by incubating affinity-purified DM with DR1 followed by SDS-PAGE analysis. Mock-purified DM and I-A^d (purified with an MKD6 monoclonal antibody affinity column by parallel procedures) had no activity in peptide release assays, excluding a role for detergent or other components. Peptides were synthesized by fluorenyl methoxycarbonyl chemistry with a Ranin Symphony multiple peptide synthesizer and labeled with biotin at the α-amino group before or after deprotection and cleavage by reaction with biotin-amido caproate *N*-hydroxysuccinimide as described (*14*). All binding and dissociation reactions were done in 0.1 M citrate-phosphate buffer (pH 5.0) with 0.2% NP-40 containing protease inhibitors (binding buffer). DR-biotin-peptide complexes were formed by incubation of 500 nM DR with 5 μM biotin-peptide complex for 18 hours at 37°C. For dissociation, preformed complexes were diluted 1:10 and incubated with 100 μM unlabeled MAT(17–31) peptide fragment (sequence, SGPLKAEIAQRLEDV) (*15*) or HSP(3–13) with or without DM. After neutralization, the complexes were captured on L243-coated microtiter wells at 4°C and biotin-peptide complexes were measured with streptavidin-europium as described (*8*).
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11. The affinity of each peptide was determined by measuring the capacity of unlabeled peptides to inhibit binding of biotin-MAT(17–31) to DR1 or biotin-HSP(3–13) to DR3. Values are normalized relative to CLIP(89–100), which is assigned an arbitrary IC_{50} value of 1.0.
12. Dissociation rates were measured in the absence of DM for most of the peptides represented in Fig. 2A. Complexes were formed by incubation of 500 nM purified DR1 or DR3 with 2.5 μM biotin-peptide for 18 to 72 hours. Samples were diluted and incubated with 100 μM HA(306–318) (DR1) or HSP(3–13) (DR3) in binding buffer at pH 5 and 37°C. At various time points, samples were placed on ice and the pH was neutralized with 1 M tris-HCl (pH 7.5) to prevent further dissociation. DR-biotin-peptide complexes were quantified by the europium fluorescence immunoassay.
13. The total rate of peptide dissociation is the sum of catalyzed and uncatalyzed rates. The catalyzed rate is equal to k_2 [DRp•DM]. Under conditions where peptide dissociation is irreversible, application of the steady approximation gives
$$v = \frac{k_2[DM]_{total} \times [DRp]}{K_m + [DRp]} + k_{in}[DRp]$$
where v = initial reaction velocity and $K_m = (k_{-1} + k_2)/k_1$.
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15. Abbreviations for 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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20. Solid-phase HA(306–318) was labeled through the α-amino group with fluorescein isothiocyanate (FITC) before deprotection and cleavage. Other peptides, including MBP(90–102) (sequence, HFFKNIVTPR-TPA) (*15*), were labeled with FITC after cleavage. Preformed DR1-peptide complexes were diluted to 1 μM total DR1 and incubated at 37°C in 0.1 M citrate-phosphate buffer (pH 5) and 0.2% NP-40 in the presence of 0.1 to 0.2 mM unlabeled HA(306–318) and the indicated concentration of affinity-purified DM. At various times, 10-μl samples were removed and DR1-FITC-peptide complexes were quantified with a Tosohaas TSK GFC 200 high-performance size-exclusion column (7.8 × 150 mm) and a Shimadzu RF-10A fluorometer with 490-nm excitation and detection at 520 nm. The column buffer was 50 mM phosphate, 150 mM NaCl, 1 mM dodecyl β-D-maltoside (pH 7.0) (*16*). DR eluted between 3.2 and 3.8 min with a flow rate of 1 ml/min.

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