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Immunology

Conformational variants of class II MHC/peptide complexes induced by N- and C-terminal extensions of minimal peptide epitopes

(HLA-DR/invariant chain/hemagglutinin/polymer)

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ABSTRACT **Class II MHC molecules are known to exist in conformational variants. "Floppy" and "compact" forms of murine MHC molecules, for example, are discriminated by their migration behavior on SDS/PAGE and represent empty and ligand-loaded forms. Here we show that formation of distinctly faster-migrating ligand complexes (F-forms) rather than the normal compact (C-) forms of HLA-DR1 or -DR4 results from extensions of minimal peptide epitopes (such as HA306-318 or IC106-120) by ≈10 amino acids at either the N or the C terminus. Two similar but distinct F-forms (F_I and F_{II}) were detected, depending on the site of the extension. Both F-forms were characterized by increased surface hydrophobicity and reduced SDS-stability. Native gel separations and size exclusion chromatography indicated that the F-forms had increased hydrodynamic radii compared with the C-form and an apparent size similar to that of empty MHC molecules. The regions on the ligand overhangs responsible for the effect began at a distance of ≈5 amino acids on either side of the epitopes, comprised 4–8 amino acids (i.e., a total overhang of 9–14), and did not have a particular sequence preference. The possible functional significance of these forms is discussed.**

Most proteins undergo changes in conformation on binding to substrates or other ligands because of the newly formed bonds between these molecules. Sometimes, conformational changes are also required to signal the change in status. In receptor proteins, the binding of the ligand is often indicated by an altered tertiary structure, causing shifts in the affinity of the receptor to other proteins initiating signal transduction or endocytosis (1, 2).

The association of MHC molecules with peptide ligands also causes some alterations in conformation (3–6). Peptide-loaded MHC complexes are well characterized, and crystallographic studies of class I and class II MHC/peptide complexes indicate a similar spatial structure of the two classes of MHC molecules (7, 8). Within the two classes, only relatively slight shifts in the arrangement of the secondary structure elements of the peptide-binding groove have been found (9–12). However, indirect evidence suggests that MHC/peptide complexes are also likely to exhibit more significant differences. Conformational variants of class II MHC molecules were reported, for example, in response to the pH of the environment (13–15) or in the form of unstable kinetic intermediates appearing before the formation of stable peptide complexes (16). Recently, relatively stable, interconvertible conformers of some MHC/peptide complexes were detected (17), and at least two different conformational states of empty MHC molecules were described, one characterized by nonreceptiveness to loading with peptide ligands (18, 19).

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In the present work, another distinct form of class II MHC/peptide complexes has been detected. After loading class II MHC molecules with long peptides (23- to 33-mer) instead of the minimal length core binding epitopes usually found in the binding groove of MHC molecules (13- to 15-mer), stable but distinctly altered forms of the ligand complexes were found. These complexes differ from the compact (C-) form in a faster migration behavior during SDS/PAGE (F-forms) and show some properties similar to those of "empty" molecules.

MATERIALS AND METHODS

MHC Class II Ligands. Most of the ligands contained the sequence of the binding epitopes HA306-318 (PKYVKQNTLKLAT) (20) or IC106-120 (KMRMATPLLMQLPM). Synthetic peptide ligands and constructs bridged by tetraethylene glycol (teg), the equivalent of ≈7 amino acids (J.M., K.F., O.R., T. Walk, J.L.S., and G.J., unpublished work), were produced by using solid phase Fmoc/tBu chemistry (in teg-linked constructs, for technical reasons, a Gly-residue was added to the N terminus of HA306-318). 2-Chlorotritylchloride resin (Senn Chemicals, Dielsdorf, Switzerland) was used for the preparation of peptide acids, and Rink amide resin (Rapp Polymere, Tübingen, Germany) was used for the preparation of peptide amides. Fmoc-Ats-OH (for the addition of C-terminal teg residues) and Fmoc-Atg-OH (all other positions) were prepared as described elsewhere (21). The products were dissolved in tert-butyl alcohol/water (4:1) and were lyophilized and purified on a C18-HPLC-column. For biotinylated polypeptide ligands, NHS-LC-biotin (Pierce) was attached on the N terminus of an additional spacer sequence (SGSG) at the N-terminal end of the peptide. In teg-linked biotinylated constructs, the amino acid spacer was replaced by a teg-unit.

Production of MHC Class II Molecules. Soluble HLA-DR1 (DRA1*0101, DRB1*0101) and -DR4 molecules (DRA1*0101, DRB1*04011) were prepared as described from S2-cells transfected with vectors encoding the class II MHC molecules (22).

Binding of Peptide Ligands to Soluble HLA-DR1 Molecules and SDS/PAGE Separation. Binding studies were carried out by incubating 3.75 μl of HLA-DR1 (1 mg/ml) with 0.5 μl of ligand (1 mg/ml) in PBS for 16–48 h at 37°C. In most experiments, 4–7% ethanol was added to accelerate the binding. SDS/PAGE separation was done on a 4–15% Tris-glycine gradient gel (Bio-Rad) at 4°C. Samples were loaded on the gel without prior boiling by using a nonreducing loading buffer. For native gel separations, sample buffer and Tris-glycine running buffer without SDS were used. A protein standard of 25–150 kDa (Novagen) was used as the apparent molecular weight (MW_{app}) marker, and the protein bands on the gels were visualized by silver staining.

ELISA. Biotinylated peptide (0.5 μg) ligand was added to HLA-DR1 (3.75 μg) and was incubated at 37°C overnight. In

Abbreviation: MW_{app}, apparent molecular weight.

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some experiments, pH was adjusted by the addition of 1 μl of 250 mM phosphate/citrate buffer. The mixtures were added to ELISA plates previously coated with an α-HLA-DR1 monoclonal antibody (L243, American Type Culture Collection number HB-55) and were blocked with 2% BSA in PBS. After 1 h at 37°C, the plates were washed and incubated for 30 min at room temperature with streptavidin coupled with horseradish peroxidase (Sigma). Peptide/HLA-DR1 complexes were detected by using the TMB substrate (Kirkegaard & Perry Laboratories) and were measured at 450 nm with a microplate reader (MRX, DYNEX Technologies, Chantilly, VA).

Digestion with Cathepsin S. For digestion experiments with cathepsin S, MHC/ligand complexes were preformed by incubating 1 μg of ligand with 3.75 μg of HLA-DR1 at pH 6.0 (50 mM citrate/phosphate buffer) at 37°C overnight. Cathepsin S (0.2 μg), previously activated by incubating the proenzyme for 3 h at pH 4.5 in the presence of 1 mM DTT and 0.5 mM EDTA, was added to the binding mixtures. After digesting for 1 h at 37°C, the pH was raised to 7.5, and the incubation was continued for 30 min at 37°C. Samples then were visualized by SDS/PAGE.

HLA-DM-Mediated Ligand Release Experiments. For release experiments with soluble HLA-DM (22, 23), the pH was adjusted with 50 mM citrate/phosphate buffer to 5.0. After the preincubation of biotinylated peptide ligands with HLA-DR1 overnight, HLA-DM was added to the reaction mixture together with an excess of unlabeled HA306-318 peptide (3 mg/ml) and was incubated for 3 h at 37°C. Samples were analyzed by ELISA as described above.

Size Exclusion Chromatography. For size exclusion chromatography experiments, HLA-DR1 (100 μg) was loaded for 48 h with peptide ligand (30 μg) in PBS/5% EtOH at 37°C. The size exclusion was carried out with a BioLogic FPLC (Bio-Rad) on a Superdex 200 HR 10/30 column (Amersham Pharmacia) with PBS as eluent. A low molecular weight protein mixture (Amersham Pharmacia) and PBS-solutions of BSA (American Bioanalytical, Natick, MA), hen egg albumin (Calbiochem), carbonic anhydrase (Calbiochem), and soybean trypsin inhibitor (Calbiochem) were used as standards.

RESULTS AND DISCUSSION

SDS/PAGE Analysis of HLA-DR/Ligand Complexes. In a previous study, the association of soluble class II MHC molecules to long protein-like polypeptide oligomers was seen to result in the formation of unusual MHC/ligand complexes (24). The most obvious difference from the normal "compact" (C-) complex was a significantly reduced MW_{app} during SDS-PAGE analysis, i.e., a faster migration despite the real increase in MW. The same drop in the MW_{app} also was observed with synthetic dimeric peptide ligands (Fig. 1). In these constructs, two HA306-318 peptide epitopes were linked together by a tetraethylene glycol spacer (teg). After loading HA306-318-dimers (i.e., HA306-318-teg-HA306-318-teg) onto soluble HLA-DR1 or -DR4 molecules (Fig. 1, D lanes), ligand complexes were formed that migrated significantly faster than the C-complexes loaded with the single peptide HA306-318 (Fig. 1, P lanes). The MW_{app} of these fast-migrating F-complexes translated into a value of only ≈40 kDa, compared with >60 kDa for the compact form (the calculated value for the MW of a soluble HLA-DR1/HA306-318 complex is ≈45 kDa). Additionally, with HLA-DR1/HA306-318-teg-dimer, a split of the band representing the ligand complex into doublet bands (38 kDa and 42 kDa) was observed. Only one band (38 kDa) was detected with HLA-DR4, although it appeared on close examination to actually represent a narrow doublet band.

The HLA-DR1-binding capability of one of the two HA306-318 epitopes in the dimeric ligand constructs was abrogated by a substitution of the main anchor residue Y308 (24, 25) (Fig. 2*A*). By replacing one of the two epitopes in the HA-dimer with Y308D-substituted HA306-318 (YD), HA306-318 ligands were designed, which carried defined N-terminal (YD-HA) or C-terminal (HA-YD) peptide segment overhangs. SDS/PAGE of the HLA-DR1/ligand complexes revealed that each of the YD-HA- (Fig. 2*A*, lane N_{YD}) and the HA-YD- (lane C_{YD}) loaded MHC molecules produced only a single band. These complexes comigrated with either one or the other of the two doublet bands of the HA-HA-loaded complexes (lane D), indicating that the slower-migrating F_I-band represents the ligand complex with an N-terminal overhang, and the faster migrating F_{II}-band represents the ligand complex with a C-terminal overhang.

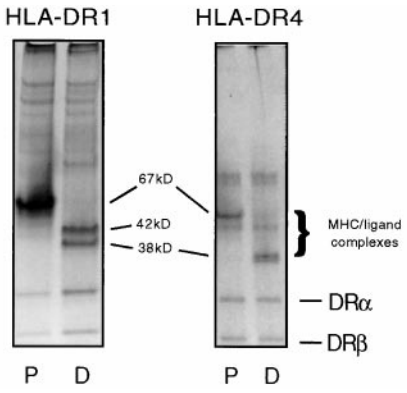

FIG. 1. Apparent molecular weight (MW_{app}) of class II MHC/ligand complexes loaded with monomeric or dimeric ligand constructs. (*A*) Soluble HLA-DR1 or -DR4 molecules loaded with HA306-318 peptide (P lanes) or dimeric ligand constructs consisting of two HA306-318 epitope units linked by a tetraethylene glycol spacer (D lanes) were separated by SDS/PAGE. The MW_{app} indicated in the figure refers to bands representing distinct MHC/ligand complexes. Gels were visualized by silver staining.

Because the formation of F-forms was apparently caused by amino acid residues located in the overhanging peptide segments, truncations were introduced to narrow down the approximate position of the effector sites (Fig. 2*B*). The separation of the series of N-terminal truncations (Fig. 2*B Left*) revealed that F_I is still formed if the overhang contains only 10 of the 14 amino acids of the YD-overhang (lane N_{10}). The additional elimination of 5 more amino acids, however, led to the formation of the C-form (Fig. 2*B Left*, lane N_5). The effect could not be reversed by extending the polyethylene glycol linker with a second teg-unit [which has a length of ≈7 amino acids (Fig. 2*B Left*, lane N_5')]. Also on the C-terminal side (Fig. 2*B Right*) an extension with a fragment of 10 amino acid residues was sufficient to produce the F_{II}-complex (lane C_{10}) whereas a 5-aa-long extension resulted in the formation of the C-form (lane C_5). In contrast to the N-terminal side, here the introduction of a second teg-unit could compensate for the loss of the 5 amino acids. With two teg-units as spacer, the 5-aa-long fragment effectively stabilized the F_{II}-complex (Fig. 2*B Right*, lane C_5').

Thus, as a rough approximation, the sites responsible for the constitution of the F_I- and F_{II}-forms comprised 5–10 amino acid residues and appeared to be located at a distance of 5–10 amino acids on either side of the 13-mer epitope. The effects were not limited to the artificial dimeric ligand constructs because the same results were obtained with "natural" polypeptide ligands in which HA306-318 was extended by 20 amino acids of the sequence of the hemagglutinin protein (i.e., HA286-308 and HA306-338; Fig. 2*C*). The association of the N-terminal-extended HA286-318 peptide to the HLA-DR1 molecule resulted in the formation of an F_I-complex (Fig. 2*C*, lane P_N) whereas the C-terminal-extended HA306-338 peptide formed the F_{II}-complex (lane P_C).

To test for potential requirements of particular sequences, teg-linked HA306-318 ligands with randomized C-terminal overhangs were used (HA-X_{15}). With these ligands (Fig. 2*D*, lane X_{15}) the same sharp F_{II}-band was detected as with the naturally extended HA306-338 peptide (lane P_C) or the HA-dimer (lane D). Only very little staining was evident at the respective position

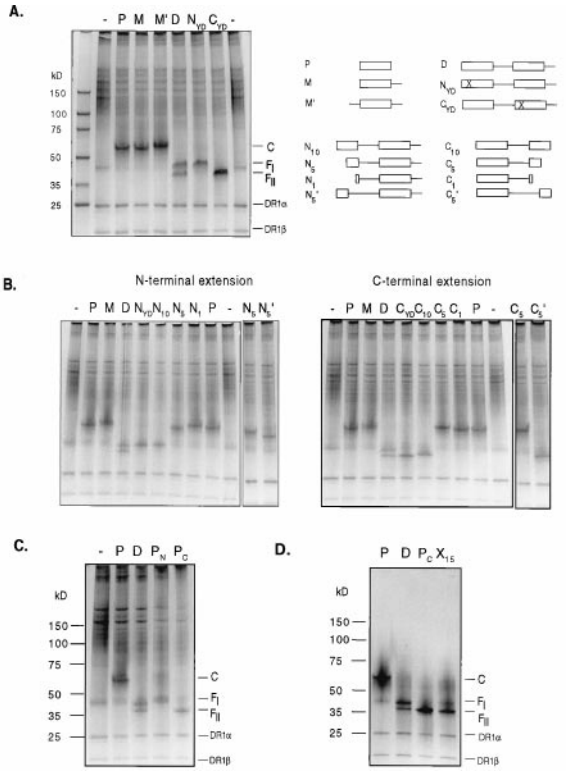

FIG. 2. Induction of F_I- and F_{II}-complexes by amino acid overhangs of the ligand. Soluble HLA-DR1 molecules were loaded with ligand constructs containing the epitope HA306-318. The structure of the ligands is depicted schematically on the upper right panel. The box represents the HA306-318 binding epitope, and the line represents a tetraethylene glycol unit used to connect the epitopes. The cross in the N_{YD}- and the C_{YD}-ligand marks a substitution of the main anchor residue Y_{308} with D in one of the two HA306-318 epitopes, which abrogates binding to the HLA-DR1 molecule. N_{10}-N_1 and C_{10}-C_1 represent dimeric ligand constructs with truncations in the N- or C-terminal epitope. (*A*) Formation of F_I- and F_{II}-ligand complexes with teg-linked peptide constructs with defined N- or C-terminal polypeptide extensions. HLA-DR1/ligand complexes, loaded with monomeric (lanes P, M, and M′) and dimeric HA306-318 ligand constructs (lanes D, N_{YD}, and C_{YD}), were separated by SDS/PAGE. The position of bands representing the compact C-form (associated with the monomeric ligands) as well as of the two distinct F_I- and F_{II}-forms (associated with the dimeric ligands) are indicated. (*B*) To estimate distance and size of the polypeptide region responsible for the formation of the F_I- and F_{II}-complexes, HLA-DR1 molecules were loaded with truncated forms of the dimeric HA306-318 construct. The series of N-terminal truncations (*Left*) consists of HA306-318 peptides linked by a teg spacer with the extension VKQNTLKLAT (lane N_{10}), LKLAT (lane N_5), and T (lane N_1). In addition, a ligand also was used in which the LKLAT overhang was connected by two instead of one teg-units (lane N_5', shown on the right side of the gel). For the series of C-terminal truncations (*Right*), overhangs were used with the sequence GPKYVKQNTL (lane C_{10}), GPKYV (lanes C_5 and C_5'), and G (lane C_1). On both gels, empty HA-DR1 molecules (−) and ligand complexes preformed with the HA306-318 peptide (P lanes), the monomer (M lanes), and the dimer (D lanes) are shown as a reference. (*C*) Formation of F-complexes with naturally extended polypeptide ligands. HLA-DR1 molecules were loaded with peptides extended by 20 amino acids of the natural sequence of the hemagglutinin protein on the N-terminal side (HA286-318; lane P_N) and on the C-terminal side (HA306-338; lane P_C) of the HA binding epitope. SDS/PAGE separation is shown in comparison to the HA306-318-loaded complex (lane P) and the complex loaded with the dimer (lane D). (*D*) Formation of an F_{II}-complex by a ligand with randomized overhang. HLA-DR1 molecules were loaded with an HA306-318 epitope, which (through a teg-spacer on the C-terminal side) was connected to a 15-aa-long peptide with random sequence (HA-X_{15}). The overhang was produced by a sequential synthesis using an amino acid pool (G, A, P, V, I, L, M, Y, F, S, T, N, Q, D, E, K, R, H). The HLA-DR1/HA-X_{15} ligand complex (lane X_{15}) is shown in comparison to the complex loaded with the HA306-318-peptide (lane P), the dimer (lane D), and the HA306-338 peptide (lane P_C).

of the C-form. A randomized copolymer of the amino acids Y, E, A, and K of different lengths revealed similar results. Copolymer I peptides (26) are known to bind promiscuously to most class II MHC molecules (27) and are reported to be effective in the suppression of certain autoimmune diseases (26). Binding of these copolymer I-like peptides to soluble HLA-DR1 produced mainly C-complexes but also F_I- and F_{II}-complexes as soon as the ligands reached a sufficient length (data not shown).

Most amino acid combinations are apparently effective in the induction of the F-forms. So far, only one amino acid sequence $(GSPS)_3$ has been found that, attached to the N terminus of the HA-epitope, did not prevent the formation of the C-form (data not shown). However, at least a peptide backbone seems to be required because overhangs generated with building blocks other than α-amino acids, such as tetraethylene glycol (teg) or β-alanine/6-aminohexanoic acid, failed to stabilize the F-form (data not shown).

HLA-DR1 Complexes with Ligands Containing the IC106-120 Epitope. The invariant chain epitope IC106-120 occupies the binding groove of class II MHC molecules during the transport and maturation of class II MHC chains (11, 28–30). Because this peptide must be removed before the loading of the MHC molecules, it binds to the HLA-DR1 molecule with a relatively low affinity. To evaluate the effect of N- and C-terminal extensions on class II MHC/ligand complexes formed with low affinity ligands, some of the previous experiments were repeated with ligand constructs containing IC106-120 (Fig. 3).

First, HLA-DR1/ligand complexes formed with HA306-318 (HA) and IC106-120 (IC) were compared (Fig. 3*A*). No significant difference was observed in the MW_{app} of the two C-complexes formed with the peptides (Fig. 3*A*, P lanes). Also with dimeric constructs (Fig. 3*A*, D lanes), distinct F-complexes with the reduced MW_{app} were detected. However, in contrast to the HA-HA-ligand complexes, only a single F_{II}-complex could be detected with the IC-IC-ligand. The F_I-complex was apparently missing. Binding experiments with dimeric hybrid ligands YD-IC (Fig. 3*B Left*, lane N_{YD}) and IC-YD (lane C_{YD}) revealed that the F_{II}-complex was formed only with the ligand with the N-terminal overhang. The C-terminal extended IC-YD construct, in fact, did not produce any detectable stable complex after SDS/PAGE.

To confirm this result, binding experiments were carried out with polypeptide ligands extended by the natural sequence of the invariant chain molecule (Fig. 3*B Right*). Analogous to the synthetic hybrid IC/YD ligands, the F_{II}-complex was formed with N-terminal-extended IC97-120 ligand [the CLIP fragment produced naturally during the degradation of the MHC-bound invariant chain protein (28); Fig. 3*B Right*, lane P_N] but not with the C-terminally extended IC106-135 (lane P_C). The latter, instead, produced a unique band with a MW_{app} of ≈75 kDa, approximately double the MW_{app} of the F-forms. Separations on native gels (Fig. 3*C*) revealed that the irregular behavior of the HLA-DR1 complexes loaded with the two C-terminally extended IC ligands is a consequence of the influence of SDS. Without SDS, both complexes migrated similarly to the respective N-terminally extended forms (Fig. 3*C Lower*).

Apparently, the C-terminal YD-extension converts the otherwise stable IC106-120/HLA-DR1-complex into a highly SDS-sensitive form. "SDS stability" has originally been used as marker for structural maturity of the MHC/peptide complex (16). Recently, however, it has been attributed to the accessibility of SDS molecules to certain pockets in the MHC binding cleft to SDS (31). In this light, the C-terminal YD-extension could allow SDS molecules to enter the binding cleft by altering its conformation or by loosening its association to the ligand.

Characterization of F- and C-forms of HLA-DR1/Ligand Complexes. The MW_{app} determined by SDS/PAGE usually matches well to the actual molecular weight of a protein. In most cases, this applies even if the protein has not been denatured

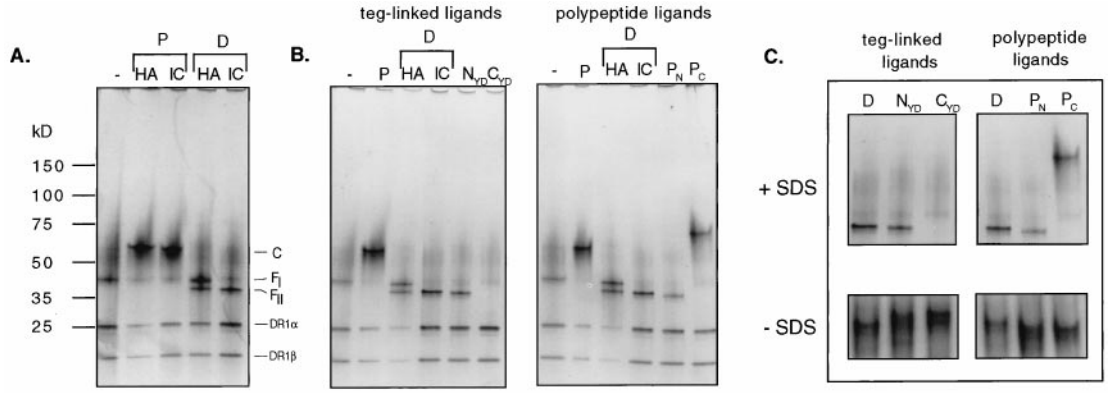

FIG. 3. Formation of HLA-DR1/ligand complexes with ligand constructs containing the IC106-120 epitope. (*A*) Comparison of HLA-DR1/ligand complexes formed with the HA306-318 and IC106-120 ligand constructs. The MHC molecules were loaded with the peptides HA306-318 and IC106-120 (P lanes) or with dimeric teg-linked tandem constructs HA-HA or IC-IC (D lanes). The position of bands representing the C-form or the F_I- or F_{II}-form is indicated. (*B*) SDS/PAGE separation of ligand complexes loaded with IC106-120 binding epitopes with defined N- and C-terminal overhangs. For the left gel, IC ligands were used, which, through the teg-spacer, were extended by the nonbinding Y308D-substituted HA306-318 epitope [N-terminal, YD-IC (lane N_{YD}); C-terminal, IC-YD (lane C_{YD})]. The right gel shows complexes formed with natural polypeptide ligands extended by the amino acid sequence of the invariant chain protein [N-terminal, IC97-120 (lane P_N); C-terminal, IC106-135 (lane P_C)]. (*C*) Comparison of the migration of extended IC106-120 ligands in the absence of SDS. Gel separations are shown that were carried out in the presence (*Upper*) or absence (*Lower*) of SDS.

before the separation. The MW_{app} of class II MHC/ligand complexes, however, in particular that of the C-form, differs significantly from the calculated value of ≈45 kDa [MW_{app} (C-form) ≈ 65 kDa; MW_{app} (F-form) ≈ 40 kDa]. To test whether the migration anomaly is caused by differences in the accessibility to SDS molecules, HLA-DR1/ligand complexes were separated by PAGE in the absence of SDS. Without SDS (Fig. 4*A Lower*), the relative migration was reversed as compared with separations in the presence of SDS (*Upper*). C-forms of HLA-DR1 molecules loaded with HA306-318 or IC106-120 peptide (Fig. 4*A Upper*, P lanes) now migrated faster than F-forms loaded with the extended dimeric teg-linked ligand constructs (D lanes). The same observation also was made with the other extended ligands (data not shown). The faster migration of the C-forms in the absence of SDS indicates smaller hydrodynamic radii, presumably caused, as the terminology suggests, by a more compact conformation of these ligand complexes. This matches the faster migration of F-forms observed in the presence of SDS, which suggests a looser conformation of the F-complexes providing a higher accessibility to the SDS molecules.

The differences in the migration during SDS/PAGE had previously been noticed for the class II MHC/ligand complexes formed with the short and the extended IC peptides (31, 32). Although the relevance of this observation had been disputed, it has been used as an indication for different conformations of the two IC ligand complexes (32). Another parameter potentially effected by the ligand overhangs is the pH stability of the complex (Fig. 4*B*). An earlier study indicated that natural polypeptide extensions at the N terminus of IC106-120 destabilized this ligand complex at relatively acidic pH (33). However, HLA-DR1/ligand complexes formed with the HA peptide or teg-linked dimeric constructs exhibited only relatively subtle difference with regard to their pH stability (Fig. 4*B*). Slightly more intense bands of the F-forms detected after the incubation at pH 6.0 suggested a slight increase in the pH-stability compared with the C-forms. The almost identical behavior of the IC102-120 and the IC97-120 also illustrates that, at least on HLA-DR1, a proposed "self-release" of the extended ligand (34) was barely noticeable (Fig. 6*B*).

SDS sensitivity induced by the ligand overhangs was most dramatic in the case of the SDS-unstable IC-YD ligand complex

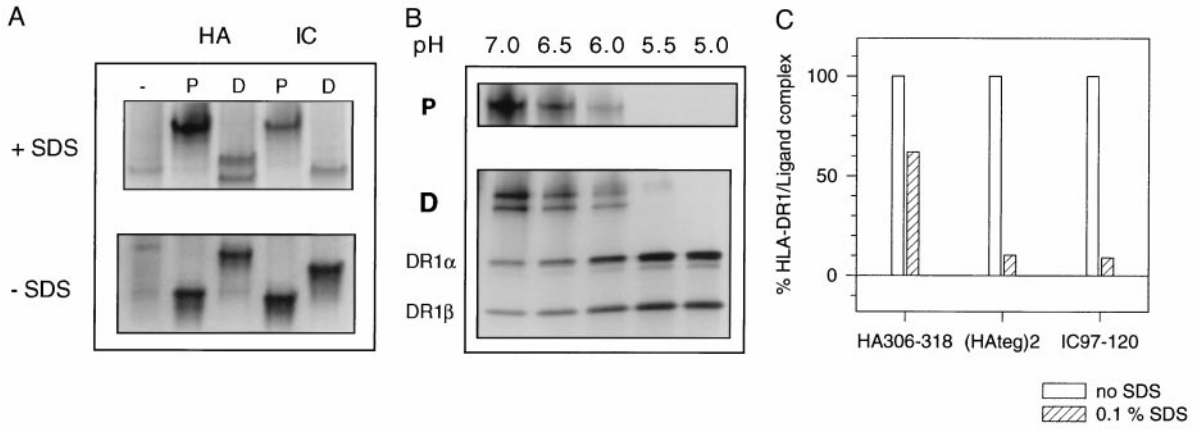

FIG. 4. Characterization of the F- and C-forms of HLA-DR1/ligand complexes. (*A*) Surface hydrophobicity. The migration behavior of HLA-DR1/ligand complexes was compared under native conditions and in the presence of SDS. Soluble HLA-DR1 molecules were loaded with the HA306-318 or the IC106-120 peptide (P lanes) or with dimeric constructs HA-HA or IC-IC (D lanes). Gels of the MHC/ligand complexes are shown that were separated in the presence (*Upper*) or in the absence (*Lower*) of SDS. (*B*) pH stability. HLA-DR1/ligand complexes were loaded with HA306-318 peptide (P lanes) or the dimer (D lanes) at a pH ranging from 5.0–7.0 (indicated above the lanes) and were analyzed by SDS/PAGE. (*C*) SDS stability. HLA-DR1 molecules, preloaded with the biotinylated ligands HA306-318, HA-HA, or IC97-120, were incubated for 3 h at 37°C in the absence (open bars) or presence (hatched bars) of 0.1% SDS. The amount of stable MHC/ligand complex was determined after the incubation by ELISA.

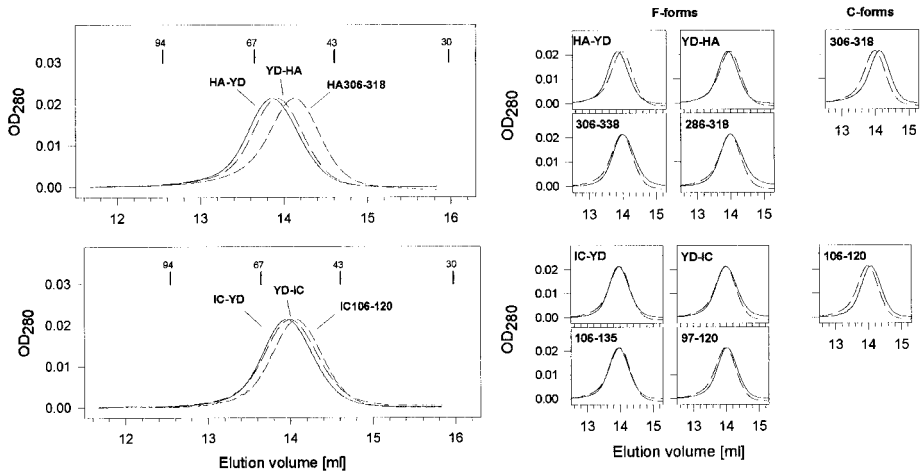

FIG. 5. Determination of the apparent size of F- and C-forms of HLA-DR1/ligand complexes by size exclusion chromatography. The elution profiles of HLA-DR1/ligand complexes formed with ligands containing the HA306-318 (*Upper*) and IC106-120 epitope (*Lower*) are shown. In the left panels, the elution of peptide-loaded C-forms is compared with the elution of the F-forms generated with the teg-linked YD hybrid dimers (indicated in the figure). As a reference, elution volumes of marker proteins and their molecular weights are indicated. The right panels show a comparison of the elution of the ligand-loaded forms (solid lines) to the elution of empty HLA-DR1 preparations (dashed lines).

(Fig. 3*C*, C_{YD} lanes). Although MHC/ligand complexes formed with the other extended ligands still could be detected after SDS/PAGE separations, an increased SDS sensitivity also was found for these complexes after a prolonged incubation at 37°C with 0.1% SDS. The amount of F-complexes recovered after the incubation was usually lower than the amount of recovered C-complexes (Fig. 4*C*).

The separation on native gels indicated differences in the hydrodynamic radii of the C- and the F-forms. This could be confirmed by size exclusion chromatography, which revealed a smaller apparent size of the compact forms (Fig. 5). Both the IC106-120-loaded and, in particular, the HA306-318-loaded HLA-DR1 molecule had a higher elution volume than the respective F-complex loaded with extended ligands (Fig. 5 *Left*). Of interest, the two C-forms also eluted behind preparations of empty HLA-DR1 molecule (Fig. 5 *Right*). The F-complexes, in contrast, eluted at almost the same elution volume as the empty molecules. A similarity between empty forms and F-complexes also was suggested by circular dichroism experiments (data not shown). Similar optical rotatory dispersion values were found at 200–210 nm whereas the optical rotatory dispersion values of C-forms were slightly elevated.

It is practically impossible to exclude that a fraction of the preparation of empty molecules is already loaded with ligands. In fact, SDS/PAGE separations of the empty HLA-DR1 preparations used for these experiments revealed a band similar to the F-complexes (e.g., Fig. 3*A*). Empty HLA-DR1 is generally considered to be SDS-unstable, but, recently, a conversion into a SDS-stable form could be achieved by a single-point mutation in the P1-pocket (31). Of interest, this mutant complex migrated very similarly to the complex found in our empty preparations. A resistance to protease digests aimed at the removal of ligand overhangs might actually suggest the absence of ligands in these nonmutated stable complexes (see Fig. 6*A*), but it is not clear yet whether it actually represents an empty form or a "contamination" with loaded MHC molecules.

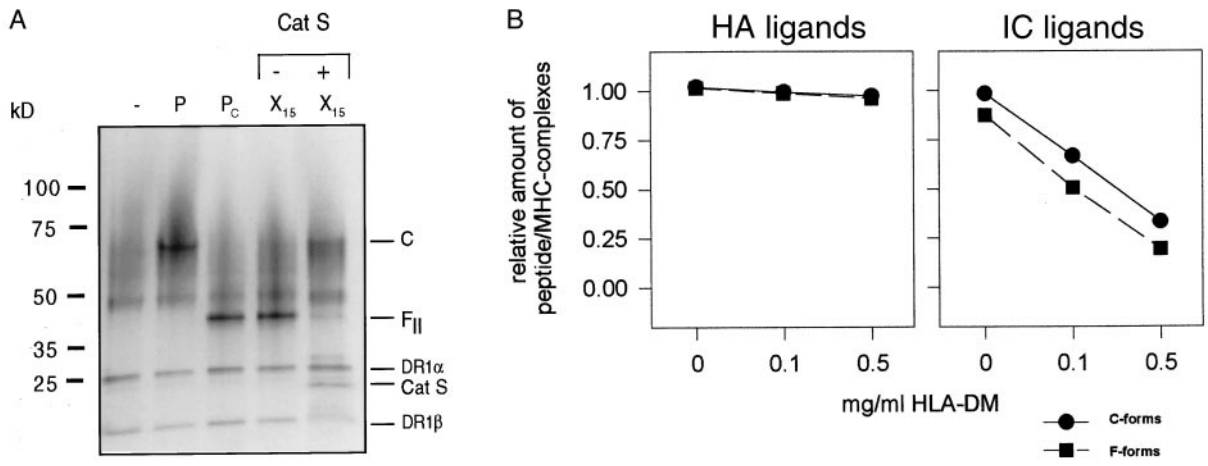

FIG. 6. Effect of cathepsin S and HLA-DM on C- and on F-forms of HLA-DR1/ligand complexes. HLA-DR1/ligand complexes were incubated with cathepsin S or soluble HLA-DM. (*A*) Conversion of an F-complex into the C-form by cathepsin S digest. F_{II}-complexes, preformed by loading the HA-X_{15} ligand onto the HLA-DR1 molecules, were subjected to cathepsin S digest to remove the random peptide overhang. The gel shows the SDS/PAGE analysis of HLA-DR1/HA-X15 ligand complexes (lanes X_{15}) before (−) and after (+) the treatment with cathepsin S. Also shown are the complexes formed with the HA306-318 peptide (lane P) and the HA306-338 peptide (P_C). (*B*) HLA-DM mediated release of ligands from C- or F-ligand complexes. F- (closed square) or C-HLA-DR1/ligand complexes (closed circle) were formed with biotinylated ligands containing the HA306-318 (*Left*) or the IC106-120 binding epitope (*Right*). HA306-318 and IC106-120 were used for the formation of C-complexes, and the F complexes were generated with the HA teg-dimer or the IC97-120 peptide. The ligand complexes were incubated with the indicated amounts of soluble HLA-DM, and the release of ligands was measured by ELISA by determining the amount of stable MHC ligand complexes left after the incubation.

Functional Studies with Components of the Processing Machinery. The conformational changes accompanying the conversion from F- to C-forms might play a role during antigen processing. The F-conformation could indicate the status of an "unfinished" MHC/peptide complex associated with a long precursor peptide and might provide docking sites for chaperones or other proteins involved in transport or processing of antigens. So far, at least three key components involved in processing have been described: cathepsins (30), HLA-DM (22), and, more recently, HLA-DO (35–37). Of these components, cathepsin S (38) and HLA-DM (22, 23) were available for functional studies using the different HLA-DR1/ligand complexes (Fig. 6).

Cathepsin S was identified as one of the key proteases active during antigen processing. This enzyme is known to be responsible for providing a few defined cuts necessary to release the invariant chain protein (38) but also may participate in the general cleavage of peptide ligands. *In vitro*, the enzyme was found to be effective in the degradation of a number of synthetic peptides (data not shown). Although not all of the extended peptide ligands were attacked, the protease was able to degrade the random 15-aa-long polypeptide overhang of the HA-X_{15} ligand. Preloaded on HLA-DR1 molecules, the enzyme effectively removed this overhang indicated by the conversion of the F_{II}-complex into the C-form (Fig. 6*A*). The conversion could be prevented by the addition of E-64 inhibitor [specific for Cys-proteases, such as cathepsin S (39)], and the same effect also was seen with a mixture of carboxypeptidases X and Y instead of cathepsin S (data not shown).

HLA-DM catalyzes the release of low-affinity peptide ligands from the MHC binding groove. One of its major function is the removal of IC106-120. Lack of expression of HLA-DM results in the accumulation of IC106-120-associated MHC molecules on the cell surface (40). A recent study suggested that HLA-DM molecules may interact through the contact with hydrophobic surface areas with the class II molecule (41). However, despite the increase in hydrophobicity observed for F-forms, no difference in the HLA-DM-mediated release of ligands from these complexes was observed. No release was detected from any of the complexes loaded with short or extended HA306-318 ligands, and also, no enhancement in the release of IC97-120 compared with the IC106-120 peptide was observed (Fig. 6*B*).

Thus, relatively short regions in polypeptide overhangs proximal to the binding site of peptide ligands effectively prevented the formation of compact class II MHC/ligand complexes. They arrested the MHC molecule in a conformation, which, in some of its physical properties, resembled an empty MHC molecule. The sequence of the effector regions in the ligand overhang could be relatively random. They apparently interacted with one or more regions on the class II MHC molecule sensitive to contact with the overhang. This region(s) is located close to the peptide-binding groove and was reached by relatively short extensions from both ends of the binding groove. Although the induction of F-conformations is likely to be an allosteric effect, at least in principle, the receptor site(s) also could be located inside the binding groove. In any case, the identification of the exact location of this region(s) is of particular interest. It represents an area(s) that not only can alter the conformation of the MHC molecule but also can potentially destabilize the peptide/MHC interaction, which, in turn, might trigger the release of low-affinity peptide ligands.

It remains to be seen what role these ligand-dependent conformations play during antigen processing. Although no effect on the HLA-DM mediated exchange was detected, it was shown that cathepsins, at least, can convert the F-form into the C-form. The differences in the conformation of the ligand complexes might, therefore, be important for the interaction with other components of the class II MHC antigen processing pathway.

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