Identification of the Lateral Interaction Surfaces of Human Histocompatibility Leukocyte Antigen (HLA)–DM with HLA–DR1 by Formation of Tethered Complexes That Present Enhanced HLA–DM Catalysis

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Abstract

Human histocompatibility leukocyte antigen (HLA)–DM is a major histocompatibility complex (MHC)–like protein that catalyzes exchange of antigenic peptides from MHC class II molecules. To investigate the molecular details of this catalysis we created four covalent complexes between HLA–DM and the MHC class II allele DR1. We introduced a disulfide bond between the naturally occurring cysteine $\beta 46$ on HLA–DM and an engineered cysteine on the end of a linker attached to either the NH_2- or the COOH terminus of an antigenic peptide that is tightly bound on DR1. We find that when DM is attached to the NH_2 terminus of the peptide, it can, for all linker lengths tested, catalyze exchange of the peptide with a half-life a few minutes (compared with uncatalyzed $t_{1/2} > 100$ h). This rate, which is several orders of magnitude greater than the one we obtain in solution assays using micromolar concentrations of HLA–DM, is dominated by a concentration independent factor, indicating an intramolecular catalytic interaction within the complex. A similar complex formed at the COOH terminus of the peptide shows no sign of DM-specific intramolecular catalysis. Restrictions on the possible interaction sites imposed by the length of the linkers indicate that the face of DR1 that accommodates the NH_2 terminus of the antigenic peptide interacts with the lateral face of HLA–DM that contains cysteine $\beta 46$.

Key words: MHC • antigen presentation/processing • antigens/peptides • catalysis • kinetics

Introduction

Newly synthesized major histocompatibility complex class II molecules contain in their peptide binding groove a portion of the invariant chain I_i (1). The I_i segment must then be exchanged with antigenic peptides destined for presentation on the cell surface (2). This exchange occurs in specialized endosomal compartments and is facilitated by a nonclassical MHC class II molecule named HLA–DM (3–5) (henceforth denoted as DM).* Peptide exchange from MHC class II molecules is very slow and inefficient in the absence of DM, both in vivo and in vitro (6–9). Acceleration of this exchange by DM follows Michaelis-Menten kinetics, and is proportional to the intrinsic rate of peptide dissociation (10). The structure of DM, determined by x-ray crystallography (11, 12), shows a fold similar to that of classical MHC class II molecules, but with the striking absence of a peptide binding groove. The structure provides no clear answers to the mechanism of catalysis, however. Recent biochemical and kinetic evidence implicate a previously suggested alternative conformation of the MHC molecule that is an intermediate in the peptide loading pathway and can exchange peptide rapidly (13, 14). It has been proposed that DM may act either by stabilizing this intermediate MHC conformation (15) or by accelerating the interconversion of the stable MHC state to this conformation (16). Nevertheless, virtually nothing is known at a molecular level about the interaction of DM

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    *Abbreviations used in this paper: DM, HLA–DM; DR1, HLA–DR1.

with MHC molecules or any related conformational changes.

Both DM and HLA-DR1 (a class II MHC molecule, henceforth denoted as DR1) are heterodimers with each chain contributing about half of the total molecular weight of the protein. Each chain has a large extracellular region, a single transmembrane segment, and a small cytoplasmic domain. Full-length, B cell–derived, detergent-solubilized DM stimulates in vitro peptide exchange from full-length HLA-DR class II molecules (5, 8). Nanomolar concentrations of DM are sufficient to achieve physiologically relevant rates of exchange in these experiments. By contrast, micromolar concentrations of soluble recombinant DM (lacking the transmembrane segment and cytoplasmic tail) are needed to obtain a similar exchange rate on soluble DR (4). The difference in rates has been attributed to the effect of colocalization of full-length DM and DR on detergent micelles or lipid bilayers and not to specific interactions of the transmembrane or cytoplasmic domains of the two proteins (17). Indeed, theoretical calculations of the effect of anchoring interacting proteins on lipid bilayers estimate an enhancement of up to 10^6-fold in their interactions, due to high local concentrations, excluded volume by other proteins and entropic effects (18).

To investigate the DR1/DM interaction in the absence of the lipid bilayer or detergent micelles we created a series of tethered complexes and screened for the ability of DM to catalyze peptide exchange within each complex. Screening for productive interaction as a function of attachment position and tether length in DM/DR1 complexes provides an opportunity to investigate the general structural features of the interaction and to identify surfaces in each molecule that contact each other during catalysis. The tether (linker) was in all cases attached to cysteine β46 of DM, which is located on a conserved surface patch of the molecule, and which is therefore a candidate for interaction with DR1. Although this area of DM is not the only one conserved between different species, screening of different linker lengths can be used to investigate whether the site of interaction on DM is located proximally or distally to the attachment point of the tether. Two linkage sites were explored on DR1: the NH2 terminus and the COOH terminus of the bound peptide. These two sites are located on opposite faces of DR1 and therefore allow us to determine which is the lateral face of DR1 that interacts with DM.

We have constructed a series of covalently linked soluble DM/soluble DR complexes by introducing a disulfide bond between the naturally occurring cysteine β46 of DM and a cysteine introduced on the peptide bound to DR1. We show that in complexes in which DM is linked through the NH2 terminus of the peptide, catalytic activity is retained and peptide dissociation is very rapid, presumably because of the high concentration of DM in the vicinity of DR1 as induced by the covalent linkage. Cross-linking thus simulates, in the absence of detergents and full length molecules, the high local concentrations induced by anchoring the proteins on a lipid bilayer. Variation of the linker length has only minimal effects on catalysis, indicat-

ing saturation of the catalytic capacity of DM in these complexes. No catalytic effect was found when DM was attached to the DR/peptide complex through the COOH terminus of the peptide, indicating that DM interacts with the lateral face of DR1 that is adjacent to the NH2 terminus of the bound peptide, in agreement with previously published mutational data (7, 19). When DM is attached to the NH2 terminus of the peptide its catalytic ability is retained even for linkers as small as 3 amino acids, indicating that DR1 contacts the lateral face of DM to which the linker is attached (on cysteine β46).

Materials and Methods

Expression and Purification of Proteins. Recombinant HLA-DR1 was produced and purified as previously described (4, 20). Briefly, HLA-DR1 was secreted from a stably transfected Schneider 2 insect cell line. Expression was induced by 1 mM $CuSO_4$, and the cell supernatant was collected after 4–5 d. The supernatant was cleared by centrifugation and loaded onto a LB 3.1 antibody column, washed with PBS, and eluted with CHAPS at pH 11.5. The fractions were immediately neutralized with Tris buffer at pH 8, concentrated, and the buffer was exchanged with PBS. For peptide loading, 1 mM PMSF, 1 mM EDTA, 0.02% NaN_3, and a 20-fold excess of peptide were added to the protein, and the mixture was filter sterilized and incubated at 37°C for 72 h. The peptide-loaded DR1 was further purified on a Sepharose S200 size exclusion column (Amersham Pharmacia Biotech), concentrated to 2–10 mg/ml, and stored at 4°C for a few days or at −80°C for longer periods of time.

Recombinant HLA-DM was also expressed in a stably transfected S2 insect cell line as described previously (11) and purified by size exclusion chromatography (Sepharose S200; Amersham Pharmacia Biotech) and anion exchange chromatography (MonoQ; Amersham Pharmacia Biotech). The protein was typically stored in aliquots at −80°C, but it was found to be stable at 4°C for several weeks.

Peptide Synthesis and Activation. All peptides used in this study are modifications of the highly antigenic hemagglutinin peptide HA (sequence: PKYVKQNTLKLAT) modified at the NH2- or COOH terminus with linker extensions bearing a single cysteine residue near the end of the linker (see Table I). The linker extensions were not found to affect the ability of the peptides to bind DR1, or the stability of DR1-peptide complexes as judged by SDS-PAGE (data not shown). Peptides synthesized by solid-phase Fmoc chemistry were deprotected, purified by reverse-phase HPLC, and analyzed by electrospray mass spectrometry to confirm purity and integrity. Peptide concentrations were determined by absorption at 280 nm, using an extinction coefficient of 1,215 $M^{-1}cm^{-1}$. As the single cysteine residue on each peptide was prone to oxidation, the purified peptides were dissolved in Tris buffer, pH 8.0, to a final concentration of ∼1 mM, and reacted with a twofold excess of dithiothreitol (DTT) for 30 min at room temperature. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was added in a fourfold excess over DTT and allowed to react for 30 min at room temperature to yield the peptide–TNB adduct. The peptide–TNB adduct was purified on a Superdex Peptide HR 10/30 size exclusion column (Amersham Pharmacia Biotech) and kept at −80°C until needed.

Construction of DM–Peptide Adduct. Purified DM was found to contain 0.1 to 0.5 free cysteines per mol protein, depending on the preparation, possibly because the naturally occurring cysteine

at position 46β was partially oxidized and blocked by a small molecule. Treatment of DM with 1–2 mM DTT for 1 h at room temperature followed by repurification by size-exclusion chromatography to separate the excess DTT, yielded protein that contained 0.8–0.9 free cysteines per mol protein and had activity comparable to the untreated protein (data not shown). The DTT-treated DM was stable for weeks at 4°C with cysteine β46 remaining reduced. The DM–peptide conjugate was made by mixing the DTT-treated DM with a 10-fold excess peptide–TNB adduct and allowing them to react for 2 h at room temperature. The progress of the reaction could be followed by native gel electrophoresis, because the peptide has a net charge of +3 and thus imparts a lower electrophoretic mobility to DM when it is linked on it. The yield of this reaction varied from 50–80%. The product, DM–peptide adduct, was purified on size exclusion chromatography (Sephadex Peptide; Amersham Pharmacia Biotech) to separate any free peptide that would interfere with later steps. The DM–peptide adduct was found to be able to fully substitute for DM in the catalysis of CLIP peptide exchange of DR1 using the fluorescence polarization assay described below (data not shown) indicating that the derivatization of cysteine 46β with a peptide did not affect DM activity.

It is possible to construct the DM–peptide–DR complex by mixing the DM–peptide adduct either with empty DR1 produced in insect cells or with DR1 complexed with a weakly binding peptide. Because of limitations in the amount of DM–peptide adduct that could be made, we found it more efficient to preload DR1 with a readily available weakly binding peptide and then exchange it out with DM–peptide. Therefore, a two step strategy was adopted that exploited the low affinity for DR1 in the presence of streptavidin of an NH$_2$-terminally truncated and biotinylated HA peptide, bYHA (see Table I). This peptide can be loaded with high yield onto insect expressed DR1, but can be exchanged-out easily if it is complexed with streptavidin through its biotin moiety. This induced weak affinity was exploited for the construction of the DM–peptide–DR complex with high yields. bYHA–DR1 complex was mixed with a 2–4 molar excess of streptavidin and a 2–3-fold excess of DM–peptide adduct. After overnight incubation, the mixture was separated on a sepharose S200 size exclusion column; SDS-PAGE electrophoresis confirmed that the largest peak (at around 90 kD) was the DM–peptide–DR complex. In most cases the complex was further purified on a MonoQ column (Amersham Pharmacia Biotech) eluted with a 0.025 to 0.5M NaCl gradient at pH 7.5.

Measurement of Complex Dissociation. To measure the effect of the proximity of DM and DR1 in all complexes constructed, we devised a simple kinetic assay. Purified complex (1–20 μM) was mixed with a large excess of HA peptide (2 mM) and the mixture incubated at pH 5.0 at 37°C. At specific time intervals small aliquots of the reaction mixture were withdrawn, diluted 1:10, mixed with native gel electrophoresis sample buffer, and immediately frozen on dry ice. All aliquots were kept frozen until they were analyzed by native gel electrophoresis on a 10% polyacrylamide gel. The amount of complex that had dissociated at each time point was estimated by densitometry of the band corresponding to the complex on the gel, after staining with Coomassie Blue. The density of the band at each time point was corrected for the gel background and divided by the density of a control band of undissociated complex, to normalize to the fraction of complex remaining. The normalized value was plotted versus time. The data were fit using a single exponential decay model: $Y = Span*exp(-K*X) + Plateau$, which produced the time constant K for the decay of the complex in the presence of a

large excess of free peptide HA. The half-life of the dissociation is given by $t_{1/2} = \ln(2)/K$, where $\ln(2)$ is the natural logarithm of 2. Each measurement was repeated 3–5 times to allow calculation of standard errors.

Construction of the Streptavidin/HA/DR1 Complexes. Biotinylated versions of the HA peptide (see Table I) were constructed by either reacting the NH$_2$ terminus of the peptide with biotin-X succinimidyl ester (Molecular Probes) before deprotection (for construction of the bHA peptide) or with biotin-X-maleamide (Molecular Probes) after deprotection (for the b6GHA and HA6Gb peptides). In both cases the biotinylated peptides were purified by reverse-phase HPLC and their correctness confirmed by electro-spray mass spectrometry. 100-fold excess of each peptide was mixed with purified insect DR1 and incubated at 37°C for 3 d to load the peptide onto DR1. The DR1/peptide complex was further purified by size exclusion chromatography. All streptavidin/HA/DR1 complexes were constructed in situ by mixing a 2:1 molar ratio of streptavidin (Molecular Probes) with the appropriate DR1/peptide complex. The formation of the complexes was confirmed by native PAGE by the appearance of slower migrating bands compared with DR1/peptide, streptavidin, or streptavidin/peptide controls (not shown). The dissociation of these complexes upon the addition of excess HA peptide was studied in a manner identical to the dissociation experiments for DM/DR1 complexes described above, with only the slowest migrating band being taken to be representative of the streptavidin/DR1 complex.

Measurement of Peptide Dissociation from DR1. Peptide dissociation from DR1 was measured by ELISA using peptides carrying a biotin moiety covalently linked to their NH$_2$-termini by a 6 carbon linker. The biotin linker was attached before deprotection by reacting with biotin-X succinimidyl ester (Molecular Probes). The biotinylated peptide was loaded onto insect-produced DR1 as described above, and the complex was purified. Its dissociation was followed by incubating the purified DR1/peptide with a 100 to 1,000-fold excess unbiotinlyated peptide at pH 5.0 and at 37°C in the presence of different concentrations of DM. At specific time points, a small aliquot was extracted from the reaction mixture, diluted in ELISA dilution buffer (PBS with 0.3% BSA) and frozen. After the end of the time course all aliquots were thawed and loaded on an ELISA plate coated with the LB3.1 antibody, and allowed to bind for 1 h at 4°C. The plate was washed three times with PBST (15 min each wash) and streptavidin conjugated with horseradish peroxidase was added and allowed to bind for 1 h at 4°C. The plate was washed five times with PBST, and ABTS was added for detection. The developed color was measured using a ELISA plate reader with a filter at 495 nm. The data were plotted as signal versus time and fit to a single exponential decay model to extract the observed rate of dissociation. The observed rates were plotted versus DM concentration and fit to a linear model.

For the measurement of the catalytic activity of DM when tethered onto the COOH terminus of the HA peptide that was bound onto DR1, a fluorescence polarization assay way used, as the DR1 attached on DM would interfere with the ELISA assay described above. Either the CLIP or the HA peptide were synthesized with a cysteine residue at position P5 (peptides CLIP-P5C and HA-P5C in Table I) and labeled with Alexa-C5-maleamide (Molecular Probes) by mixing an equimolar amount of the peptide and the reagent in PBS and incubating for 30 min at room temperature. The labeled peptides were purified on a reverse-phase C18 column and the products of the reaction confirmed by electro-spray mass spectrometry. The peptides were

loaded onto purified insect cell DR1 by addition of a 100-fold excess of the peptide, incubating at 37°C for 3 d and purifying the DR1/labeled peptide by size-exclusion chromatography. 10 nM of DR1 loaded with the labeled peptide were incubated in the presence of 1 mM HA peptide at pH 5.0 with varying concentrations of DM, or with the DM/DR1 complex where DM is tethered to the COOH terminus of the peptide and the fluorescence polarization signal of the mixture was followed over time (excitation was at 490 nm and emission at 520 nm) using an Analyst AD (LJL Biosystems) plate reader. Upon incubation with unlabeled peptide the labeled peptide is competed out of the DR1 binding site leading to a decrease of its fluorescence polarization signal. Peptide exchange kinetics followed by this method were found to be essentially identical to results obtained with the ELISA method described above.

Results

Assembly of the DM–Peptide/DR1 Complex. A series of four covalent complexes between DM and peptide-loaded DR1 was assembled by forming a disulfide bond between a cysteine at the NH$_2$ or COOH terminus of the HA peptide and the naturally occurring cysteine β46 on DM. This DM–peptide adduct was then loaded onto DR1. DR1 molecules secreted from insect cells are free of tightly bound antigenic peptides and can easily be loaded with peptide by prolonged incubation (2–3 d) at 37°C (21). Although it is possible to load the DM–peptide adduct onto DR1 in this way (Fig. 1 A, last lane), the process requires an excess of the peptide for satisfactory yield. Because the amount of peptide is limited in our case by the amount of the covalently attached DM, we adopted a strategy in which DR1 is preloaded with a weakly bound peptide that can be exchanged out by the DM–peptide adduct. In order for such a competition to be effective, the affinity of DM–peptide must be higher than the affinity of the preloaded peptide. The CLIP peptide (PVSKMRMATPLLMQA) has been reported to bind DR1 with an affinity lower than the HA peptide (22) and free HA peptide will exchange DR1-bound CLIP peptide with high yield. The exchange of CLIP for DM-HA gave poor yields of DM–peptide/DR1 complex, however, even after a 3-d incubation (Fig. 1 A, lane 4). Thus, it appears that the affinity of DM-HA for DR1 is lower than the affinity of HA for DR1 and possibly even lower than the affinity of CLIP for DR1. Covalently attached DM to the HA peptide must therefore catalyze the peptide dissociation very efficiently, presumably because of the high effective concentration induced by the linkage, thus inducing an observed lower affinity for the attached peptide.

The DM–peptide adduct can be loaded onto DR1 with high yield by exploiting the observed reduction of affinity of the truncated and biotinylated bYHA peptide (Table I) in the presence of streptavidin. DR1 can be loaded with high yield with the bYHA peptide, forming a stable complex from which the peptide exchanges slowly (Fig. 1 B, compare lanes 2 and 4 and lanes 2 and 6 and 7). In the presence of streptavidin, however, the resulting streptavidin/biotin-YHA/DR1 complex dissociates rapidly in the
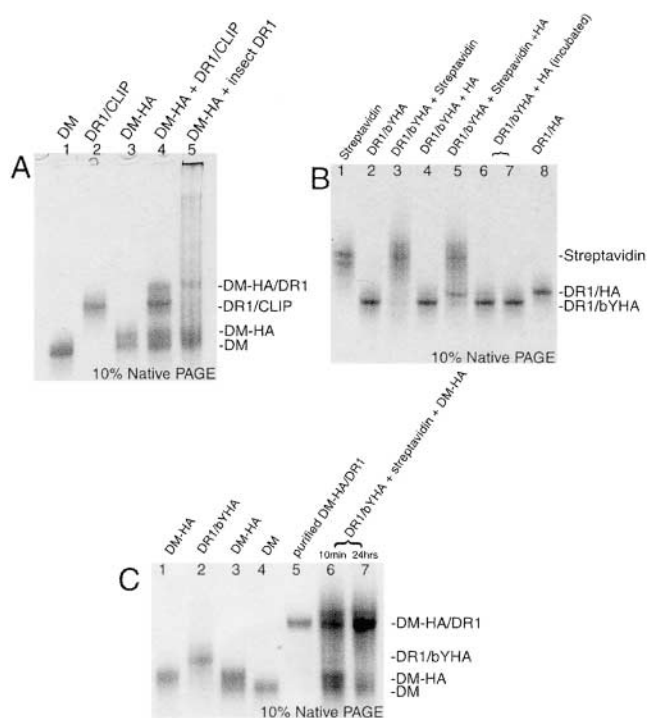

Figure 1. Analysis of different methods of assembly of DM–peptide/DR1 complexes by native PAGE. (A) DM–peptide/DR1 complex can be assembled either by loading of DM–peptide onto insect cell produced empty DR1 or by competing out a preloaded CLIP peptide. DM–peptide adduct was mixed either with purified DR1-CLIP or with insect cell produced unloaded DR1, and incubated for 3 d at 25°C. The samples were analyzed on 10% native PAGE. Lane 1, DM; lane 2, DR1/CLIP; lane 3, DM–peptide adduct; lane 4, DM–peptide/DR1-CLIP mixture after incubation; lane 5, DM–peptide/insect DR1 mixture after incubation. (B) Exchange of bYHA peptide bound on DR1 by free HA peptide is accelerated by the binding of streptavidin. Lane 1, streptavidin; lane 2, DR1/bYHA; lane 3, streptavidin mixed with DR1/bYHA; lane 4, DR1/bYHA mixed with HA peptide; lane 5, streptavidin and HA peptide mixed with DR1/bYHA; lanes 6 and 7, DR1/bYHA mixed with HA and incubated at 37°C for 1 h; lane 8, DR1/HA. (C) The DM–peptide/DR1 complex can be assembled with high yield by competing out prebound bYHA peptide on DR1 with the aid of streptavidin. DR1 loaded with the bYHA peptide was mixed with a twofold excess streptavidin and a 1.5-fold excess DM–peptide adduct. The sample was split in two and was either incubated for 10 min or 24 h at 25°C. Lane 1, DM–peptide; lane 2, DR1/bYHA; lane 3, DM–peptide (different preparation); lane 4, DM; lane 5, purified DM–peptide/DR1 complex; lane 6, mixture after 10 min incubation; lane 7, mixture after 24 h incubation.

presence of free peptide (Fig. 1 B, compare lanes 2 and 5). We speculate that this rate enhancement is due to effects on k_{on} and possibly on k_{off}, because of steric hindrance from the bulky streptavidin (MW 60,000 Da) attached to the biotin near the peptide P1 side-chain. Mixing of the streptavidin/biotin-YHA/DR1 complex with the DM–peptide adduct, gave DM–peptide/DR1 in high yield (Fig. 1 C, compare lanes 2 and 6 and 7). This technique can be used effectively for the high yield loading of any weakly binding peptide that is not in large supply.

A summary of the reaction steps used to assemble the DM–peptide/DR1 complex is shown in Fig. 2. In the first step the cysteine-containing HA peptide (usually oxidized upon storage) was reduced by DTT and modified by

Table I. *Peptides Used in This Study*

Peptide name	Sequence
HA	PKYVKQNTLKLAT
Biotin-HA (or bHA)	Biotin-GPKYVKQNTLKLAT
Biotin-YHA (or bYHA)	Biotin-YVKQNTLKLAT
Biotin-6GC-Biotin (or b6GHA)	Biotin-CGGGGGGPKYVKQNTLKLAT
HA-6GC-Biotin (or HA6Gb)	PKYVKQNTLKLATGGGGGGC-Biotin
2G-HA	GCGGPKYVKQNTLKLAT
6G-HA	GCGGGGGGPKYVKQNTLKLAT
11-HA	GCGGSGGGSGGSGPKYVKQNTLKLAT
HA-6G	PKYVKQNTLKLATGGGGGGCG
CLIP	PVSKMRMATPLLMQA
BCLIP	Biotin-PVSKMRMATPLLMQA
CLIP-5PC	PVSKMRMACPLLMQA
HA-5PC	PKYVKQCTLKLAT

The residues that bind into the P1 specificity pocket of DR1 are shown in bold, and the cysteines used for linking to DM are underlined.

DTNB to protect and activate the cysteine. Cysteine β46 on DM was also found to be partially or totally oxidized depending on the preparation, but when reduced by DTT and repurified by size exclusion chromatography it remained reduced during storage at 4°C (based on standard DNTB assays; reference 23, and data not shown). Reaction of the reduced DM with the TNB activated peptide yielded the DM–peptide adduct with high yield (step 2) as judged by native gel electrophoresis (Fig. 1 C, compare lanes 1 and 4). Insect cell–secreted DR1 was loaded with b-YHA peptide, purified, and mixed with streptavidin (step 3) just before the final reaction. The DM–peptide adduct was mixed with the streptavidin/b-YHA/DR1 complex, yielding the DM–petide/DR1 complex (step 4)
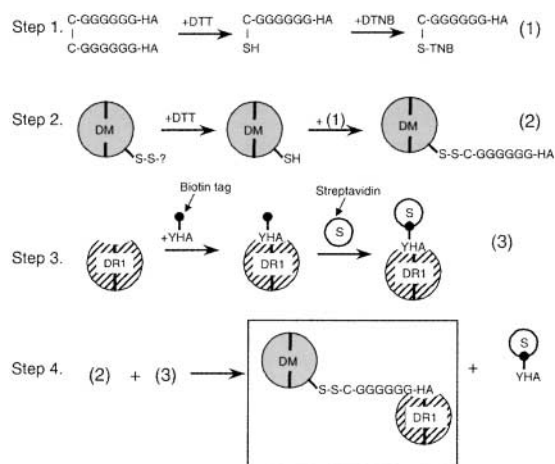

Figure 2. Assembly of the DM–peptide/DR1 complex. Cartoon diagram of the four step synthetic strategy used to produce the DM–peptide/DR1 complex in high yield. Presented here for the 6G-HA peptide (Table I).

which could be purified away from streptavidin and unreacted DM–peptide by size exclusion chromatography where it runs at ∼90 kD (Fig. 3, peak 1). Typical yields of the reaction were over 90% as judged by the obvious absence of DR1 running as a monomer (∼50–60 kD) in size-exclusion chromatograms of the mixture (Fig. 3, peak 2 and lane 6). The contents of the complex were confirmed by SDS-PAGE and shown to include both DM and DR1 in an apparent 1:1 ratio (Fig. 3, lane 5, and inset in chromatogram).

Peptide Dissociation in DM–Peptide/DR1 Complexes. The kinetics of the dissociation of DM–peptide/DR1 complexes can be followed by native gel electrophoresis. Dissociation of the complex in this case indicates dissociation of the peptide since the peptide is covalently attached to DM. Reaction with excess free peptide pushes the equilibrium toward the dissociated complex and allows measurement of the dissociation rate of the peptide attached to DM (Scheme I):

$$\text{DM-linker-HA} \ldots \text{DR1} + \text{HA} \leftrightarrow \text{HA} \ldots \text{DR1} + \text{DM-linker-HA.}$$
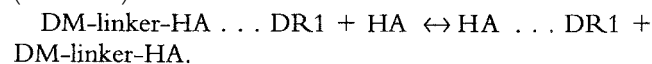
(Scheme I)

Gels showing dissociation of DM–peptide/DR1 complexes, as well as for streptavidin/biotin-peptide/DR1 complexes, are shown in Fig. 4. The DM–peptide/DR1 complex and the streptavidin/biotin–peptide/DR1 run as the lowest mobility band on a native gel compared with DM-peptide or DR1, and this band slowly disappears upon incubation with a large excess of free HA peptide. Different complexes show different dissociation kinetics. The complexes with DM attached to the NH$_2$ terminus of the peptide (Fig. 4, A–D) have rapid dissociation kinetics (half-lives of 10–40 min); the complex with DM attached to the COOH terminus of the peptide (Fig. 4 E) and com-
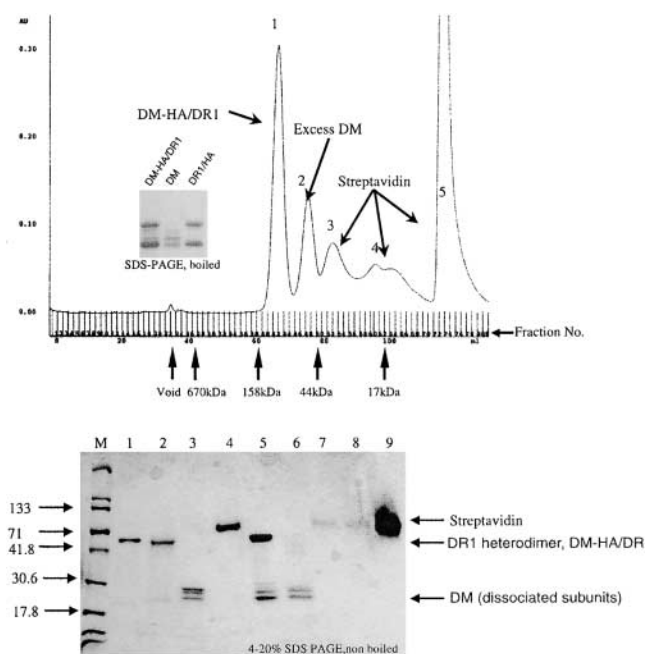
Figure 3. Purification and analysis of the DM–peptide/DR1 complex. (Top panel) DR1 preloaded with bYHA peptide was mixed with a four-fold excess of streptavidin and then with a 1.5-fold excess of DM–peptide. The mixture was incubated overnight and separated on Sephadex S200 (Amersham Pharmacia Biotech). Positions of molecular weight standards (Bio-Rad Laboratories) are indicated with arrows bellow the chromatogram. (Inset) SDS-PAGE showing the composition of the purified complex compared with equimolar amounts of DM and DR1 controls. Samples were heated to 100°C for 5 min before loading. (Bottom panel) Analysis of DM-pepitde/DR1 complex; SDS-PAGE (4–20%), nonreducing, nonboiled. Lane M, prestained molecular weight standards (Bio-Rad Laboratories). Lane 1, previously purified DM–peptide/DR complex. Lane 2, DR1/bYHA. Lane 3, purified DM. Lane 4, streptavidin. Lane 5, fraction no. 43 (peak 1) from above. Lane 6, fraction no. 48 (peak 2). Lane 7, fraction no. 53 (peak 3). Lane 8, fraction no. 61 (peak 4). Lane 9, fraction no. 73 (peak 5). Note that the DM–peptide/DR1 complex is SDS resistant (lane 1 or lane 5) when the sample is not boiled but falls apart to the DM–peptide and DR1 components when the sample is boiled (inset in chromatogram).

plexes between streptavidin and DR1 (Fig. 4, F–H) have slow dissociation kinetics (half-lives of several hundred minutes). The topmost band of each gel was scanned, and the data were plotted as the fraction of complex remaining versus incubation time (Fig. 5). It is worthwhile to note that in all cases where DM was attached onto the NH_2 terminus of the peptide, complex dissociation followed single exponential decay kinetics, indicating homogeneity in the behavior of the complex and thus of the catalytic efficiency of the attached DM.

The rapid dissociation kinetics observed require DM to be attached to the peptide, as attachment of streptavidin did not lead to a rate enhancement of such a magnitude. Dissociation experiments were also performed using biotinylated versions of the HA peptide (Table I) bound to streptavidin through the attached biotin. The biotin was attached through a 6 carbon linker to either the NH_2- or the COOH terminus of a peptide containing either a single amino acid extension over the HA peptide (for the NH_2
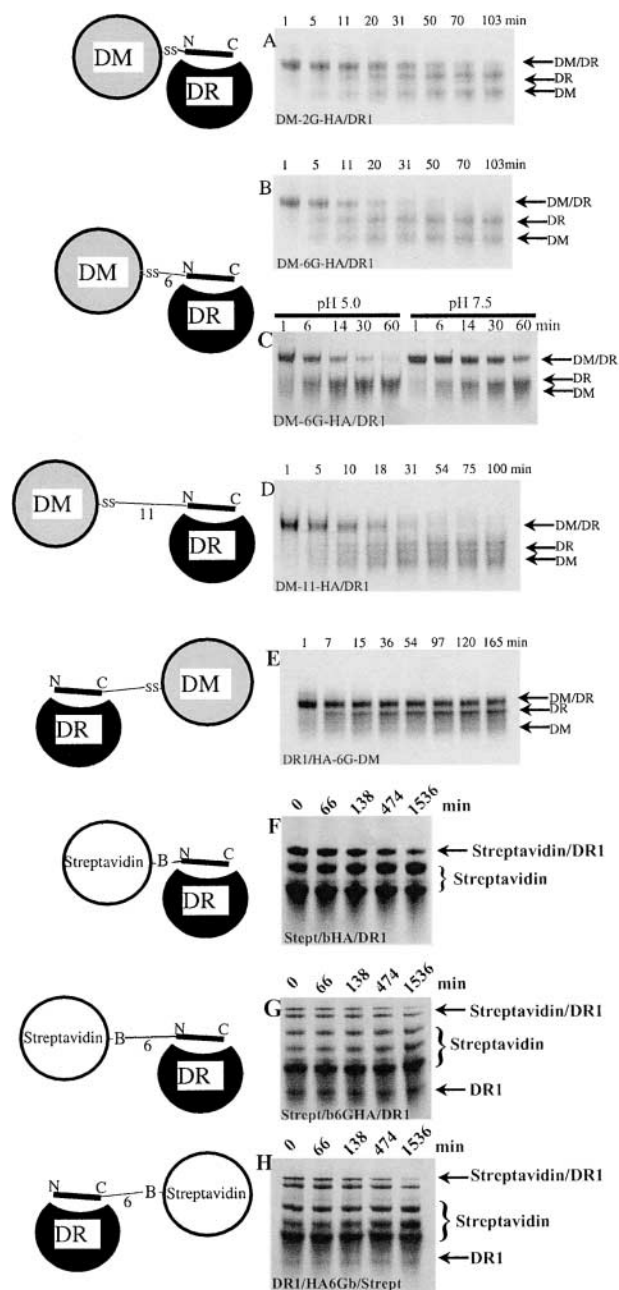

Figure 4. Time course of the disassembly of DM/DR1 and streptavidin/DR1 complexes followed by native gel electrophoresis. (Left column) Cartoon representations of complex tested. (Right column) Native gel showing time points of the dissociation of each complex upon the addition of excess free HA peptide. In every case the top most band (or bands) corresponds to the DM/DR complex (gels A–E) or the streptavidin/DR complex (gels F–H). Note the difference of dissociation rate between gels A–C and E, and the effect of the solution pH on the dissociation rate in gel D. Complex analyzed is indicated at the bottom of each gel ("/" indicates a noncovalent interaction, where "−" indicates a covalent interaction. The NH_2 terminus of the peptide is considered to be on its left and the COOH terminus on its right).

terminus) or a six amino acid extension (for the NH_2- and COOH terminus) as indicated in Table I. All streptavidin/biotin-peptide/DR1 complexes dissociated slowly in the presence of excess free peptide (Fig. 4, gels F-H). Thus, at-
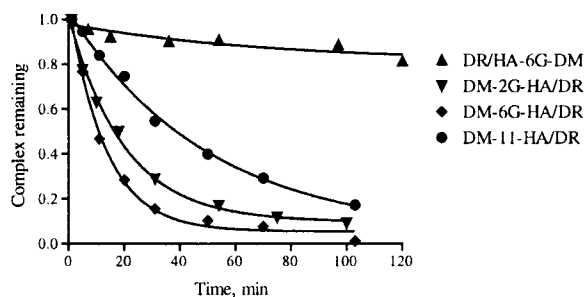
Figure 5. Dissociation kinetics for DM/DR complexes. The intensity of the topmost band of panels A, B, D, and E (see Fig. 4) was measured and quantitated by densitometry and plotted versus time. The data were fit to a single exponential decay model to produce the rate and the half-life of dissociation.

Figure 6. Dependence of peptide dissociation from DR1 on DM concentration. Circles, peptide linked to DM through its NH_2 terminus; triangles, peptide linked to DM through its COOH terminus; diamonds, peptide not linked to DM. For the case where the peptide is linked on DM through its COOH terminus or not linked at all, the data were fit using a simple linear model and the results of the fit are indicated in the graph. The same was not possible for the case where the peptide is linked to DM through its NH_2 terminus since no clear dependence on the concentration was found; in that case the drawn line is only given as a visual aid.

tachment of DM, but not of any other protein, to the NH_2 terminus of the peptide induced the enhanced dissociation. Furthermore, the rate of dissociation of the NH_2-terminally linked DM/DR1 complex was found to depend on pH in a manner similar to solution catalysis by DM. The half-life of dissociation of the DM-6G-HA/DR1 complex was found to be 90 min at pH 7.5 compared with 14 min at pH 5.0 (Fig. 4 C), almost sixfold slower. This is in accordance to previous reports that have identified pH 5.0 to be the optimal for the catalytic activity of DM (which is also the physiologically relevant pH, as it is the pH of the endosomes where DM exerts its physiological function; reference 8). These evidence taken together, strongly suggest that the interaction of DM with DR1 in the NH_2-terminally tethered complexes is mechanistically similar to the solution interaction.

Rapid dissociation of DM-peptide/DR1 complexes was observed only when DM was attached to the NH_2 terminus of the peptide and not to the COOH terminus. When DM was attached to the NH_2 terminus of the peptide the dissociation half-lives were between 12 and 14 min for a 6 or 11 amino acid linker and 37 min for a small 2 amino acid linker. When DM was attached to the COOH terminus of the peptide, the dissociation half-life was significantly slower and depended on the concentration of the complex itself in a manner similar to the dependence of HA peptide dissociation from DR1 on the concentration of soluble (untethered) DM as measured by an ELISA assay (Fig. 6). Extrapolation of this dependence to infinite dilution (intercept with the y-axis in Fig. 6) should describe the intrinsic (intramolecular) dissociation rate for this molecule and was calculated to be 5×10^{-4} min^{-1} which corresponds to a half-life of 1,386 min. This rate is two orders of magnitude slower than the rate measured for the NH_2-terminally linked DM/DR complex and is very similar to the dissociation rates of the streptavidin/DR1 complexes that have half-lives of 591 to 825 min (Fig. 7). This suggests that no DM-specific intramolecular effect is at work here. Nevertheless, this rate is faster than the rate of release of untethered peptide from DR1 which was calculated to be 3×10^{-5} min^{-1} by an ELISA assay described in Materials and Methods (Fig. 6, filled diamonds), which indicates that
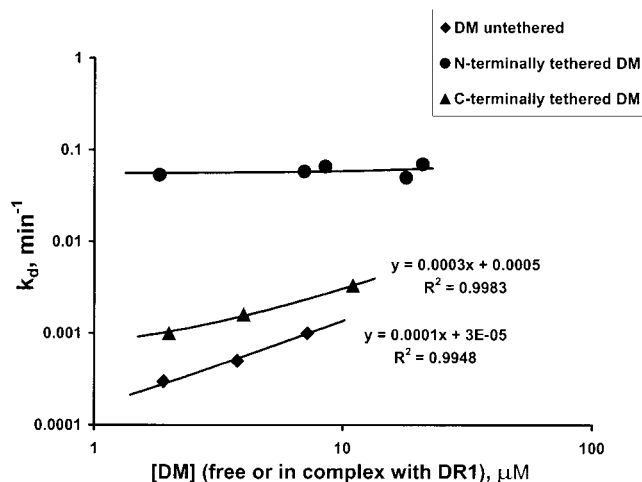
the tethering of a protein to any of the ends of the peptide does present a small effect in the dissociation kinetics of the peptide. However, this effect seems to be brought about by either DM or streptavidin when tethered to the COOH terminus of the peptide suggesting that it is not related to the catalytic mechanism of DM. Furthermore the concentration dependence of the COOH-terminally linked DM/DR1 complex suggests that DM in this complex can catalyze in trans the dissociation of peptides from other DR1 molecules but not from the one to which it is attached. The ability of the COOH-terminally linked DM to catalyze peptide exchange in trans is independently confirmed by the finding that this complex can catalyze the dissociation of the DR1-bound peptide in a separate peptide exchange assay (fluorescence polarization assay as described in Materials and Methods; data not shown) to an extent similar to catalysis by free, soluble DM. These observations suggest that any acceleration of peptide release seen in the COOH-terminally linked DM/DR1 complex compared with the dissociation of the untethered peptide, is in part due to nonspecific tethering effects and in part to intermolecular catalysis by DM attached to other complexes. We therefore conclude that when DM is tethered on DR1 through a linker attached to the COOH terminus of the peptide, it is unable to interact with the tethered DR1 in a normal catalytic manner, but the flexibility of the linker allows it to interact with other DR1 molecules in solution.

Dissociation kinetics of NH_2-terminally linked DM–peptide/DR1 complexes appear to be independent of the concentration of the complex, for the range of concentrations examined, from 1 to 20 µM. A 10-fold dilution of the complex results in a similar degree of dissociation for a
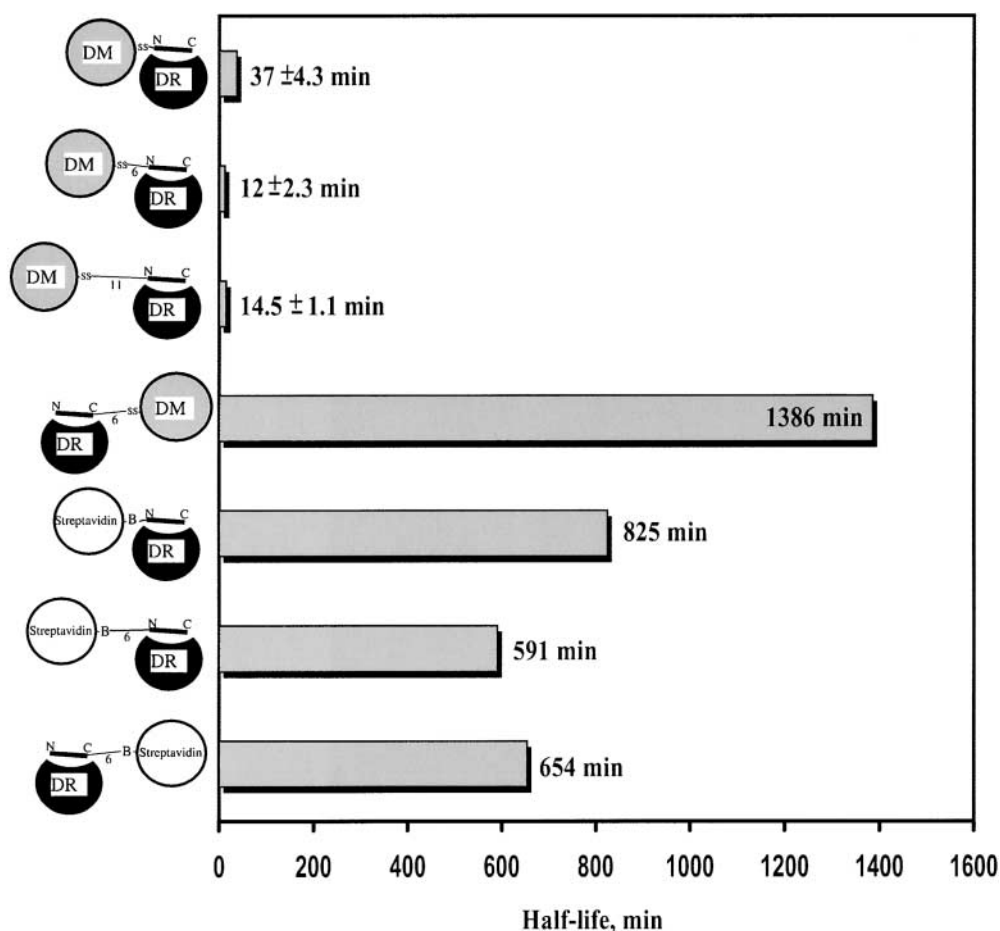
Figure 7. Summary of half-lives of intrinsic peptide dissociation for the DM–peptide/DR1 complexes, and Streptavidin/peptide/DR1 constructs. Standard deviations indicated are a result of 3–5 different measurements. On the left is a cartoon representation of the constructs, where the solid thick line represents the peptide bound on DR1 and the thin line the linker between the peptide and DM or Streptavidin. As the dissociation of the complex where the peptide is linked to DM through its COOH terminus is dependent on the concentration (Fig. 6), the half-life shown here is calculated by extrapolating the concentration dependence to infinite dilution (intercept with the y-axis in Fig. 6). The half lives or dissociation of the streptavidin/DR1 complexes were estimated from gels F–H of Fig. 4.

20 min incubation at 37°C (data not shown), whereas a 10-fold dilution of a bimolecular reaction would be expected to yield a 10^2 decrease in rate. The dissociation rates for a series of DM-6G-HA/DR1 concentrations are shown in Fig. 6 (filled circles). The y-axis intercept is the rate at infinite dilution, where intermolecular effects are not observable; the dissociation rate is virtually unchanged over the concentrations tested with a y-axis intercept accounting of almost, if not all, of its magnitude, indicating that the dissociation is catalyzed predominantly intramolecularly. Although some intermolecular (concentration dependent) component similar in magnitude to the COOH-terminally linked complex cannot be excluded by these experiments its role in the dissociation of the NH$_2$-terminally linked complex is certainly secondary. The dominance of the concentration-dependent component in the dissociation of the NH$_2$-terminally linked DM/DR1 peptides strongly suggests that it is an intramolecular event specific to DM (as tethering a different protein does not lead to such an effect) that leads to the fast kinetics observed.

Discussion

Four stable, tethered, DM/DR1 complexes were constructed and analyzed for enhanced peptide dissociation rates from DR1. When DM was attached to the NH$_2$ terminus of the DR1-bound peptide we found rapid kinetics of peptide dissociation. By contrast, when DM was attached to the COOH terminus of the DR1-bound peptide dissociation rates were slow. Similar slow dissociation kinetics were found when an unrelated protein, streptavidin, was attached to either the NH$_2$ or COOH terminus of the peptide. Furthermore, the dissociation kinetics for the complexes where DM was tethered to the NH$_2$ terminus of the peptide were found to be largely concentration independent, suggesting that it is an intramolecular interaction that is responsible for the rapid kinetics. The rapid kinetics measured for the NH$_2$-terminally linked complexes can be attributed to the high local concentrations induced by the covalent linkage. A simple calculation based on the spherical volume that DM can occupy because of linker restrictions (10, 30, and 45Å assumed linker lengths) indicates local DM concentrations of 40, 1.5, and 0.5 mM, respectively. Further restrictions because of steric clashes between the two proteins would decrease the available volume that DM can move into and increase the local concentration "visible" to DR1 even further. Extrapolation to high DM concentrations of the observed rates of peptide dissociation in solution assays using untethered DR1 and DM (Fig. 6, filled diamonds) shows that half-lives of dissociation of a few minutes,

such as those observed in the tethered complexes, can be expected at DM concentrations of ~1 mM. Finally, the pH of the solution was found to affect the rate of dissociation of the tethered complexes in a manner similar to the effect of pH on the catalytic ability of detergent solubilized DM (8). These results, taken together, suggest that the interaction between DM and DR1 in the NH_2-terminally tethered complexes is mechanistically similar to the normal interaction in solution that leads to enhanced peptide dissociation.

For the three NH_2-terminally tethered complexes studied, linker length seems to have only a marginal effect on dissociation kinetics. A small increase in dissociation rate was seen when the linker length was decreased from 11 amino acids to 6 amino acids, but this is much less than expected from the threefold increase in local concentration (from 0.5 to 1.5 mM) based on the calculation above. Furthermore, the dissociation rate decreased about threefold when the linker length was decreased to only 2 amino acids but still remained at least 10-fold higher than the rate expected by intermolecular catalysis at the concentrations used in the experiment. This small decrease of the dissociation rate for the shortest linker, compared with the longer linkers, is presumably due to some steric interference on the DM/DR1 interaction imposed by the limited flexibility of a short connection. Overall, lack of clear correlation between linker lengths and dissociation rates might indicate a saturation of the catalytic activity of DM in very high concentrations. This saturation might be attributed either to K_M saturation (the enzyme interacts with the substrate at 1:1 ratio) or to the possibility that DM accelerates only a part of the peptide exchange pathway. In the latter case, the overall peptide dissociation kinetics would become limited by another step in the pathway when DM concentrations are very high. Indeed, a recent study by Zarutskie et al. (16) suggests that DM accelerates the equilibrium between two peptide/DR1 conformations only, one of which is able to release the peptide. It is not unreasonable to expect that under very high DM concentrations, the interconversion between those two conformations would become faster than the peptide release and the observed rate would then be the rate of peptide release from the rapidly releasing conformation. This condition would, of course, signify that in the DM-peptide/DR1 complexes described here, the dissociation rates seen are the fastest possible rates of HA peptide dissociation that can be induced by DM. Because of the large excess of free peptide used in the dissociation experiments, it is reasonable to assume that DM will catalyze the dissociation of the complex it is part off only once, and therefore the fastest rates calculated here (0.07 min^{-1}) would also be the turnover number for DM catalysis of peptide dissociation from DR1. These rates are more than sufficient for peptide exchange in vivo and probably represent an upper limit of DM catalysis in the cell, where a high local concentration of DM is achieved by membrane tethering and compartmentalization.

Fig. 8 presents a schematic representation of DM and DR1 linked by a two-glycine linker through the NH_2 terminus of the HA peptide bound on DR1. Both proteins are oriented in such a manner that the transmembrane tails (not shown) would be located at the bottom of the picture. Even with such a small linker length, it is reasonably easy to position both proteins so that no steric clashes occur between them. The linker is modeled in a fully extended form to reveal maximum degree of freedom that it can al-
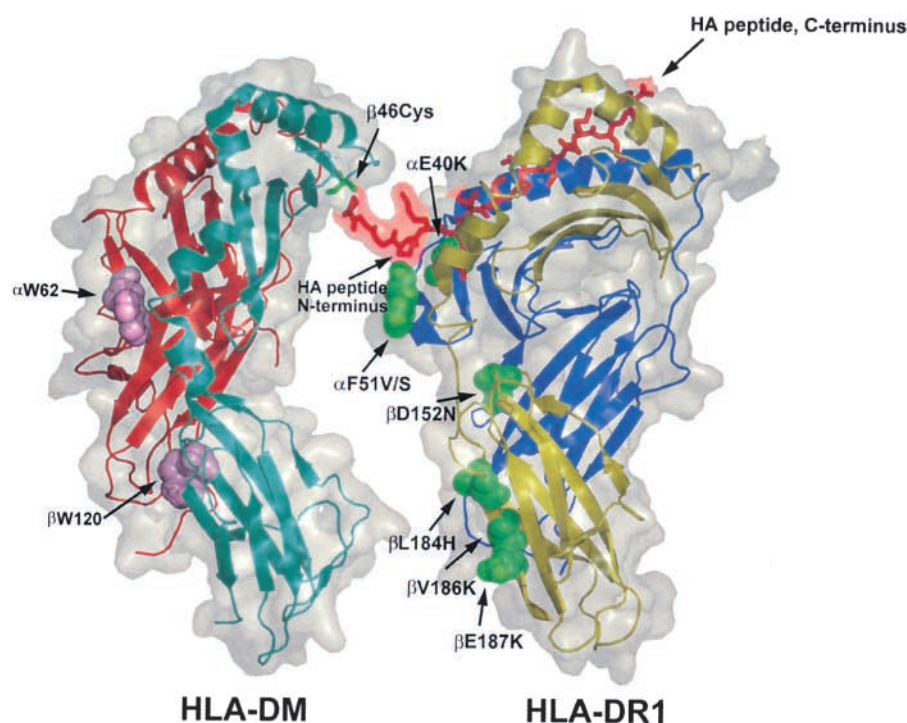

Figure 8. Schematic model of HLA-DM (left, red ribbon: chain A; cyan ribbon: chain B) linked by a disulfide bond from cysteine B46 (green sticks) to a cysteine at the NH_2 terminus of the HA peptide (red sticks) bound on HLA-DR1 (right, blue ribbon: chain A; yellow ribbon: chain B). The linker from the NH_2 terminus of the peptide to the bonded cysteine is two glycines (peptide 2G-HA, Table I) and is modeled here in an extended conformation. Solvent surfaces for both DM and DR1 are shown in gray. Note the lack of clashes between DM and DR1, even for such a small linker. Mutations on DR1 that have been found to interfere with DM interaction are indicated as green spheres (reference 19). Tryptophans on DM that were previously speculated to interact with DR1 are shown in pink (reference 11). Figure was made with PyMol (reference 28) using PDB coordinate files 1HDM and 1DLH.

low in the interactions between DM and DR1. The finding that the interaction between DM and DR1 seems to be mechanistically the same compared with the untethered proteins, even for such a short linker, imposes great restrictions in the possible surfaces of interactions on both proteins. The requirement for DM to be tethered to the NH_2 terminus of the peptide in order to catalyze its exchange efficiently in combination with the requirement of both proteins to be tethered on the same membrane suggests that it is the left lateral face of DR1 (as presented on Fig. 8) that interacts with DM. This conclusion is in agreement with a recent mutagenesis study (19), which identified mutations that abolish DM/DR1 interactions as probed in cell assays (the mutants identified in this study are indicated with green spheres in Fig. 8). Furthermore, the short linker identifies the surface of DM that interacts with DR1 as being the lateral face of DM that contains cysteine β46 (the right side of DM as presented in Fig. 8). It has been previously speculated that two solvent-exposed tryptophans on DM (α62 and β120, shown in pink spheres in Fig. 8) might reside in the interface of the DM/DR1 complex (11). These tryptophans reside on the opposite face of DM than cysteine β46, however, and it is thus virtually impossible to rotate DM so that they come in contact with DR1 while still satisfying the small linker restrictions. Our results therefore suggest that the lateral face of DM that interacts with DR1 is opposite from the one previously speculated. The requirement for the proximity of cysteine β46 to the NH_2 terminus of the bound peptide also mechanistically implicates the part of the binding groove of DR1 that accommodates the NH_2 terminus of the peptide in the DM/DR1 interaction. The P1 pocket of DR1 (the pocket of the binding groove that accommodates the tyrosine of the HA peptide) is a very important binding determinant for the peptide. Along with hydrogen bonds around it, is possibly a target region for DM (15, 24–26). A mechanism for DM action that would be in agreement with the present data would involve recognition and binding by DM of an altered conformation of DR1 where the region that accommodates the NH_2 terminus of the peptide and the P1 side-chain is either partially unfolded or in a metastable conformation that breaks some of the interactions with the peptide. Interactions with the lateral face of DM, in the vicinity of cysteine β46, could stabilize this conformation by either substituting some of the interactions between peptide and DR1 that were lost during the conformational change or by creating new ones. This DR1 conformation could be either the intermediate recognized before as a fast peptide exchanger (14, 27) or a transition state leading to that intermediate. In either case, stabilization of that transient conformation of DR1 by DM would result in an apparently enhanced rate of peptide dissociation. Soluble, tethered complexes where the DM/DR1 interaction is saturated, like the ones presented here, in conjunction with the knowledge of the interacting surfaces on both proteins, can be powerful tools for "locking" the transient interaction of DM with DR1. This would make the isolation of the high energy conformational state of DR1 possible, opening the way toward a detailed understanding of the structural events of the mechanism of DM catalysis.

References

1. Ghosh, P., M. Amaya, E. Mellins, and D.C. Wiley. 1995. The structure of an intermediate in class II MHC maturation: CLIP bound to HLA-DR3. *Nature*. 378:457–462.
2. Roche, P.A., and P. Cresswell. 1990. Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. *Nature*. 345:615–618.
3. Kelly, A.P., J.J. Monaco, S.G. Cho, and J. Trowsdale. 1991. A new human HLA class II-related locus, DM. *Nature*. 353: 571–573.
4. Sloan, V.S., P. Cameron, G. Porter, M. Gammon, M. Amaya, E. Mellins, and D.M. Zaller. 1995. Mediation by HLA-DM of dissociation of peptides from HLA-DR. *Nature*. 375:802–806.
5. Denzin, L.K., and P. Cresswell. 1995. HLA-DM induces CLIP dissociation from MHC class II alpha beta dimers and facilitates peptide loading. *Cell*. 82:155–165.
6. Morris, P., J. Shaman, M. Attaya, M. Amaya, S. Goodman, C. Bergman, J.J. Monaco, and E. Mellins. 1994. An essential role for HLA-DM in antigen presentation by class II major histocompatibility molecules. *Nature*. 368:551–554.
7. Guerra, C.B., R. Busch, R.C. Doebele, W. Liu, T. Sawada, W.W. Kwok, M.D. Chang, and E.D. Mellins. 1998. Novel glycosylation of HLA-DRalpha disrupts antigen presentation without altering endosomal localization. *J. Immunol.* 160:4289–4297.
8. Sherman, M.A., D.A. Weber, and P.E. Jensen. 1995. DM enhances peptide binding to class II MHC by release of invariant chain-derived peptide. *Immunity*. 3:197–205.
9. Roche, P.A. 1995. HLA-DM: an in vivo facilitator of MHC class II peptide loading. *Immunity*. 3:259–262.
10. Weber, D.A., B.D. Evavold, and P.E. Jensen. 1996. Enhanced dissociation of HLA-DR-bound peptides in the presence of HLA-DM. *Science*. 274:618–620.
11. Mosyak, L., D.M. Zaller, and D.C. Wiley. 1998. The structure of HLA-DM, the peptide exchange catalyst that loads antigen onto class II MHC molecules during antigen presentation. *Immunity*. 9:377–383.

12. Fremont, D.H., F. Crawford, P. Marrack, W.A. Hendrickson, and J. Kappler. 1998. Crystal structure of mouse H2-M. *Immunity*. 9:385–393.

13. Sadegh-Nasseri, S., and H.M. McConnell. 1989. A kinetic intermediate in the reaction of an antigenic peptide and I-Ek. *Nature*. 337:274–276.

14. Dornmair, K., B. Rothenhausler, and H.M. McConnell. 1989. Structural intermediates in the reactions of antigenic peptides with MHC molecules. *Cold Spring Harb. Symp. Quant. Biol.* 54:409–416.

15. Chou, C.L., and S. Sadegh-Nasseri. 2000. HLA-DM recognizes the flexible conformation of major histocompatibility complex class II. *J. Exp. Med.* 192:1697–1706.

16. Zarutskie, J.A., R. Busch, Z. Zavala-Ruiz, M. Rushe, E.D. Mellins, and L.J. Stern. 2001. The kinetic basis of peptide exchange catalysis by HLA-DM. *Proc. Natl. Acad. Sci. USA*. 98:12450–12455.

17. Weber, D.A., C.T. Dao, J. Jun, J.L. Wigal, and P.E. Jensen. 2001. Transmembrane domain-mediated colocalization of hla-dm and hla-dr is required for optimal hla-dm catalytic activity. *J. Immunol.* 167:5167–5174.

18. Grasberger, B., A.P. Minton, C. DeLisi, and H. Metzger. 1986. Interaction between proteins localized in membranes. *Proc. Natl. Acad. Sci. USA*. 83:6258–6262.

19. Doebele, C.R., R. Busch, M.H. Scott, A. Pashine, and D.E. Mellins. 2000. Determination of the HLA-DM interaction site on HLA-DR molecules. *Immunity*. 13:517–527.

20. Dessen, A., C.M. Lawrence, S. Cupo, D.M. Zaller, and D.C. Wiley. 1997. X-ray crystal structure of HLA-DR4 (DRA*0101, DRB1*0401) complexed with a peptide from human collagen II. *Immunity*. 7:473–481.

21. Stern, L.J., and D.C. Wiley. 1992. The human class II MHC protein HLA-DR1 assembles as empty alpha beta heterodimers in the absence of antigenic peptide. *Cell*. 68:465–477.

22. Sato, A.K., J.A. Zarutskie, M.M. Rushe, A. Lomakin, S.K. Natarajan, S. Sadegh-Nasseri, G.B. Benedek, and L.J. Stern. 2000. Determinants of the peptide-induced conformational change in the human class II major histocompatibility complex protein HLA-DR1. *J. Biol. Chem.* 275:2165–2173.

23. Ellman, G., and H. Lysko. 1979. A precise method for the determination of whole blood and plasma sulfhydryl groups. *Anal. Biochem.* 93:98–102.

24. McFarland, B.J., C. Beeson, and A.J. Sant. 1999. Cutting edge: a single, essential hydrogen bond controls the stability of peptide-MHC class II complexes. *J. Immunol.* 163:3567–3571.

25. McFarland, B.J., J.F. Katz, C. Beeson, and A.J. Sant. 2001. Energetic asymmetry among hydrogen bonds in MHC class II*peptide complexes. *Proc. Natl. Acad. Sci. USA*. 98:9231–9236.

26. Bryant, P.W., P. Roos, H.L. Ploegh, and A.J. Sant. 1999. Deviant trafficking of I-Ad mutant molecules is reflected in their peptide binding properties. *Eur. J. Immunol.* 29:2729–2739.

27. Cresswell, P., J.S. Blum, M.S. Marks, and P.A. Roche. 1989. Structural and functional aspects of HLA class II glycoproteins and the associated invariant chain. *Cold Spring Harb. Symp. Quant. Biol.* 54:309–318.

28. DeLano, W.L. The PyMol molecular Graphics System. DeLano Scientific, San Carlos, CA. http://www.pymol.org.