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Editing of the HLA-DR–peptide repertoire by HLA-DM

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Antigenic peptide loading of classical major histocompatibility complex (MHC) class II molecules requires the exchange of the endogenous invariant chain fragment CLIP (class II associated Ii peptide) for peptides derived from antigenic proteins. This process is facilitated by the non-classical MHC class II molecule HLA-DM (DM) which catalyzes the removal of CLIP. Up to now it has been unclear whether DM releases self-peptides other than CLIP and thereby modifies the peptide repertoire presented to T cells. Here we report that DM can release a variety of peptides from HLA-DR molecules. DR molecules isolated from lymphoblastoid cell lines were found to carry a sizeable fraction of self-peptides that are sensitive to the action of DM. The structural basis for this DM sensitivity was elucidated by high-performance size exclusion chromatography and a novel mass spectrometry binding assay. The results demonstrate that the overall kinetic stability of a peptide bound to DR determines its sensitivity to removal by DM. We show that DM removes preferentially those peptides that contain at least one suboptimal side chain at one of their anchor positions or those that are shorter than 11 residues. These findings provide a rationale for the previously described ligand motifs and the minimal length requirements of naturally processed DR-associated self-peptides. Thus, in endosomal compartments, where peptide loading takes place, DM can function as a versatile peptide editor that selects for high-stability MHC class II–peptide complexes by kinetic proofreading before these complexes are presented to T cells.

Keywords: antigen presentation/kinetic proofreading/ mass spectrometry/MHC class II/peptide binding assay

Introduction

Classical major histocompatibility complex (MHC) class II molecules are heterodimeric glycoproteins that are specialized in presenting peptides on the surface of antigen-presenting cells for recognition by $CD4^+$ T cells (Germain *et al.*, 1994). Newly synthesized class II α and β chains associate with a type II glycoprotein, the invariant chain (Ii), forming a nonameric $(\alpha\beta Ii)_3$ complex in the ER (Roche *et al.*, 1991). Ii binds to class II molecules by virtue of a domain encoded by exon 3 (Freisewinkel *et al.*, 1993; Bijlmakers *et al.*, 1994; Romagnoli *et al.*, 1994), termed CLIP (class II associated Ii peptide) (Riberdy *et al.*, 1992), assisted by Ii regions flanking the CLIP segment on both sides (Vogt *et al.*, 1995). This association protects the peptide-binding groove of class II molecules from premature occupancy by endogenous peptides or by unfolded polypeptides in the ER (Busch *et al.*, 1996). In addition, a targeting motif in the cytosolic domain of Ii is responsible for efficient delivery of class II αβIi complexes from the *trans*-Golgi network to acidic endosomal–lysosomal organelles (Bakke and Dobberstein, 1990; Lotteau *et al.*, 1990). In these compartments class II molecules encounter peptides generated by proteolysis from self-proteins of the endocytic pathway or from internalized exogenous antigens (Chicz *et al.*, 1993). Loading with these peptides, however, cannot occur unless Ii is proteolytically degraded (Roche and Cresswell, 1991) and the intermediary class II-associated fragment CLIP removed (Avva and Cresswell, 1994). After CLIP has been exchanged for cognate peptide, class II molecules are sorted to the cell surface.

CLIP as a peptide is thought to occupy the peptide-binding groove of class II dimers (Ghosh *et al.*, 1995). Thus, comparable with the situation *in vitro*, where the dissociation of prebound self-peptides is known to prolong the association rate of synthetic peptides to >20 h (Buus *et al.*, 1986), the removal of CLIP appears to be the rate-limiting step for loading *in vivo*. At least two mechanisms have evolved to solve this problem. (i) CLIP has low affinity for certain class II alleles, such as the murine A^k, and therefore dissociates very rapidly (Brooks *et al.*, 1994). In addition, enhanced dissociation at endosomal pH is mediated by the N-terminal segment of CLIP (Urban *et al.*, 1994; Kropshofer *et al.*, 1995a), resulting in partial loading with self-peptides of certain human class II alleles in the absence of HLA-DM (Kropshofer *et al.*, 1995b). (ii) Most efficiently, CLIP is removed by a non-classical class II molecule, HLA-DM (Fling *et al.*, 1994; Morris *et al.*, 1994).

HLA-DM is composed of two subunits, α and β, which are both encoded in the MHC class II region (Cho *et al.*, 1991; Kelly *et al.*, 1991). Unlike classical class II molecules, HLA-DM does not seem to function as a peptide receptor (Denzin and Cresswell, 1995; H.Kropshofer, in preparation) and it carries a cytoplasmic internalization signal preventing it from being expressed on the cell surface (Lindstedt *et al.*, 1995; Marks *et al.*, 1995). Instead, HLA-DM is localized to MHC class II-containing endocytic MIIC compartments (Sanderson *et al.*, 1994), where it transiently binds to HLA-DR molecules (Sanderson *et al.*, 1996). During this transient

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interaction CLIP is believed to be removed, as evidenced by *in vitro* studies (Denzin and Cresswell, 1995; Sherman *et al.*, 1995; Sloan *et al.*, 1995). Further studies have shown that HLA-DM not only enhances dissociation of CLIP and of LIP, a 22 kDa CLIP-containing processing intermediate of Ii (Denzin and Cresswell, 1995), but it also accelerates the off-rate of a peptide different from CLIP, the immunodominant epitope from myelin basic protein (MBP), bound to DR1 (Sloan *et al.*, 1995). In contrast, another epitope, the DR1-restricted peptide HA(307–319) from influenza hemagglutinin revealed to be DM insensitive (Sloan *et al.*, 1995). Based on this limited observation it has been speculated that DM may function as a peptide editor that selects a certain subset of αβ–peptide complexes for presentation on the cell surface. However, it remained to be established whether the MBP peptide mentioned above was an exception and the function of DM mainly restricted to removal of CLIP, or whether DM may in general proofread self-peptides different from CLIP.

In this report we provide evidence that DM can act as a highly versatile peptide editor, with the kinetic stability of class II–peptide complexes determining whether a peptide is DM resistant or not: low-stability complexes are good substrates for DM-mediated peptide release, whereas high-stability complexes are DM insensitive. This mechanism selects for presentation of long-lived class II–peptide complexes and at the same time minimizes the number of class II molecules that may lose unstably bound peptide *en route* to or after reaching the cell surface.

Results

HLA-DR molecules from lymphoblastoid cell lines carry self-peptides that are DM sensitive

In endosomal compartments of antigen-presenting cells HLA-DM removes CLIP from the intermediary DR–CLIP complexes so that loading with peptides can occur. In order to see whether all these peptides would be resistant to the action of DM, HLA-DR molecules affinity-purified from lysates of two different EBV-transformed lymphoblastoid cell lines were incubated for 16 h under endosome-like conditions (pH 5.0, 37°C) in the absence or presence of affinity-purified HLA-DM. Putatively released peptides were removed by ultrafiltration and the whole set of bound self-peptides, including those that may have rebound to DR, was eluted by treatment with trifluoroacetic acid and analyzed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). Although this technique does not allow for measurement of the absolute amount of peptides, it is suitable for monitoring changes in the composition of peptide mixtures if one compares the relative height of signals. In the case of DR3, DM treatment left the majority of signals of the MS peptide spectrum unchanged (Figure 1A), but a considerable number of the signals, including those corresponding to CLIP, have been strongly reduced or have vanished. These observations suggest that DM has released a certain subset of self-peptides, including CLIP, whereas most of the peptides were not susceptible to DM-mediated removal. These findings were reproducible with different DR3 preparations (unpublished data), and similar results were obtained with purified DR2 molecules (Figure 1B): a set

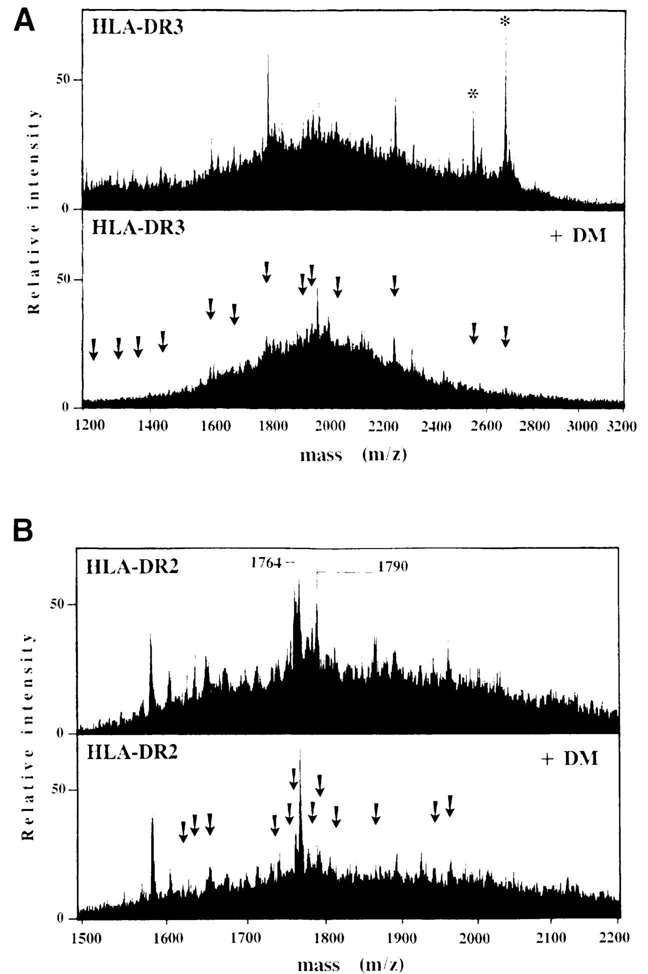

Fig. 1. DM-mediated release of naturally processed self-peptides bound to HLA-DR3 and -DR2. Mass spectrometric analysis of DM-sensitive self-peptides. HLA-DR3 (**A**) and HLA-DR2 (**B**) molecules (5 μM each) purified from the Epstein–Barr-virus-transformed B cell line COX and LD2B respectively, were incubated for 16 h at pH 5.0, 37°C ± HLA-DM (50 nM). Self-peptides that remained bound to DR3 and DR2 were released by acid treatment and analyzed by MALDI-MS. DM-sensitive peptide species are indicated by arrowheads. The peaks marked by asterisks in the upper DR3 spectrum correspond to CLIP variants CLIP(82–104) (m/z = 2562) and CLIP(81–104) (m/z = 2675) (Kropshofer *et al.*, 1995b). The signals with m/z = 1764 and m/z = 1790, marked by their masses in the DR2 spectrum, share the same masses with self-peptides CLIP(90–104) and HLA-A3(134–149) respectively (cf. Table I). The profiles are representative of two to three independent experiments.

of peptides appears to have been released due to their DM sensitivity. Consistent with that, we found by RP-HPLC a correspondingly increased amount of dissociated peptides in the supernatant of DR2 incubated with DM (unpublished data). Together, these findings suggest that DM is able to release a subset of DR-associated non-CLIP self-peptides.

Low-stability confers DM sensitivity

Two of the mass spectrometry signals of the peptides with marked DM susceptibility, characterized by mass-to-charge ratios of m/z = 1764 and 1790 (cf. Figure 1B), correspond to two previously described dominant DR2-associated self-peptides, namely the CLIP variant CLIP(90–104) (Chicz *et al.*, 1993) and the HLA-A3-derived peptide A3(134–149), abbreviated as A3 peptide

Table I. Effect of HLA-DM on the off-rate of DR2-associated self-peptides

Peptide		m/z[a]	Off-rate[b] (min)	
			–DM	+DM
HLA-DQw6(43–58)[c]	DVGVY RAVTPQGRPDA	1700	2400 ± 240	2400 ± 360
HLA-A3(134–149)[c]	TAADM AAQITKRKWEA	1790	35 ± 5[f]	5 ± 1
CLIP(90–104)[d]	KM RMATPLLMQALPM	1764[e]	190 ± 20[f]	30 ± 5
HLA-DQw6(43–58)/47-Met	DVGVM RAVTPQGRPDA		140 ± 8	45 ± 3
HLA-A3(134–149)/138-Tyr	TAADY AAQITKRKWEA		1320 ± 120	1380 ± 240

[a]Theoretical mass-to-charge ratio of the given peptides, equivalent to signals in the MALDI-MS spectrum of DR2 (Figure 1A).
[b]The off-rates are given as half-times of dissociation, $t_{1/2}$, of the respective DR2a–AMCA-peptide complexes ± HLA-DM and determined by HPSEC as described in Materials and methods. Values are means ± error of the mean from two individual experiments.
[c]DR2-associated self-peptides (Vogt *et al.*, 1994); the putative P1 anchor residue of each peptide is underlined and used for the alignment.
[d]DR2-associated CLIP truncation variant (Chicz *et al.*, 1993).
[e]m/z of CLIP(90–104) with two oxidized Met residues, as described previously (Sette *et al.*, 1992).
[f]From these short half-lives one might expect that both peptides dissociated quantitatively during the 16 h incubation of the mass spectrometry experiment (cf. Figure 1A). However, owing to the high concentration of DR–peptide complexes (5 μM), rebinding of peptide is likely to occur. This is less likely in the HPSEC assay where the DR–peptide complexes have a much lower concentration (50 nM).

(Vogt *et al.*, 1994; Table I). To confirm the DM sensitivity of both peptides, we measured their off-rates directly in a peptide binding assay based on high performance size-exclusion chromatography (HPSEC): CLIP(90–104) and the A3 peptide bound with low kinetic stability to purified DR2a, as reflected by their time of half-maximal dissociation: $t_{1/2}$ = 190 min and 35 min respectively, at pH 5.0 and 37°C (Table I). Consistent with the mass spectrometry data, DM accelerated the off-rates of both peptides 6- to 7-fold. As a control, we tested another self-peptide previously described for DR2, DQw6(43–58), abbreviated as DQw6 peptide henceforward (Vogt *et al.*, 1994), and found that it formed long-lived complexes with DR2a ($t_{1/2}$ = 2400 min) which were unaffected by DM (Table I).

We next attempted to elucidate whether certain peptides, such as CLIP or the A3 peptide, bear some common structural features that make them susceptible to DM-mediated removal. Peptides are known to bind to class II molecules by virtue of 'anchor' side chains at defined positions (e.g. P1) that reach into pockets of the peptide-binding groove. The alignment of CLIP(90–104) and the A3 peptide according to the anchor motif described for DR2a (Vogt *et al.*, 1994) shows that both peptides share the amino acid Met at the putative P1 anchor position (cf. Table I). Therefore, we replaced Met138 of the A3 peptide by Tyr that has been reported to be a strong P1 anchor residue for DR2a (O'Sullivan *et al.*, 1990). The mutant A3/138-Tyr bound with ~40-fold higher stability ($t_{1/2}$ = 1300 min) and, most important, turned out to be DM resistant (Table I). In accordance with this observation, substitution of the strong P1 anchor Tyr47 of the DQw6 peptide by Met rendered the mutant DQw6/47-Met less stable and sensitive to DM, as indicated by the accelerated off-rate upon addition of DM (Table I). We conclude that fast off-rates of peptides correlate with DM sensitivity and that this can be influenced by the nature of the P1 anchor amino acid of the peptide.

DM-sensitive peptides display reduced IC_{50} values

Given the fact that DM accelerated the off-rate of the A3 peptide, DM should also influence the inhibitory potential (IC_{50}) of this peptide in a competition assay against a reporter peptide that is DM insensitive, as DM would constantly remove a certain fraction of the bound A3 peptide so that it would be expected to become a weaker competitor. This was actually the case (Table II): the IC_{50} of the A3 peptide competing against labeled DQw6 peptide on DR2a increased ~6-fold upon addition of DM, whereas the IC_{50} of the DM-insensitive DQw6 peptide remained unchanged. Likewise, DM left the IC_{50} of the DM-resistant mutant A3/138-Tyr unaltered, whereas it increased the IC_{50} of the DM-sensitive mutant peptide DQw6/47-Met nearly 6-fold. These data show that determination of IC_{50} values in the presence and absence of DM is a valid approach to discriminate between DM-insensitive and DM-sensitive peptides.

To see whether, for another DR allele, naturally processed peptides would behave in a similar way to the A3 peptide, we compared the IC_{50} values of six DR1-associated self-peptides in presence and absence of DM using influenza hemagglutinin peptide HA(307–319), short:HA peptide, as a reporter. The HA peptide has been described to be unaffected by DM (Sloan *et al.*, 1995; cf. Table III). The IC_{50} values of four self-peptides, derived from HLA-A2, bovine fetuin, SP3 and from a serine protease, were not influenced by DM indicating that they resisted DM-dependent release (Table II). In contrast, DM led to a 7-fold increase of the IC_{50} of the transferrin receptor peptide and, as expected, to a 15-fold higher IC_{50} of CLIP(81–105). Thus, wild-type B cells under steady state express DR molecules carrying CLIP as well as other DM-sensitive peptides that are likely to be substrates for DM-mediated editing.

Influence of P1 anchor mutants of HA peptide on DM editing

In order to assess whether the P1 anchor effect seen with DR2a-associated self-peptides can be extended to other alleles and peptides, we chose the HA peptide (Figure 2A) because it is known from three-dimensional structural analysis of the DR1–HA peptide crystal that the side chain of Tyr309 occupies pocket P1 of the DR1 peptide binding groove (Stern *et al.*, 1994). With the aim to imitate more thoroughly the conditions under which loading and editing by DM take place *in vivo*, we developed a novel mass spectrometry assay based on small peptide libraries. In

Table II. Effect of HLA-DM on the IC_{50} of DR2a- and DR1-associated self-peptides

Allele	Peptide		IC_{50}[a] (μM)	
			–DM	+DM
DR2a	HLA-DQw6(43–58)	DVGVY RAVTPQGRPDA	0.6	0.7
	HLA-DQw6(43–58)/47-Met	DVGVM RAVTPQGRPDA	4.0	22.8
	HLA-A3(134–149)	TAADM AAQITKRKWEA	2.8	18.1
	HLA-A3(134–149)/138-Tyr	TAADY AAQITKRKWEA	0.7	0.5
DR1	CLIP(81–105)[b]	LPKPPKPVSKM RMATPLLMQALPMG	0.1	1.5
	HLA-A2(103–120)[b]	VGSDW RFLRGYHQYAYDG	0.8	0.8
	bovine fetuin(56–73)[b]	YKHTL NQIDSVKVWPRPP	0.4	0.5
	transferrin receptor(680–696)[b]	RVEY HFLSPYVSPKESP	1.2	8.4
	self-peptide SP3(1–16)[c]	AILEF RAMAQFSRKTD	0.3	0.2
	trypsin-like serine protease(239–252)[d]	GPGVY ILLSKKHLN	2.1	2.0

[a]Relative affinities are given as the concentration of the respective competitor peptide where 50% inhibition was obtained. AMCA-labeled DQw6 peptide (1 μM) on DR2 and HA peptide (1 μM) on DR1 were used as reporter peptides and their binding determined by HPSEC. Each peptide was tested in two to three independent experiments.
[b]Chicz *et al.* (1992).
[c]Kropshofer *et al.* (1992).
[d]Kropshofer (1993).

this assay mixtures of peptides that are distinguishable by their masses are co-incubated with purified class II molecules with and without DM. After removal of unbound peptides by ultrafiltration, DR-bound peptides are released by acid elution and monitored by mass spectrometry. Thus, comparison of the mass profiles of the peptides allows us to detect DM-mediated editing directly without any need for peptide labeling.

Tyr309 of the HA peptide was mutated non-conservatively by Asp or conservatively by the aliphatic amino acids Val and Met. Saturating amounts of the three mutant peptides and the wild-type HA peptide were co-incubated with DR1 in the absence and presence of DM. In order to deal with the *in vivo* substrate of DM, we used DR1 prepared from T2.DR1 transfectants which, owing to the DM defect in T2 cells, is exclusively occupied by CLIP variants (unpublished data). The upper panels of Figure 2B show the composition of the input mixture and the lower panels those peptides that could be recovered from DR after incubation with or without DM. In the absence of DM, DR1 clearly favored the wild-type HA peptide (Figure 2B, middle panel of P1), but it also bound the aliphatic mutants 309-Val and 309-Met, albeit to a lesser extent. However, no 309-Asp was recovered. These findings are consistent with published IC_{50} values, where substitution of an aromatic P1 anchor residue by an aliphatic residue markedly decreased and replacement by a charged residue abolished the inhibitory potential of the respective peptides (Hammer *et al.*, 1994). However, in the presence of DM the profile of bound peptides was changed (Figure 2B, bottom panel of P1): no binding of the 309-Met mutant was seen and binding of the 309-Val mutant was reduced so that the wild-type HA peptide became the dominant species. When SEB was used as a control instead of DM, the resulting spectrum was comparable with the one obtained without DM (see inset Figure 2B, P1 panel). A further control involving Ii-derived peptides shows that the height of peptide peaks correlates with the relative amount of input peptide allowing us to interprete these MS spectra in a semi-quantitative manner (Figure 2C). Together, these data demonstrate that introduction of suboptimal residues at

Table III. DM sensitivity of lysine scanning mutants of HA peptide on DR1

Peptide		IC_{50}[a] (μM)	
		–DM	+DM
HA(307–319)	PKYVKQNTLKLAT	1.1	1.2
309-Lys	--K----------	>100	>100
310-Lys	---K---------	2.0	2.2
312-Lys	-----K-------	1.5	2.0
313-Lys	------K------	1.1	1.1
314-Lys	-------K-----	>100	6.0
315-Lys	--------K----	5.0	5.2
317-Lys	----------K--	1.1	0.8
318-Lys	-----------K-	2.6	2.5

[a]cf. footnote a to Table II.

the P1 anchor position can be sufficient to lose DM resistance, suggesting that DM editing aims at eliminating low-stability binder provided that high-stability binders are available.

Influence of other anchors on editing by DM

Next, we introduced mutations at the anchor positions P4, P6 and P9 of the HA peptide (Figure 2A). According to the crystallographic analysis of the DR1–HA peptide complex, the pockets accommodating the above anchor side chains in the DR1 binding groove are smaller and more permissive than the P1 pocket and contribute less to the tight binding of the HA peptide (Stern *et al.*, 1994). Editing by DM was also found with mutants of these minor anchors (Figure 2B). For example, the P9 HA mutant 317-Tyr, that bound comparably with the wild-type peptide in the absence of DM, was found to be disfavored by DM, as shown by the reduced height of the mass spectrometry signal. Interestingly, at the P6 position the opposite effect of DM was observed: replacement of 314-Thr by Lys led to a strong reduction in binding, consistent with the DR1 binding motif that predicts only small side chains to be tolerated at the P6 anchor position (Hammer *et al.*, 1993). However, in the presence of DM, binding of the HA/314-Lys mutant was improved (positive

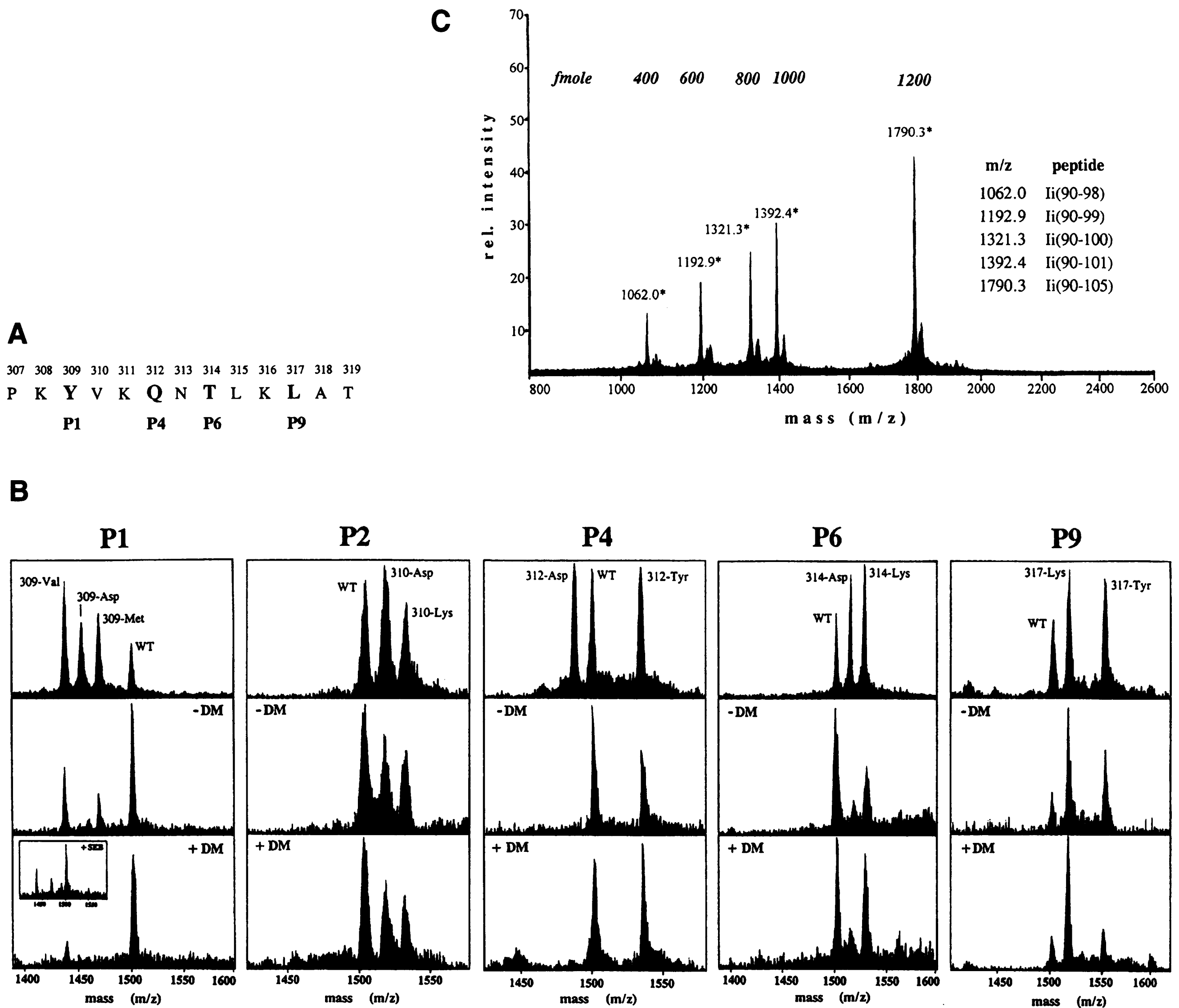

Fig. 2. Anchor mutations of HA(307–319) peptide conferring DM sensitivity or DM insensitivity. (**A**) Anchor residues of the HA peptide. The anchor positions P1, P4, P6 and P9, essential for high-stability binding to DR1, as described by the three-dimensional structural analysis of the DR1–HA peptide crystal (Stern *et al.*, 1994), are marked by bold letters. (**B**) Mass spectrometric analysis of HA peptide anchor mutants bound to DR1 in the absence and presence of DM. Purified DR1 (1 μM) from T2.DR1 transfectants was incubated with the indicated mixtures of wild-type HA peptide (WT) and single-residue anchor mutants (25 μM each) for 20 h at pH 5.0, 37°C ± DM (50 nM). In the upper panel of each row the input peptide mixture is given. DM-mediated editing follows from ratio changes of the signal intensities comparing the spectra generated without DM (middle panel) and with DM (bottom panel). As a control, P1 anchor mutants and DR1 were co-incubated with the superantigen staphylococcal enterotoxin B (SEB) (2 μM; see inset of the P1 bottom panel). Bound peptides were analyzed by MALDI-MS. One of two or three experiments with similar results is shown. (**C**) Height of MS signals correlates with the amount of input peptide. 400–1200 fmole of the indicated Ii-derived peptides were analyzed by MALDI-MS.

editing). This observation was confirmed by determining the IC_{50} values of HA lysine scanning mutants versus wild-type HA peptide in the HPSEC competition assay (Table III): DM reduced the IC_{50} of 314-Lys by at least one order of magnitude, but did not show any influence on the IC_{50} of other lysine mutants. One explanation for positive editing observed with the 314-Lys mutant may be that binding of DM to DR leads to a transient enlargement of the P6 pocket so that binding of 314-Lys is kinetically favored compared with WT-HA peptide. Editing by DM can also involve anchor position P4: first, positive editing by DM occurred at position P4 when 312-Gln was substituted by Tyr, although the effect was not as strong as the one seen at P6 (Figure 2B); second, the DM-sensitive DR1-restricted epitope IM(18–29) from influenza matrix protein became DM resistant either by replacement of the P1 anchor Leu-20 by Tyr or by substitution of the P4 anchor Glu-23 by Leu (Table IV).

Table IV. DM sensitivity of influenza matrix protein IM(18-29) on DR1

Peptide		IC_{50}[a] (μM)	
		–DM	+DM
IM(18–29)	GPLKAE IAQRLE	1.4	11.5
IM(18–29)/20-Tyr	GP<u>Y</u>KAE IAQRLE	0.8	0.8
IM(18–29)/23-Leu	GPLKA<u>L</u> IAQRLE	1.0	1.0

[a]cf. footnote a to Table II.

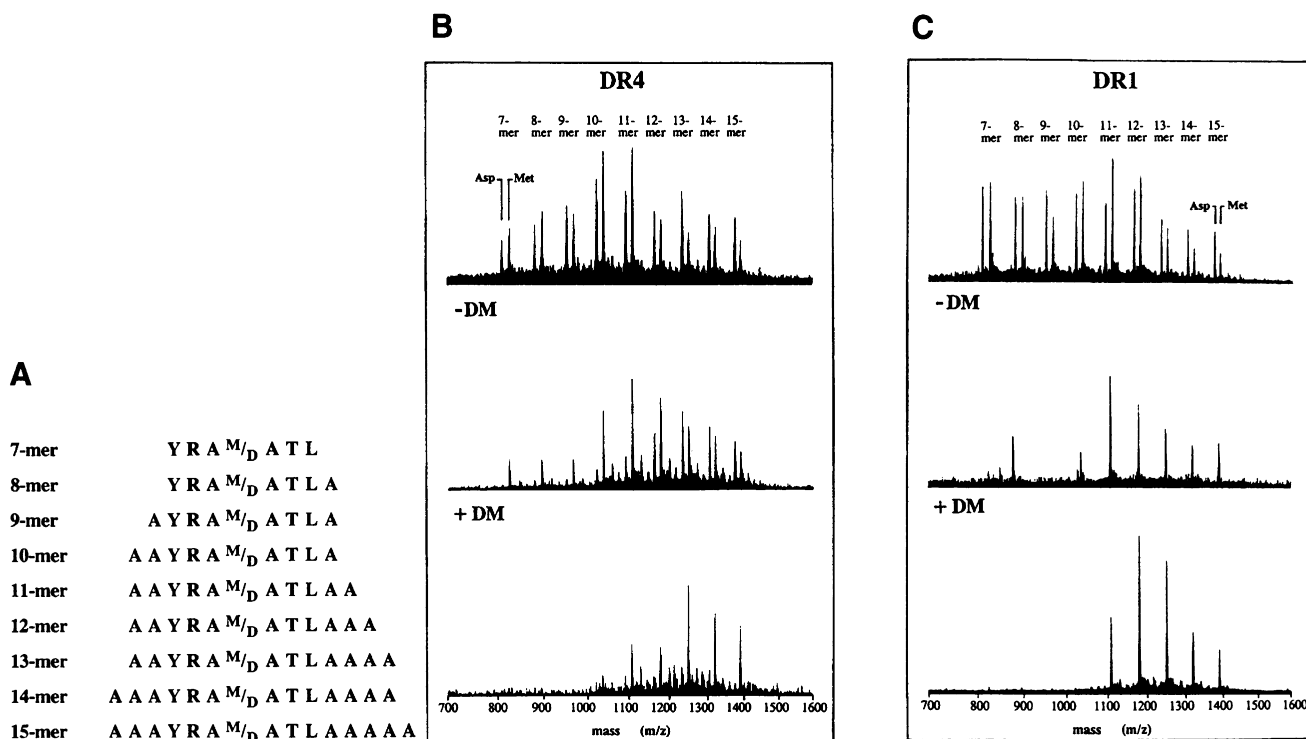

Fig. 3. Peptide length selection by DM. (**A**) Sequences of the designer peptide library. The 7-mer core sequence $YRA^{M}/_{D}ATL$ containing Asp or Met at position P4 was optimized for binding to DR4Dw4 (Hammer *et al.*, 1994). (**B** and **C**) Mass spectrometric analysis of the peptide library bound to DR4 and DR1 ± DM. DR4 (3 μM) and DR1 (1 μM) purified from T2.DR4 and T2.DR1 transfectants respectively, were incubated with the peptide library under the conditions described above (see legend to Figure 2B). The peptide library contained each peptide in saturating amounts (20–50 μM). The stoichiometry of the peptides was different for both alleles, as indicated in the uppermost panel.

As a control, we investigated mutants at the non-anchor position P2 of the HA peptide. The side chain at P2 is oriented away from the DR1 binding site, as shown by the crystal structure (Stern *et al.*, 1994). Both mutants, 310-Asp and 310-Lys, were revealed to be DM resistant (Figure 2B). Together these observations imply that editing by DM is not restricted to a certain structural element of a peptide, such as the P1 anchor, since anchor residues along the whole length of a peptide can influence DM-mediated loading.

DM selects for a minimal peptide length

Our findings suggest that DM somehow senses the kinetic stability of class II–peptide complexes. As it is known from X-ray crystallography that hydrogen bonds between residues of the binding groove and the peptide main chain contribute substantially to the stability of the complex, the length of a peptide might also determine whether or not a peptide is DM sensitive. Therefore, we designed a peptide library containing 7- to 15-mer length variants of the core sequences $YRA^{M}/_{D}ATL$ carrying Ala-based N- and C-terminal tails (Figure 3A). Both core sequences have been designed for high-affinity binding to DR4Dw4 (Hammer *et al.*, 1994). The peptide library was offered to different DR alleles and DM-mediated loading analyzed by mass spectrometry. Figure 3B shows that in the absence of DM, DR4 prefers the P4-Met variant of the library, especially the 10- to 12-mers. Binding of the P4-Asp variants was also observed provided that they were at least 10 amino acids long. In contrast, in the presence of DM, binding of P4-Asp variants and of 7- to 10-mers was prevented, so that 13- to 15-mers became the dominant species on DR4. Comparable editing effects were also found on DR1 (Figure 3C), although the input peptide mixture used here contained higher amounts of the smaller peptide variants (upper panel; Figure 3C): again, DM prevented binding of peptides smaller than 11 residues thereby skewing the repertoire towards the longer peptides. The only difference between the DR1 and DR4 spectra is that DR1 does not tolerate binding of the P4-Asp variants, even in the absence of DM, agreeing with the HA mutant experiments (Figure 2B) and with earlier reports (Hammer *et al.*, 1992). DM editing comparable with that described for DR1 was also found on DR2a (unpublished data). In summary, peptides with a core sequence suitable for binding to several DR alleles revealed to be subject of DM-mediated release as long as they were shorter than 10–11 residues.

Binding of DM to DR1 correlates with DM susceptibility of the offered peptide

One model explaining how DM editing may be accomplished involves a direct interaction between DM and class II molecules (Sanderson *et al.*, 1996; Vogt *et al.*, 1996). Therefore we incubated DR1–CLIP complexes from T2 cells with DM and saturating amounts of P1 anchor mutants of HA peptide that displayed differential susceptibility to DM editing (cf. Figure 2B) and investigated whether DM bound differently to the resulting DR1 complexes. The putative DM–DR complexes were immunoprecipitated with the anti-DM mAb DM.K8 and analyzed by Western blotting using the anti-DRα mAb 1B5. Figure 4A shows that DR1 was efficiently co-precipitated with DM in the absence of added peptide as well as in the presence of

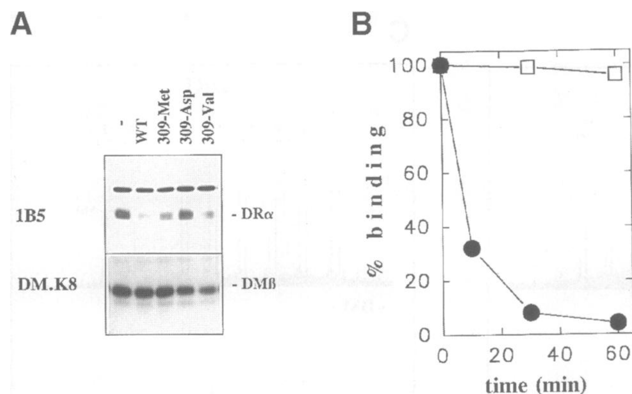

Fig. 4. DM favors binding to DR in the presence of low-stability peptides. (**A**) DR1–CLIP complexes (50 nM) from T2.DR1 transfectants were co-incubated with the indicated HA peptides (50 μM) and DM (50 nM) at pH 5.0, 37°C for 48 h. Immunoprecipitations were carried out with anti-DMβ mAb DM.K8. Immunoprecipitated material was analyzed by SDS–PAGE and Western blotting. Blots were probed with the anti-DRα mAb 1B5 (upper panel) and anti-DM mAb DM.K8 (lower panel). The contaminating band with apparent MW 50 kDa results from reactivity of the conjugated anti-mouse Ig with DM.K8 heavy chain eluted from the beads. (**B**) DR1–CLIP complexes lose CLIP very rapidly at pH 5.0, 37°C in the presence of DM. The dissociation kinetics was measured by HPSEC, as described in Materials and methods, using DR1–AMCA-CLIP(81–105) (●, 50 nM) or DR1–AMCA-HA complexes (□, 50 nM) and DM (10 nM).

the non-binding HA mutant 309-Asp. The presence of 309-Met or 309-Val, which were shown to be susceptible to removal by DM (cf. Figure 2B), led to moderate co-precipitation of DR1 molecules. In contrast, only very small amounts of DR1 were recovered in the presence of the DM-resistant HA wild-type peptide. It should be noted that maximal intensity of the DRα bands was attained after 40 h of co-incubation of DR and DM. The respective intensities were significantly weaker after 6 h of incubation, but we observed the same ratio of intensities as described above (unpublished data). Since CLIP dissociated from DR1 very rapidly in the presence of DM ($t_{1/2}$ ~5 min, Figure 4B), and since we did not succeed in detecting any peptide eluted from the precipitated DM–DR complexes (unpublished data), it is most likely that the co-precipitates reflect 'empty' DR αβ dimers associated with DM. Together, these findings show that the efficiency of binding of DM to DR molecules correlates with the DM susceptibility of the offered peptide.

Discussion

In this study we provide evidence that human DR class II molecules that have been loaded with peptide can undergo an editing process, with HLA-DM acting as the editor: only those class II–peptide complexes that exceed a certain threshold of kinetic stability are tolerated, otherwise the peptide will be released and exchanged for another one. Thus, in living wild-type APCs under steady state a sizeable fraction of class II–peptide complex is DM sensitive, namely those that have not yet encountered DM or have not yet been loaded with a DM-resistant peptide. We estimate that this fraction covers 10–25% of the DR molecules in the lymphoblastoid cell lines COX and LD2B, which were investigated here in more detail (Figure 1). Typical representatives of such DM-sensitive self-peptides are CLIP as well as other peptides, e.g. a HLA-A3-derived peptide on DR2 (Tables I and II) and a transferrin receptor peptide on DR1 (Table II). The majority of the DR–peptide complexes, however, appeared to be DM resistant. Long-lived DR–peptide complexes are commonly referred to as 'mature' αβ dimers (Sadegh-Nasseri *et al.*, 1994; Kropshofer *et al.*, 1995a), and investigators have used the stability in SDS as a measure of 'maturity' or longevity of αβ–peptide complexes. Consistent with this, only a small effect of DM on the SDS stability of mature αβ dimers from another B lymphoblastoid cell line was observed (Denzin and Cresswell, 1995). In this context, however, one should bear in mind that SDS stability of αβ dimers is not well understood: it does not correlate with the affinity of a peptide (Verreck *et al.*, 1996), and it does not necessarily reflect DM resistance. Examples demonstrating discordances include the DM-sensitive A3 peptide that is able to form SDS-stable complexes with DR2 molecules as efficiently as the DM-insensitive DQw6 peptide (unpublished data), and CLIP(81–105) which is readily released from DR1 by DM (Sloan *et al.*, 1995; Table II), although DR1–CLIP is stable in SDS (Chicz *et al.*, 1992).

So far two peptides have been reported to be DM sensitive, namely CLIP and MBP peptide (Sloan *et al.*, 1995), but until now it was unclear which properties of peptides render class II–peptide complexes DM susceptible. Several observations in this study demonstrate that it is not the affinity under steady state but the kinetic stability that confers DM sensitivity: (i) the DQw6 and the A3/138-Tyr peptides bind stably to DR2a, as indicated by their slow off-rates, and both remain stably bound in the presence of DM, whereas peptides with a high intrinsic off-rate, such as the A3 and the DQw6/47-Met peptide, were DM sensitive (Table I); (ii) CLIP(81–105) binds to DR1 with a 10-fold higher affinity than HA peptide as determined by IC_{50} measurements (Tables II and III), but CLIP was readily released by DM, as reflected by a 15-fold increase in the IC_{50}, whereas HA peptide was not, (iii) similarly, the influenza matrix protein peptide IM(18–29) and the mutant IM(18–29)/23-Leu exhibited almost the same IC_{50}, but the wild-type peptide was released by DM whereas the mutant was not (Table IV). The fact that DM-mediated removal does not correlate with the peptide's affinity explains why DM is able to release CLIP from murine alleles, such as A^d or A^b, that form high-affinity complexes with CLIP, as estimated by IC_{50} measurements (Sette *et al.*, 1995). The destabilizing effect of the CLIP N-terminus, previously described for DR molecules (Kropshofer *et al.*, 1995a,b), may help to explain the high DM susceptibility of CLIP.

Our study also elucidates the structural requirements of the peptide that are essential for DM resistance. From the X-ray structural analysis of the DR1–HA peptide crystal (Stern *et al.*, 1994), and from *in vitro* binding studies, it was evident that the stability of a class II–peptide complex is determined by two types of interaction: first, by the number of hydrogen bonds involving the peptide main chain and residues of the whole peptide binding cleft, and second, by attracting and repelling forces between so-called 'anchor' side chains of the peptide and specificity pockets in the MHC binding groove. Recurring residues

at the anchor positions are part of the peptide sequence specificity or 'motif'. Such motifs have been deduced for many human DR alleles by alignment of self-peptides and/or systematic *in vitro* binding studies (Hammer *et al.*, 1992, 1993; Kropshofer *et al.*, 1992, 1993; Vogt *et al.*, 1994; Rammensee *et al.*, 1995). However, in several instances the currently available class II motifs do not allow the prediction of DM-resistant epitopes, as shown by the following examples: the DR2-associated A3 self-peptide (Table I) and the DR1-restricted epitope IM(18–29) (Table IV) fitted the respective DR2a and DR1 binding motifs very well, but both were DM sensitive. Conversely, the stable HA peptide became DM-releasable upon introducing the P1 anchor residues Met or Val at P1 (Figure 2B). Thus, although the crystal structure of the DR3–CLIP complex has revealed that Met91 of CLIP fits into the P1 pocket of DR3 equally well as does Tyr309 into the P1 pocket of the DR1–HA peptide crystal (Ghosh *et al.*, 1995), for these DR alleles, aliphatic P1 anchors such as Met, Leu or Val may not confer sufficient stability. Aliphatic P1 anchors are only tolerated by DM provided other pockets of the groove are occupied by strong anchors, as shown with the DM-insensitive bovine fetuin peptide (Table II) and IM(18–29)/23-Leu (Table IV). In both cases pocket P4 is occupied by Leu, one of the optimal P4 anchors for DR1 (Hammer *et al.*, 1993). DM editing could also be observed with HA peptide mutants involving the P9 anchor position (Figure 2B), whereas this was not the case at positions like P2 (Figure 2B; Table III) or P7 (Table III) where the side chains are directed away from the groove (Stern *et al.*, 1994). From these data we conclude that the peptide structure as a whole determines whether the peptide will be susceptible to DM editing, thereby rendering it unlikely that DM-sensitive peptides share a common DM recognition signal that confers DM susceptibility.

Simulation of the *in vivo* scenario of loading by the mass spectrometry assay has revealed further constraints of DM-mediated editing that are not predictable by the available binding motifs: according to the DR1 motif established in the absence of DM (O'Sullivan *et al.*, 1991; Hammer *et al.*, 1992) only residues with small side chains, e.g. Ala, Gly, Ser, Thr, are accommodated in the P6 pocket. However, using the 314-Lys mutant of the HA peptide we could show that in the presence of DM the bulky side chain of Lys is tolerated, whereas binding was abolished in the absence of DM (Figure 2B, panel P6; Table III). Likewise, two self-peptides, SP3 and the serine protease peptide, were DM insensitive, although both are carrying bulky residues at the critical P6 position (Table II). This observation may be explained by a transient 'opening' of the P6 pocket during the DM–DR interaction so that larger side chains can be accommodated. It should be mentioned that this effect is dependent on the DR polymorphism, as it was not seen in the context of DR4 (unpublished data).

Furthermore, DM appears to select peptides that have a minimal length of ~11 residues (Figure 3). This is in accordance with a previous report wherein 11- and 12-mers were described to be the smallest DR-associated self-peptides purified from DM-positive cells (Chicz *et al.*, 1993), making it highly likely that this length restriction is a consequence of DM editing. This result was not expected in view of previous *in vitro* binding studies where even 7-mer peptides displayed high-affinity binding (Hammer *et al.*, 1994), consistent with our mass spectrometry data obtained in the absence of DM (Figure 3B). Together, the results presented here suggest that the published peptide binding motifs for MHC class II molecules, particularly those that have been deduced from *in vitro* binding studies and IC_{50} measurements, may have to be refined using DM in the respective assays. This may also apply to alleles such as A^d or A^k that have been reported to be loaded DM-independently (Brooks *et al.*, 1995; Stebbins *et al.*, 1994), as it is not excluded that DM-mediated editing takes place also in the context of these alleles. These issues await further investigation.

Finally, our data suggest that the association of DM with DR correlates with the kinetic instability of the respective DR molecules. Under steady state DM binds preferentially to those DR molecules that have been offered DM-sensitive peptides or that have lost their peptide (Figure 4). The exact nature of the precipitable DM–DR complex is not known. Most likely, it involves 'empty' DR αβ dimers and is different from the initially formed DM–DR–peptide complex. DM may bind also to DM-resistant DR–peptide complexes, but our experiments suggest that such a DM–DR association is less stable. This conclusion is reinforced by kinetic studies on DM-mediated peptide loading where low-stability DR–peptide complexes were much better competitors than high-stability complexes (Vogt *et al.*, 1996). Likewise, in plasmon resonance studies we observed better binding of DM to low-stability DR–CLIP complexes than to high-stability DR–self-peptide complexes (Vogt *et al.*, 1996). Along these lines of evidence, the explanation for the co-precipitation of DM with DR3 via the 16.23 mAb (Sanderson *et al.*, 1996) may be that the antibody 16.23 recognized the DR3 fraction carrying no peptides or DM-sensitive peptides.

In summary, the following picture emerges describing DM-mediated peptide editing *in vivo*: DM encounters DR–CLIP as well as DR–peptide complexes in acidic lysosomal or prelysosomal compartments. In this low pH environment, binding of DM to DR molecules is favored. However, the half-life of the DR–DM interaction seems to depend on the intrinsic stability of the peptide occupying the binding groove: when low-stability peptides such as CLIP are bound to the DR molecule, these peptides will dissociate more rapidly than DM, whereas high-stability peptides cause rapid dissociation of DM from DR. Mechanistically, the binding of DM to DR is likely to prolong the half-life of a transiently 'open' state of the DR molecule. This may involve subtle conformational changes in one or more of the specificity pockets of the groove leading to accelerated dissociation of short peptides or those with suboptimal anchor combinations. At this stage any peptide possessing sufficient affinity, including CLIP and other low-stability peptides, may bind into the groove. However, only peptides with optimal anchor combinations and/or sufficient length are strong enough to resist the next opening of the groove by DM and therefore will remain bound. In this case, DM will dissociate rapidly, move on to other αβ dimers and start a further round of kinetic proofreading. The outcome of the DM-mediated editing process may be influenced by a variety

of factors, such as (i) the relative amounts of DM and class II molecules in the respective intracellular compartment; (ii) the abundance of processed epitopes in such compartments; (iii) the capacity of such epitopes to bind with high stability to class II molecules; (iv) the pH of the compartment; and (v) the existence of so far unknown modulators of the DM activity. Together, the kinetic proofreading function of DM skews the peptide repertoire that is presented to T cells towards a population of stably binding peptides. In addition, DM optimizes the efficiency of antigen presentation by minimizing the number of class II molecules that are otherwise lost in the absence of DM via aggregation and degradation due to dissociation of unstably bound peptides *en route* to or at the cell surface (Germain and Rinker, 1993).

Materials and methods

Cells

The EBV-transformed homozygous B cell lines COX (DR17Dw3), LD2B (DR15Dw2) and the T×B hybrid cell line T2 transfected with the cDNA for DR1 (DRA1*0101/DRB1*0101), DR2a (DRA1*0101/DRB5*0101) or DR4 (DRA1*0101/DRB1*0401) were used as a source for the isolation of the respective DR molecules. Cell lines were maintained in RPMI 1640 with HEPES (Life Technologies, Grand Island, NY) containing 10% heat-inactivated fetal calf serum (Konco Lab Division, Wiesbaden, Germany) and cultured in roller bottles at 37°C.

Antibodies

The hybridoma cell lines L243 (anti-DRαβ; Lampson and Levy, 1980) and 1B5 (anti-DRα; Adams *et al.*, 1983) have been described. The mAb DM.K8 (anti-DM; mouse IgG1 isotype) was generated by immunizing BALB/c mice with a peptide derived from the cytoplasmic tail of the HLA-DMβ chain (sequence: TPLPGSNYSEGWHIS) coupled to KLH. For affinity-chromatography and immunoprecipitation with beads, L243 and DM.K8 were coupled to CNBr-activated Sepharose CL-4B (Pharmacia, Uppsala, Sweden) at a concentration of 3 mg of antibody per ml of gel.

Peptides

Peptides were synthesized on an AMS422 and an Advanced ChemTech 396 Multiple Peptide Synthesizer (Abimed, Langenfeld, Germany) using F-moc chemistry and were purified by RP-HPLC. Purified peptides were analyzed by laser desorption mass spectrometry and shown to be single species. N-terminal labeling with the fluorophor 7-amino-4-methylcoumarin-3-acetic acid (AMCA; Lambda, Graz, Austria) was performed as described (Kropshofer *et al.*, 1991) and used to label the following peptides: HLA-DQw6(43–58), DVGVYRAVTPQGRPDA; DQw6(43–58)/47-Met, DVGVMRAVTPQGRPDA; HLA-A3(134–149) TAADMAAQITKRKWEA; HLA-A3(134–149)/138-Tyr, TAADYAA-QITKRKWEA; CLIP(90–104), KMRMATPLLMQALPM; HA(307–319), PKYVKQNTLKLAT.

Purification of HLA-DR molecules

HLA-DR molecules were isolated from B cell lines or T2 transfectants by affinity-chromatography using anti-DR mAb L243, as described previously (Kropshofer *et al.*, 1995a).

Purification of HLA-DM

200 g of human spleen (containing ~10^{11} cells) from a patient with B-chronic lymphocytic leukemia were lysed in PBS containing 10 mM $MgCl_2$ and 1% NP-40 (Fluka) for 2 h on ice. Nuclear material was removed by centrifugation at 500 *g* for 10 min at 4°C. Further purification of the cell extract was obtained by centrifugation at 12 000 *g* for 10 min at 4°C and ultracentrifugation at 140 000 *g* for 1 h at 4°C. The lysate was applied to a Sepharose CL-4B precolumn coupled with glycine and subsequently to a Sepharose CL-4B column to which the monoclonal anti-DM antibody DM.K8 was coupled. The DM.K8 column was extensively washed with 100 mM sodium phosphate pH 8.0, containing 1.0% NP-40, followed by 100 mM sodium phosphate pH 8.0, containing 0.1% Zwittergent-12 (Calbiochem, La Jolla, CA). HLA-DM was eluted in 100 mM sodium phosphate, 50 mM sodium acetate pH 5.0, containing 0.1% Zwittergent-12. The eluted material was neutralized with 1 M NaOH and concentrated by ultrafiltration with a 20 kDa cut-off membrane (Sartorius, Göttingen, Germany). The yield was ~250 μg HLA-DM. Purity of the isolated material was >90%, as assessed by SDS–PAGE.

HPSEC binding assay

Detergent-solubilized DR molecules and the respective AMCA-labeled peptide were co-incubated in the absence or presence of solubilized DM for various periods of time at 37°C in binding buffer (50 mM sodium phosphate, 50 mM sodium citrate pH 5.0, 0.1% Zwittergent-12). Binding was quantitated by high performance size-exclusion chromatography (HPSEC) on a Superdex 75 HR 5/20 column (Pharmacia, Upsala, Sweden) equilibrated with 150 mM sodium phosphate pH 6.0, 0.1% Zwittergent-12, 15% (v/v) acetonitrile. The flow rate was 0.5 ml/min. DR–peptide complexes eluted after 2.5 min and were monitored by a fluorescence detector and a UV detector set up in series (Kropshofer *et al.*, 1995a).

Determination of IC_{50} values as a measure for affinity

Detergent-solubilized DR1 or DR2a molecules (100 nM) were co-incubated with AMCA-HA(307–319) (1 μM) or AMCA-DQw6(43–58) (1 μM) respectively, and variable amounts of competitor peptide (50 nM–100 μM) in binding buffer in the presence or absence of detergent-solubilized DM (20 nM). After 20 h of incubation at 37°C, HPSEC was performed as described above. Inhibition was calculated from fluorescence signals in the absence (F_o) and the presence (F_c) of competitor peptide: % inhibition = $(F_o–F_c)/F_o \times 100$. The concentration of competitor peptide which resulted in 50% inhibition was expressed by the IC_{50} value.

Determination of off-rates as a measure for stability

Detergent-solubilized DR molecules (500 nM) were co-incubated with the respective AMCA-peptide (10 μM) in binding buffer for 48 h at 37°C. Separation of DR–peptide complexes from unbound peptide was achieved by HPSEC. Dissociation of the resulting DR–peptide complex (50 nM) was analyzed in the absence and presence of added DM (10 nM) by HPSEC after various times of incubation at pH 5.0 and 37°C.

Mass spectrometry binding assay

Purified DR molecules (5–10 μg) were co-incubated in binding buffer with the respective mixture of synthetic peptides in the absence and presence of purified DM for 16–20 h at 37°C. To remove unbound peptides, DR–peptide complexes were extensively washed with aqua bidest in a Microcon ultrafiltration tube with a 5 kDa cutoff (Millipore). Bound peptides were eluted by incubation in 0.1% trifluoracetic acid for 0.5 h at 37°C. After separation of protein by ultrafiltration, peptides were lyophilized and prepared for MALDI-mass spectrometry by adding 1,4-dihydroxybenzoic acid in a 1:1 ratio. Spectra were recorded on a Finnigan Lasermat Vision 2000 and collected by averaging the ion signals from 20–50 individual laser shots.

Immunoprecipitation and SDS–PAGE

For immunoprecipitation of DR–DM complexes, purified DR1 (50 nM) and purified DM (50 nM) were co-incubated in the absence or presence of peptide (50 μM) in 50 mM sodium phosphate, 50 mM sodium citrate, 0.1% Zwittergent-12 pH 5.0 at 37°C. After 48 h, DM.K8 coupled to Sepharose beads was added and incubated for 4 h at room temperature. Then the beads were sedimented by centrifugation, the supernatant removed and the beads extensively washed with 100 mM sodium phosphate, 50 mM NaCl, 0.1% Zwittergent-12 pH 7.0. Immunoprecipitates were eluted in Laemmli sample buffer, analyzed by SDS–PAGE and transferred onto immobilon PVDF membranes (Millipore, Bedford, MA). Membranes were blocked in blocking reagent (Boehringer, Mannheim, Germany). Binding of the anti DRα-antibody 1B5 was detected by incubation with horseradish peroxidase-conjugated goat anti-mouse Ig (Dianova, Hamburg, Germany) followed by enhanced chemiluminescence (Pierce, Rockford, IL).

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