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Rheumatoid Arthritis (RA)-Associated HLA-DR Alleles Form Less Stable Complexes with Class II-Associated Invariant Chain Peptide Than Non-RA-Associated HLA-DR Alleles[1]

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Certain HLA-DR alleles confer strong susceptibility to the autoimmune disease rheumatoid arthritis (RA). We compared RA-associated alleles, HLA-DR*0401, HLA-DR*0404, and HLA-DR*0405, with closely related, non-RA-associated alleles, HLA-DR*0402 and HLA-DR*0403, to determine whether they differ in their interactions with the class II chaperone, invariant chain (Ii). Ii binds to class II molecules in the endoplasmic reticulum, inhibits binding of other ligands, and directs class II-Ii complexes to endosomes, where Ii is degraded to class II-associated Ii peptide (CLIP). To evaluate the interaction of Ii and CLIP with these DR4 alleles, we introduced HLA-DR*0401, *0402, and *0404 alleles into a human B cell line that lacked endogenous HLA-DR or HLA-DM molecules. In a similar experiment, we introduced HLA-DR*0403 and *0405 into an HLA-DM-expressing B cell line, 8.1.6, and its DM-negative derivative, 9.5.3. Surface abundance of DR4-CLIP peptide complexes and their susceptibility to SDS-induced denaturation suggested that the different DR4-CLIP complexes had different stabilities. Pulse-chase experiments showed CLIP dissociated more rapidly from RA-associated DR molecules in B cell lines. In vitro assays using soluble rDR4 molecules showed that DR-CLIP complexes of DR*0401 and DR*0404 were less stable than complexes of DR*0402. Using CLIP peptide variants, we mapped the reduced CLIP interaction of RA-associated alleles to the shared epitope region. The reduced interaction of RA-associated HLA-DR4 molecules with CLIP may contribute to the pathophysiology of autoimmunity in RA. ***The Journal of Immunology,*** **2001, 167: 7157–7168.**

Rheumatoid arthritis (RA)[3] is a chronic inflammatory disease of the joints thought to be the result of a $CD4^+$ T cell-driven immune response to self peptides. A key element in current models of RA pathogenesis is the observation that certain alleles of the class II MHC complex confer susceptibility to the disease (1). The MHC class II genes themselves, rather than linked genes, appear to be the inherited risk factors, based on disease association studies in humans (reviewed in Ref. 2) as well as studies of animal models of inflammatory arthritis (3–5). The specific role of class II molecules in disease pathogenesis is unknown. However, it is generally believed that MHC class II molecules confer susceptibility to RA through their involvement in critical Ag presentation events, either during thymic selection or in the periphery.

The RA-associated alleles (DRB1*0401, *0404, *0405, *0408, *1402, *0101, *0102, and *1001) share a short sequence motif, the shared epitope (SE), at residues 67–74 in the third hypervariable region of the DRB chain (6, 7). Notably, this motif is not found in closely related nonassociated alleles, implying that this region of the DR molecule is important in disease pathogenesis. This SE region influences peptide binding and T cell recognition of DR4 molecules. In the crystal structure of DR*0401 with a bound collagen peptide (CII 1168–1180) and superantigen staphylococcal enterotoxin B, the side chains of SE residues DRβ 67, 71, and 74 interact with the peptide at specificity pockets P7, P6, and P4, and the side chain of DRβ 70 is positioned to interact with the TCR (8). Peptide-binding and elution studies show that different peptides are selected by the SE^+ and SE^- alleles, with an important influence being the different charge preference of the P4 pocket of SE^+ as compared with SE^- alleles (9, 10).

In addition to allelic binding specificity, the ligands bound to class II molecules are also regulated by two accessory molecules in the class II pathway, invariant chain (Ii) and HLA-DM (reviewed in Ref. 11). Ii trimers bind to nascent MHC class II α- and β-chains in the rough endoplasmic reticulum (ER) and form nonameric $(\alpha\beta)_3Ii_3$ complexes. Ii blocks the peptide-binding groove of class II molecules, preventing the binding of ligands available in the ER (12). Ii also facilitates proper folding of class II molecules and egress from the ER, although different class II alleles vary in their dependence on Ii for transport (13–15). Class II/Ii complexes are transported via the Golgi apparatus to endosomes, where Ii is degraded to a nested set of class II-associated Ii peptides (CLIP). Some DR alleles release CLIP spontaneously, while others are dependent on an endosomally localized heterodimer, HLA-DM, to catalyze CLIP release and enable other endosomal peptides to bind to the class II molecules (16–20).

We hypothesized that three DR4 alleles, RA-associated DR*0401, DR*0404, and DR*0405, might differ from RA-nonassociated DR*0402 and DR*0403 in their interaction with Ii and its

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[3] Abbreviations used in this paper: RA, rheumatoid arthritis; B-LCL, B lymphocyte cell line; Ii, invariant chain; CLIP, class II-associated Ii peptide; ER, endoplasmic reticulum; SE, shared epitope; MF, median fluorescence.

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derivative CLIP peptides. To assess the DR-Ii interaction, we generated B lymphocyte cell line (B-LCL) cells expressing each DR4 allele and Ii. We selected B-LCL cells lacking HLA-DM so we could evaluate DR-CLIP complexes in absence of HLA-DM-mediated CLIP release. We also generated recombinant soluble DR*0401, DR*0402, and DR*0404 molecules to study the kinetics of CLIP dissociation in vitro. Our results show the RA-associated alleles DR*0401, DR*0404, and DR*0405 form relatively unstable DR-CLIP complexes, whereas the closely related RA-nonassociated alleles DR*0402 and DR*0403 form long-lived complexes with CLIP.

Materials and Methods

Cell lines

The 5.2.4 is a B-LCL that has a large homozygous deletion spanning the MHC class II region (21). Thus, 5.2.4 completely lacks expression of DR and DMB, but hemizygously expresses HLA-DP4, DOA, and DMA. The 8.1.6 is a B-LCL expressing DR*0301, DQ2, DP4, and HLA-DM. The 9.5.3 is a DM-null cell line derived from 8.1.6. Thus, 9.5.3 DR*0402-, *0403-, and *0405-transfected cells lack DM (21, 22); 8.1.6 DR*0403- and *0405-transfected cells express DM. Stable transfectants T2-DR*0401 (T2.DR4 Dw4) and T2-DR*0404 (T2.DR4 Dw14), gifts from W. Kwok (Virginia Mason Research Center, Seattle, WA), also lack HLA-DM (23, 24). B-LCL cells were maintained in RPMI 1640 with 15% BCS and 2 mM L-glutamine. Phoenix A cells, a gift from G. Nolan (Stanford University, Stanford, CA), were grown in DMEM containing high glucose and pyridoxine with 10% heat-inactivated FCS, 2 mM L-glutamine, and 100 U of penicillin G and streptomycin sulfate. Schneider-2 (S2) *Drosophila melanogaster* cells were cultured in Schneider's *Drosophila* medium containing 10% v/v FBS, 2 mM L-glutamine, and 50 μg/ml gentamicin. All media and supplements were purchased from Invitrogen Life Technologies (Carlsbad, CA).

cDNA constructs encoding full-length DR molecules

cDNA coding for full-length DRB1*0401 and DRB1*0402 was isolated from existing pSVneo plasmids encoding DRB1*0401 or DRB1*0402 cDNAs (25) and subcloned into the *Eco*RI site of the retroviral vector pBMN (a gift from G. Nolan). A cDNA coding for DRB1*0404 was PCR amplified from pACUW51-0404 (a gift from W. Kwok) using the forward primer 5′-CT GCTCGGATCCCTGGTCCTGTCCTGTTCTCC-3′ and the reverse primer 5′-CCTGTGGAATTCGCAAAGCTGGGGCAGAAGGTT-3′. The PCR product was cloned into the *Eco*RI and *Bam*HI sites of the retroviral vector pBMN. All the constructs were verified by dye-terminator sequencing performed by the Stanford Protein and Nucleic Acid Facility (Stanford, CA). The construction of a full-length DRA gene in retroviral vector pBMN-IRES-neo has been described previously (26). pSV-neo plasmids encoding either the DRA^+DRB*0403 or the DRA^+DRB*0405 cDNAs were a kind gift of G. Sonderstrup (Stanford University).

cDNA constructs encoding soluble DR molecules

cDNA coding for the ectodomains of DRB1*0402 was isolated from the DR-homozygous EBV-transformed B cell line, AL10 (gift from G. Sonderstrup), by RT-PCR (Invitrogen Life Technologies; forward primer, 5′-CT-GCTCGAATTCCCTGGTCCTGTCCTGTTCTC-3′; reverse primer, 5′-AAGAGCAGGTCGACCTAGGTCTCTGGTTCAGGAGGTGGAGTCT TGCTCTGTGCAGATTC-3′; the reverse primer attaches an epitope tag recognized by the mAb, KT3). A cDNA coding for the DRB1*0404 ectodomains was PCR amplified from pACUW51-0404 (a gift from W. Kwok) using the forward primer 5′-TGCTCCTGAATTCCCTGGTCCT GTCCTGTTCTC-3′ and the same reverse primer as for the DRB1*0402 construct. PCR products were cloned into the *Eco*RI and *Sal*I sites of pRmHA-3 (27), and constructs were verified by dye-terminator sequencing performed by the Stanford Protein and Nucleic Acid Facility.

*Generation of 5.2.4, 9.5.3, and 8.1.6 DR*04 transfectants*

Retroviral plasmid pBMN-IRES-Neo-DRA was transfected into the Phoenix A cells using a $CaPO_4$ transfection system from Invitrogen Life Technologies. The supernatant from Phoenix cells was harvested 24 h post-transfection and centrifuged at $500 \times g$ for 5 min to remove contaminating Phoenix cells, as described (28). About 5×10^6 B-LCL 5.2.4 cells were infected with a retroviral supernatant with 4 μg/ml polybrene at 32°C for 24 h. Infected B-LCL cells were transferred into fresh media after 24 h and subsequently placed under selection in 1 mg/ml G418 (Invitrogen Life Technologies). The 5.2.4-DRα cells were transduced with retroviral plasmids encoding either DRB1*0401, DRB1*0402, or DRB1*0404 genes in three separate transductions. About 5% of infected cells were positively transduced and expressed DR4 on the cell surface. DR4-expressing 5.2.4 cells were enriched by magnetic sorting using a DR-specific mAb, L243, followed by a secondary IgG-specific Ab conjugated to Dynal beads as per manufacturer's instructions (Dynal Biotech, Oslo, Norway). Magnetic sorting resulted in a population that was 60–70% DR positive. The magnetically sorted cells were stained with L243 and FACS sorted on a FACStar flow cytometer (BD Biosciences, Lincoln Park, NJ), resulting in an enriched population with 99–100% of cells expressing DR. In a separate experiment, 9.5.3 and 8.1.6 cells were electroporated with plasmids encoding either *DRA^+DRB*0403* or *DRA^+DRB*0405* cDNAs (a gift from G. Sonderstrup); transfectants were selected using 1 mg/ml G418 (Invitrogen Life Technologies).

Flow cytometry

DR4-transduced 5.2.4, 8.1.6, and 9.5.3 cells were analyzed for surface DR4 expression by flow cytometry using a panel of Abs. These Abs included a DR dimer-specific mAb L243 (29) that binds DRα (30), another DR-specific Ab ISCR3 (31), a DRB1*04-specific mAb NFLD.D1 (32), a SE-specific mAb, NFLD.D2 (33), and a CLIP-specific mAb CerCLIP.1 (34). NFLD.D1 and NFLD.D2 Abs were gifts from S. Drover. Monoclonal Abs L243 and CerCLIP were used at saturating concentrations; NFLD.D1 was used as an undiluted hybridoma supernatant. Fluorescein-labeled goat anti-mouse IgG Ab, purchased from Life Technologies, was used as secondary Ab at 1/25 dilution. Ab binding was analyzed on a BD Biosciences FACStar using the CellQuest software (BD Biosciences, San Jose, CA).

cDNA sequencing

To confirm the presence of the different alleles in DR4-transduced 5.2.4 cells, whole cell RNA was isolated using the RNeasy minikit from Qiagen (Valencia, CA). cDNA was amplified with Superscript RT-*Taq* mix (Invitrogen Life Technologies) and sequenced with ABI Prism Dye terminator cycle sequencing ready-reaction mix from PerkinElmer (Cypress, CA) performed at the Stanford Protein and Nucleic Acid Facility.

Analysis of SDS stability of DR/peptide complexes

SDS stability assays were performed essentially as described (35). Lysates of DR4 expressing 5.2.4 or 9.5.3 cells were either heated at 95°C or not, and electrophoresed on 12% acrylamide SDS-PAGE gels. Separated proteins were transferred to polyvinylidene diflouride membranes (Immobilon P; Millipore, Bedford, MA). Membranes were immunoblotted with either a DRA cytoplasmic tail-specific mAb DA6.147 (36), or a polyclonal anti-DR rabbit antisera CHAMP (37), or CLIP-specific mAb CerCLIP.1 (34), or mAb 14.23 recognizing a combinatorial epitope on the DR-CLIP complex (E. Mellins, unpublished observation). After extensive washing, membranes were incubated with HRP-conjugated goat anti-mouse IgG Ab (Invitrogen Life Technologies) followed by ECL substrate (PerkinElmer, Wellesley, MA), then exposed to film (Hyperfilm, ECL; Amersham, Arlington Heights, IL).

Cells for protein isolation

S2 cells expressing soluble recombinant HLA-DM (DMA*0101/DMB*0101) and HLA-DR*0401 (DRA*0101/DRB1*0401) have been described previously (20). S2 cells expressing soluble DR*0402 and DR*0404 were generated by cotransfecting S2 cells with pRmHA-3 vectors containing soluble DRA*0101 and soluble DRB1*0402 or DRB1*0404, and pUChs-Neo, using a calcium phosphate transfection kit (Invitrogen Life Technologies). Cells were selected in 1.5 mg/ml active G418 (Invitrogen Life Technologies) and induced for 7 days with 1 mM CuSO4, and expression was verified by Western blotting of tissue culture supernatants using an anti-DR antiserum (CHAMP; gift from L. Stern, Massachusetts Institute of Technology, Cambridge, MA) and the epitope tag-specific mAb, KT3, which recognizes the DRβ chain.

Purification of recombinant HLA-DR and DM molecules

Soluble recombinant DM was purified by FLAG epitope tag affinity chromatography, followed by Sephacryl S200-HR (Amersham Pharmacia Biotech, Piscataway, NJ) size exclusion chromatography, as described (38). The protocol for immunoaffinity purification of recombinant DR molecules was similar to that described by Gorga et al. (39). Briefly, the anti-DR mAb, L243, which recognizes assembled $\alpha\beta$ dimers, was coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) using the manufacturer's protocol. Cleared and filtered supernatants were applied to the columns and allowed to recycle. After washing in PBS, protein was

eluted with 0.1 M Tris-HCl, pH 11, and neutralized immediately with 0.2 vol 2 M Tris-HCl, pH 6.8. Protein-containing fractions determined by absorbance at 280 nm were pooled and concentrated by centrifugal ultrafiltration (Centricon-30; Amicon, Beverly, MA). Eluates were analyzed for purity by SDS-PAGE, Coomassie blue staining, and silver staining according to standard protocols; DRα and DRβ chain bands (as identified by Western blotting, as described above) comprised ≥85% of total protein. Heterodimeric assembly was assessed by native PAGE, as described (35). Soluble DR protein was quantified using the Bradford assay (Bio-Rad, Richmond, CA).

Synthesis of CLIP peptide

Human Ii fragment 81–104 CLIP (LPKPPKPVSKMRMATPLLM QALPM) unlabeled peptide, and murine Ii 85–99 M90V M98F CLIP peptide variant (KPVSQVRMATPLLFR) were synthesized by standard FMOC chemistry and purified by HPLC, and identity and purity were confirmed by mass spectrometry at Stanford Protein and Nucleic Acid Facility. Murine Ii 85–99 M90V M98F CLIP was labeled at the N termini with carboxyfluorescein, as described (40). N-terminal carboxyfluorescein-labeled human Ii 81–104 CLIP and its variants L97A CLIP (LPKPPK PVSKMRMATPALMQALPM) and T95A L97A CLIP (LPKPPKPVSK MRMAAPALMQALPM) peptides were synthesized by Research Genetics (Huntsville, AL). The underlined sequence highlights the core binding motif of the peptides.

In vitro dissociation kinetic assays

A total of 100 μM fluoresceinated f-Ii 81–104 human CLIP peptide (or its variant) was incubated with 1.7 μM soluble recombinant HLA-DR4 (DR*0401 or DR*0402 or DR*0404) in PBS (150 mM sodium chloride, 10 mM sodium phosphate, and 0.02% sodium azide) at pH 7 or in PBS acidified to pH 5.3, with 100 mM sodium citrate. The f-peptide/HLA-DR4 complex was purified as described (40). Soluble recombinant HLA-DM, 0.4 μM, was added immediately before measuring the $t = 0$ time point, where appropriate. Unlabeled competitor human Ii 81–104 CLIP peptide (10 μM) was added to all experiments. The amount of f-peptide/DR4 complex was measured using a high performance size-exclusion chromatography column connected to a Gilson fluorescence detector (Gilson, Middleton, WI) set to deliver excitation at 495 nm and to measure emission at 525 nm, as described before (40). The height of the initial peak representing the f-peptide/DR4 complex was designated F_0, and peaks measured from subsequent time points were displayed graphically as proportions of F_0 ($F{:}F_0$ ratio). The kinetics of f-peptide/DR4 dissociation was determined using single exponential curve-fitting functions in Synergy Kaleidagraph, using the following equation:

$$[p/M]/[p/M]0 = \exp[-(\ln 2/t_{1/2}) \times t]$$

where (p/M)t is concentration of f-peptide/sDR complex (M) at time t, (p/M)0 is concentration of f-peptide/sDR complex (M) at time 0, and $t_{1/2}$ is dissociation half-life (hours). For biphasic dissociation kinetics, another equation was used as described before (41). Each experiment was done at least twice, unless noted otherwise. The calculated $t_{1/2}$ was based on a nonlinear regression fit of the combined data points (typically 20–30 points) to a single or double exponential function; the limits in the confidence interval of a $t_{1/2}$ at the 95% confidence level are given by best-fit $t_{1/2} \pm 2\times$ SE (see Table II). The low SE of these $t_{1/2}$ values shows the low inherent variability of these experiments.

Pulse chase of HLA-DR molecules

Analysis of HLA-DR biosynthesis was conducted using pulse-chase immunoprecipitation, essentially as described (42). Briefly, for metabolic radiolabeling, cells were starved of Met/Cys for 25 min at 37°C and pulsed with [^{35}S]Met/Cys labeling mix (PerkinElmer) in Met/Cys-free media for specified times at 37°C and chased in the presence of 1 mM cold Met/Cys for various periods of time. Cells were lysed in buffer containing 1% Nonidet P-40; DR molecules were immunoprecipitated from precleared extracts with an anti-DR Ab L243 and protein A-Sepharose. Samples were resuspended in 2× Laemmli buffer, boiled in 0.6% SDS, 2.5% 2-ME for 10 min, and separated by 12% SDS-PAGE gels. Where noted, immunoprecipitated material was normalized for radioactivity at the time of loading on gels. Gels were fixed in 50% methanol and 10% acetic acid for 20 min, and then treated with 4% 2,5-diphenyloxazole in acetic acid (PerkinElmer) before drying and exposing to film (Hyperfilm Amersham).

Densitometry

Densitometry was performed using a Bio-Rad GS-710 densitometer. For densitometry on Fig. 6*A*, bands corresponding to DRα and Ii were grouped together for ease of assessment; the ratios presented in the results were derived from the chase time points of 5 and 15 min, when the DR bands for all three alleles were clearly visible. For densitometry on Fig. 7*A*, to quantitate the stability differences, day 3 was the most optimal time, since at this time point the CLIP bands were quantifiable by densitometry for all the three alleles, but not so beyond this.

Results

*DR*0401- and DR*0404-transfected 5.2.4 cells express fewer cell surface CLIP-DR complexes than DR*0402-transfected 5.2.4 cells*

To compare the biochemical characteristics of three closely related DR4 alleles (DR*0401, DR*0402, and DR*0404) in an identical cellular environment, we generated B-LCL 5.2.4 cells expressing the different DR4 alleles. The 5.2.4 cells have a homozygous MHC class II deletion that encompasses *DMB, DQA* and *B*, and *DRA* and *B* genes. Thus, 5.2.4 cells express DMA, but no functional DM, and express DP4 as the only endogenous class II molecules. We selected two RA-associated, SE-containing DR alleles, DR*0401 and DR*0404, and one SE-negative allele DR*0402 for initial study (Table I). These SE$^+$ alleles are strongly associated with RA, with up to 60–70% of Caucasian RA patients expressing these DR4 subtypes, as compared with 20% of controls (43). The 5.2.4 cells were transduced with DR*04-encoding retroviral vectors, and the transfectants expressed DR4 and Ii in the absence of DM and any other DR alleles. We confirmed the presence of appropriate DR4 alleles in 5.2.4 cells by RNA isolation and cDNA sequencing. Surface expression of the different DR molecules was measured by flow cytometry with monomorphic anti-DR Abs L243 and ISCR3

Table I. *Sequence comparison of DR4 alleles*[a]

			HLA-DR β-Chain Residue No.				
			Shared epitope region				
			Pocket 7	Pocket 4			Pocket 1
DRB1 Allele	Presence of SE	Susceptibility to RA	67	70	71	74	86
DR*0401	+	++++	L	Q	K	A	G
DR*0402	−	−	I	D	E	A	V
DR*0403	−	−	L	Q	R	E	V
DR*0404	+	+++	L	Q	R	A	V
DR*0405	+	+++	L	Q	R	A	G

[a] Pockets are assigned based on the crystal structure of the DR*0401-collagen peptide complex (8) and modeling using the Look program.

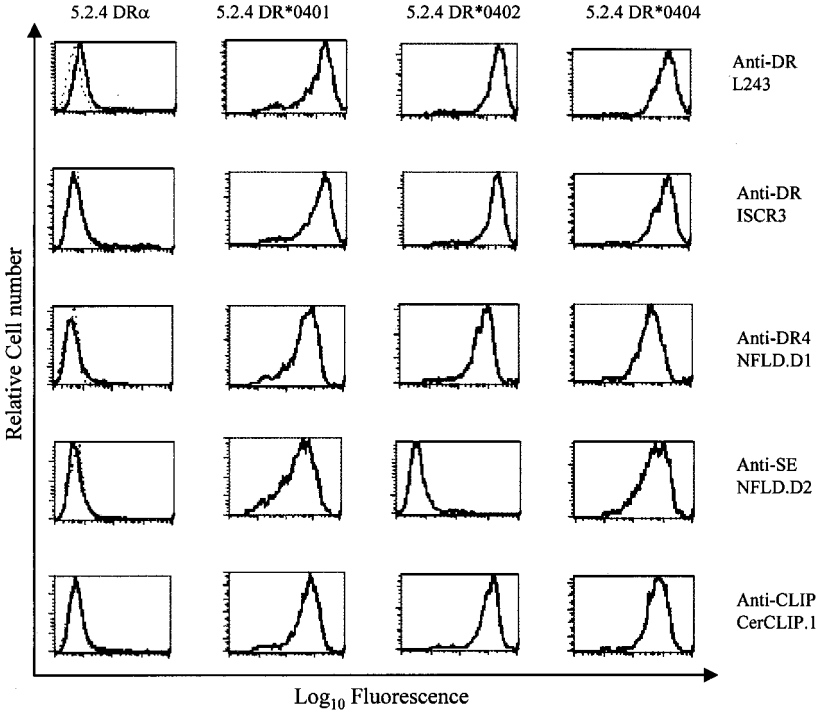

FIGURE 1. Transduced cells express the appropriate DR4 alleles on their surface. Bold lines represent binding of L243 (anti-DR), ISCR3 (anti-DR), NFLD. D1 (anti-DR4), NFLD.D2 (anti-SE), and CerCLIP.1 (anti-CLIP) Abs to DR4-expressing 5.2.4 cells. The first column, 5.2.4 cells transduced with the DRA gene alone, provides a negative control; the thin line in the first column shows the binding of secondary Ab alone. The staining of the other transfectants with the secondary Ab alone is not shown but was comparable with the staining of 5.2.4-DRA.

(Fig. 1). The uncloned, transduced 5.2.4 DR4 cells expressed similar levels of surface DR molecules. As expected, all DR4 transfectants stained positive with an anti-DR4 Ab (NFLD.D1), and the DR*0401 and DR*0404 transfectants stained positive with an Ab that recognized the SE (NFLD.D2) (Fig. 1).

To evaluate whether the three alleles interacted differently with Ii-derived CLIP peptide, we examined the surface CLIP:DR ratios for each of the DR4-expressing 5.2.4 cells. We used two different anti-CLIP Abs, CerCLIP.1 and 14.23, which recognize different determinants on the DR-CLIP complexes: CerCLIP.1 recognizes the N-terminal overhang of the CLIP peptide bound to any class II molecule (34), and 14.23 recognizes a combinatorial determinant expressed on DR-CLIP complexes (E. Mellins, unpublished data). As expected for DM-null cells, the majority of the DR molecules were associated with CLIP for all three alleles. However, we found notable differences between the alleles in the proportion of surface DR molecules associated with CLIP peptides, as reflected in the differences in the ratios of median fluorescence (MF; derived by CellQuest software from the raw FACS data; MF CerCLIP.1:MF anti-DR Ab and with MF 14.23:MF anti-DR Ab). CLIP:DR ratios were lower for DR*0401- and DR*0404-expressing cells compared with DR*0402-expressing cells. These ratios are represented as percentages, with the CLIP:DR ratio for DR*0402 designated as 100% because it was the highest ratio (Fig. 2*A*). The differences in CLIP:DR ratios were statistically significant using the unpaired *t* test with a 95% confidence interval (for CerCLIP.1, $p = 0.0011$ for DR*0401 compared with DR*0402, and $p = 0.0081$ for DR*0404 compared with DR*0402; for 14.23, $p = 0.0054$ for DR*0401 compared with DR*0402 and $p = 0.0172$ for DR*0404 compared with DR*0402). These differences were not due to different affinities of the anti-CLIP Abs for the different alleles, because we obtained similar titration curves for Ab binding to the different DR4-expressing cells (Fig. 2*B*). The reduced abundance of DR-CLIP complexes in DM-null 5.2.4 cells expressing the SE^+ alleles suggested two possible scenarios, either reduced generation of the SE^+ DR-CLIP complexes or reduced stability of these complexes.

*CLIP forms SDS-stable dimers with DR*0402 and not DR*0401 or DR*0404 molecules*

To evaluate the possibility that SE^+ DR-CLIP complexes and SE^- DR-CLIP complexes differed in stability, we evaluated the susceptibility of the complexes to SDS-induced dissociation. Most mature MHC class II-peptide complexes resist dissociation into constituent α- and β-chains in the presence of SDS detergent (44), whereas most DR-CLIP complexes dissociate under these conditions (21). The SDS-stable phenotype generally correlates with strong peptide binding for most alleles (45). To assess SDS stability of the different DR-CLIP complexes, unboiled cell lysates of 5.2.4 cells expressing either DR*0401, DR*0402, or DR*0404 were separated by SDS-PAGE and immunoblotted with the CerCLIP Ab. SDS-stable DR-CLIP complexes were detected in 5.2.4 DR*0402 cells, but rarely in 5.2.4 DR*0404-expressing cells and not at all in 5.2.4 DR*0401 cell lysates (Fig. 3*A*). Immunoblotting with a DR-specific polyclonal antisera revealed equivalent amounts of DR in each cell line and some DR*0401 and DR*0404 SDS-stable dimers (Fig. 3*B*). Thus, although DR*0401 and DR*0404 form a few SDS-stable complexes with other peptides in the absence of HLA-DM, they do not appear to form SDS-stable DR-CLIP complexes. The SDS-stable DR*0402-CLIP complexes migrated with reduced mobility reminiscent of other MHC class II-CLIP complexes that form floppy dimers (46, 47).

To confirm that SDS stability of CLIP complexes was a property of the DR4 allele and independent of other cell line-specific factors, we did a similar experiment using available DM-null cells expressing the different DR4 alleles (DR*0401 T2 cells, DR*0402 9.5.3 cells, and DR*0404 T2 cells). Abundant SDS-stable DR-CLIP complexes were detected in the DR*0402 lysate, and substantially fewer stable complexes were found in the DR*0404 and none in the DR*0401 cell lysates (Fig. 3*C*). SDS-stable DR-CLIP

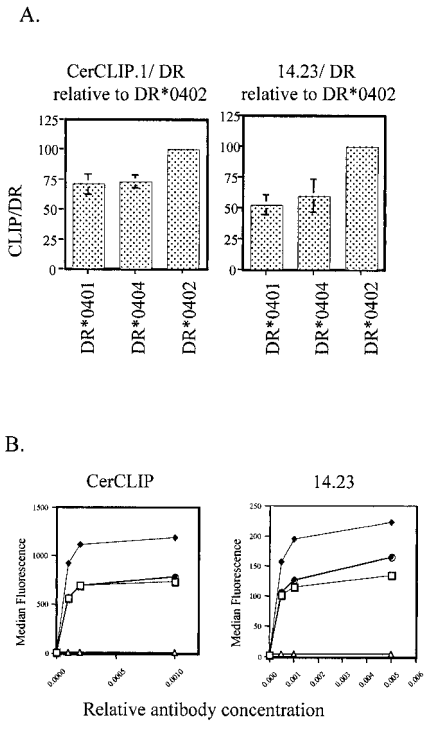

FIGURE 2. Cells expressing DR*0402 and DR*0403 have higher CLIP:DR ratios. *A,* The ratios of median fluorescence of either anti-CLIP Ab CerCLIP.1 or anti-CLIP Ab 14.23 to median fluorescence of anti-DR Abs L243 or ISCR3 are expressed in terms of percentages, with the CLIP:DR ratios of DR*0402 designated as 100%. The mean percentages are derived from three independent experiments. *B,* Cells were incubated with varying concentrations of anti-CLIP mAbs CerCLIP.1 or 14.23. The diamonds represent staining with DR*0402, circles represent DR*0404, and squares represent DR*0401-expressing 5.2.4 cells. The relative mAb concentration is given on the *x*-axis and the median fluorescence intensities are given on the *y*-axis.

dimers were detected in DR*0404-expressing T2 cells in rare experiments. In a parallel assessment of boiled cell lysates from each of the cell lines, comparable amounts of DR molecules were detected with an anti-DRα mAb, DA6.147 (Fig. 3*C*). These results showed that the SDS stability of the DR*0402-CLIP complex is allele specific and cell line independent. The finding that DR*0402 molecules formed abundant SDS-stable DR-CLIP complexes, whereas DR*0401 and DR*0404 molecules did not, implicated the SE region in the differences in DR-CLIP interaction.

*CLIP forms SDS-stable dimers with DR*0403 and not DR*0405 molecules*

To test this implication in another set of SE^+ and SE^- alleles, we measured SDS stability of RA-associated, SE^+ DR*0405 and RA-nonassociated, SE^- DR*0403 in DM-null 9.5.3 cells. We observed abundant SDS-stable DR dimers in lysates from cells transfected with DR*0403, but not in lysates from either untransfected or DR*0405-transfected cells, immunoblotted with an anti-DR Ab (Fig. 3*D*). The DR*0403 and DR*0405 alleles were evaluated only in the 9.5.3 cells. Immunoblotting with mAb 14.23, which detects DR-CLIP complexes, revealed SDS-stable DR/CLIP complexes at the same apparent molecular mass as the DR dimers in lysates of 9.5.3 cells transfected with DR*0403, but not in cells transfected with DR*0405 or untransfected cells (Fig. 3*D*). Boiled cell lysates showed comparable amounts of DRα in both DR*0403 and DR*0405 transfectants. These results further supported the conclusion that RA-associated, SE^+ DR4 alleles differ from non-RA-associated, SE^- alleles in DR-CLIP interaction, with the latter forming more SDS-stable complexes.

In vitro CLIP dissociation kinetics using soluble DR4 molecules

The flow cytometry and SDS stability experiments suggested that the DR-CLIP complexes of the three RA-associated alleles had different stabilities than the two RA-nonassociated alleles. To directly measure CLIP peptide dissociation in representative alleles, we generated soluble DR4 molecules in insect cells and isolated them by affinity purification. Soluble DR molecules were loaded with N-terminally fluoresceinated synthetic CLIP peptide (Ii 81–104), and dissociation half-times were measured at the pH of the endosomal compartments, pH 5.3, and at the pH at the cell surface, pH 7. Dissociation kinetics measured in this way have previously been shown to correlate well with peptide/MHC complex stability on live cells (22–24).

DR*0401 molecules formed a short-lived DR-CLIP complex with a $t_{1/2}$ of 2.65 h at pH 5.3 (Fig. 4*A*). DR*0402-CLIP complexes were about 10-fold more stable than the DR*0401-CLIP complex at endosomal pH 5.3. The DR*0404-CLIP complex had intermediate stability with a $t_{1/2}$ of 11.7 h. At pH 7, the DR-CLIP complexes were longer lived, but the same hierarchy of kinetic stability among the three alleles was observed (Fig. 4*B*). Thus, DR*0401 and DR*0404 form less stable DR-CLIP complexes than DR*0402 at both pH 5.3 and pH 7 (summarized in Table II).

*Mapping reduced CLIP interaction of DR*0404 using CLIP peptide variants*

Using molecular modeling, we searched for clues at the molecular level that could explain the differences in stability between CLIP-DR*0402 and CLIP-DR*0404 complexes. We hypothesized that the enhanced CLIP dissociation from DR*0404 compared with DR*0402 derived from differences in the SE region because both DR*0402 and DR*0404 are identical at all other residues. The complexes between CLIP and the two DR*04 alleles were predicted based on the crystal structure of a collagen II peptide complexed to DR*0401 (8). The Look software (Molecular Applications Group, Palo Alto, CA) was used to calculate the energy minimum of the side chain coordinates of the complexes based on the backbone coordinates of the reference crystal structure (homology modeling option) (48).

We first focused on the P4 pocket interactions between CLIP and each of the two alleles, because the P4 pocket of DR*04 alleles has been shown to be important in shaping the peptide-binding repertoire (9, 49). Based on the predicted structures, CLIP 94 Ala is placed at the P4 pocket of the alleles. CLIP 94 Ala interacts minimally with the P4 pocket of DR*0402 and DR*0404 because of its small size and nonpolar chemical structure. Therefore, it does not seem likely that the experimentally observed differences in stability between the CLIP-DR*0402 and CLIP-DR*0404 complexes are directly due to differences in the interactions of CLIP 94 Ala and the MHC molecule. However, this does not rule out the possibility that the differences in the P4 pocket structure between DR*0402 and DR*0404 (as well as other DR*04 alleles) may influence global conformation and indirectly affect the CLIP-DR*04 stability in an allele-dependent manner. Previous studies with peptide/MHC II complexes have shown that the peptide P5 side chain tends to be an important T cell contact site due to its relatively high solvent exposure (50). However, according to the predicted models, the hydroxyl group of CLIP Thr^{95} is within interaction distance of the pocket 4 residues β71 Glu of DR*0402 and β71 Arg of DR*0404 (~3 Å distance). Thus, in the CLIP-DR*0402 and *0404 complexes, the relatively short side chain of CLIP Thr^{95} is able to interact with the MHC molecules. Next, we

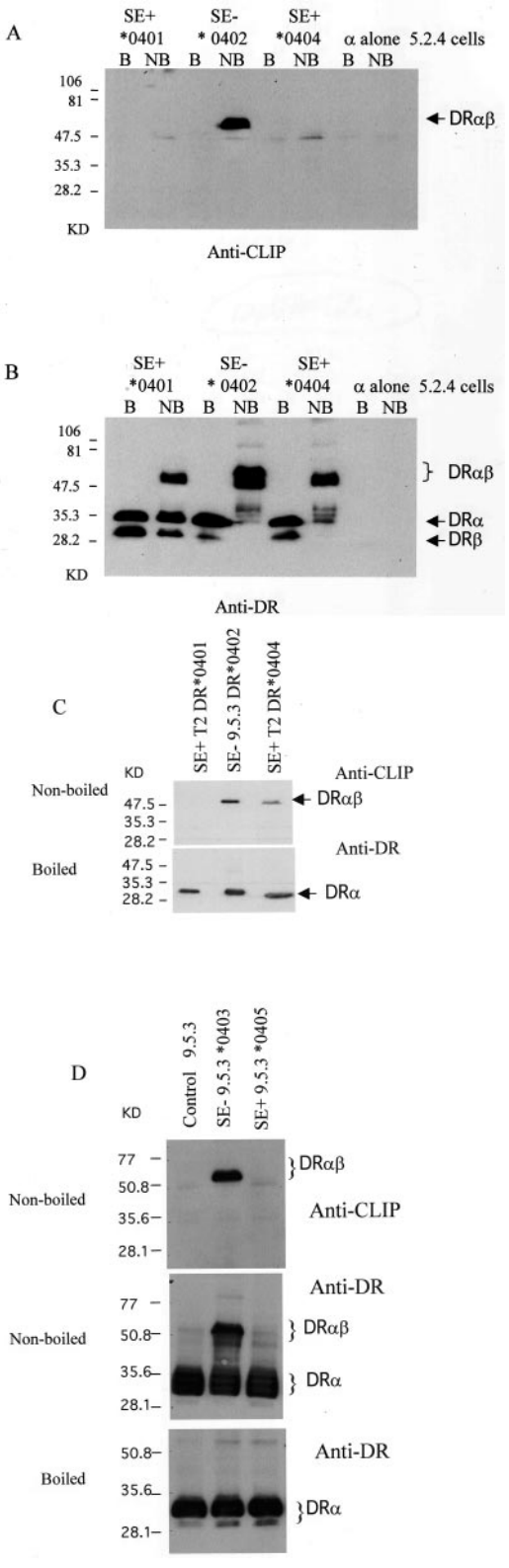

FIGURE 3. DR*0402-CLIP complexes are stable to SDS-induced dissociation. *A,* Nonboiled (NB) or boiled (B) lysates from 5.2.4 cells expressing DR*0401, DR*0402, DR*0404, or DRA alone were separated on SDS-PAGE and immunoblotted with an anti-CLIP Ab, CerCLIP.1. *B,* An identical gel to *A* was immunoblotted with anti-DR antisera, CHAMP. Arrows indicate the positions of $\alpha\beta$ dimers, α-chain monomers, and β-chain monomers. *C,* Unboiled lysates from T2-DR*0401 cells, 9.5.3-DR*0402 cells, and T2-DR*0404 cells were immunoblotted with an anti-CLIP Ab, CerCLIP.1. Boiled detergent lysates from the same cells were separated on SDS-PAGE and immunoblotted with the anti-DRα Ab, DA6.147 (shown below the anti-CLIP blot). *D,* Nonboiled lysates from 9.5.3, 9.5.3 DR*0403, and 9.5.3 DR*0405 were separated on SDS-PAGE and immunoblotted with either anti-DR-CLIP-specific mAb 14.23 (E. Mellins, unpublished data) or anti-DR mAb DA6.147. Boiled detergent lysates from same cells were separated on SDS-PAGE and immunoblotted with mAb DA6.147. Three independent experiments resulted in similar observations.

examined Pro[96] in these CLIP-DR*04 complexes, which is predicted to occupy the P6 pockets of these MHC molecules. Proline 96 is unlikely to lead to stability differences between CLIP-DR*0402 and CLIP-DR*0404 complexes because the P6 pocket structure is conserved among DR*04 alleles. Finally, we scrutinized the CLIP Leu[97] residue that, according to the molecular models, interacts with the P7 pocket of these DR*04 alleles. CLIP Leu[97] is in Van der Waals contact with β67 Leu in DR*0404 and β67 Ile in DR*0402 ($<$4 Å distance). Based on these modeling results, we mutated CLIP Thr[95] and Leu[97] to Ala, with the expectation that these mutations would diminish the differences in DR-CLIP interaction between DR*0402 and DR*0404.

We thus synthesized a variant of the CLIP peptide with alanine for leucine substitution at P7 (Ii 81–104 L97A) and measured dissociation of the CLIP variant from soluble DR*0402 and DR*0404 in vitro (Fig. 5*A*). CLIP L97A dissociated with almost identical $t_{1/2}$ lives from DR*0402 and DR*0404 at pH 7. This result argues that the SE region influences interaction with the P7 residue of CLIP at pH 7. At pH 5.3, CLIP L97A still dissociated slightly faster from DR*0404 than from DR*0402 (summarized in Table III).

To test the prediction based on molecular modeling about P5 threonine, we generated CLIP variant T95A L97A with the expectation that this variant would dissociate with similar kinetics from the DR*0402 and DR*0404 molecules. This peptide showed almost identical dissociation kinetics from DR*0402 and DR*0404 at both pH 5.3 and pH 7 (Fig. 5*B*, Table III). Thus, the P5 threonine of CLIP is involved in the peptide-MHC interaction and also contributes to differences between DR*0402-CLIP and DR*0404-CLIP complex stability.

To assess whether the influence of the SE region extended beyond these pockets, we tested dissociation of murine CLIP peptide variant M90V-M98F from DR*0402 and DR*0404. The murine CLIP peptide is identical to the human CLIP peptide in the core peptide-binding motif. According to the predicted CLIP-DR*0402 and DR*0404 structures and the crystal structure of the DR3-CLIP complex, CLIP M90 and M98 bind in the conserved P1 and P9 pockets, respectively, quite removed from the SE region (51). This peptide dissociated ~20-fold faster than wild-type CLIP peptide (Tables II and III). However, it still dissociated 2-fold faster from DR*0404 than from DR*0402, the same difference in dissociation as observed for the wild-type CLIP peptide, arguing that the SE region influences interaction with CLIP peptide residues in its immediate proximity (Fig. 5*C*, Table III).

HLA-DM enhances CLIP release from all three alleles

In the class II biosynthetic pathway, the removal of CLIP from HLA-DR is catalyzed by HLA-DM, allowing antigenic peptides to bind. To investigate whether HLA-DM catalyzes CLIP release comparably from all three alleles, we performed the CLIP dissociation reactions in the presence of 0.4 μM soluble HLA-DM. HLA-DM enhanced CLIP dissociation from all three DR4 alleles by 10- to 15-fold, resulting in $t_{1/2}$ lives of 0.5–1.5 h (Fig. 4*C*). The same hierarchy of CLIP dissociation was observed in the presence of HLA-DM, with DR*0401 forming the least stable DR-CLIP complex, followed by DR*0404, then by DR*0402 (Table II). The

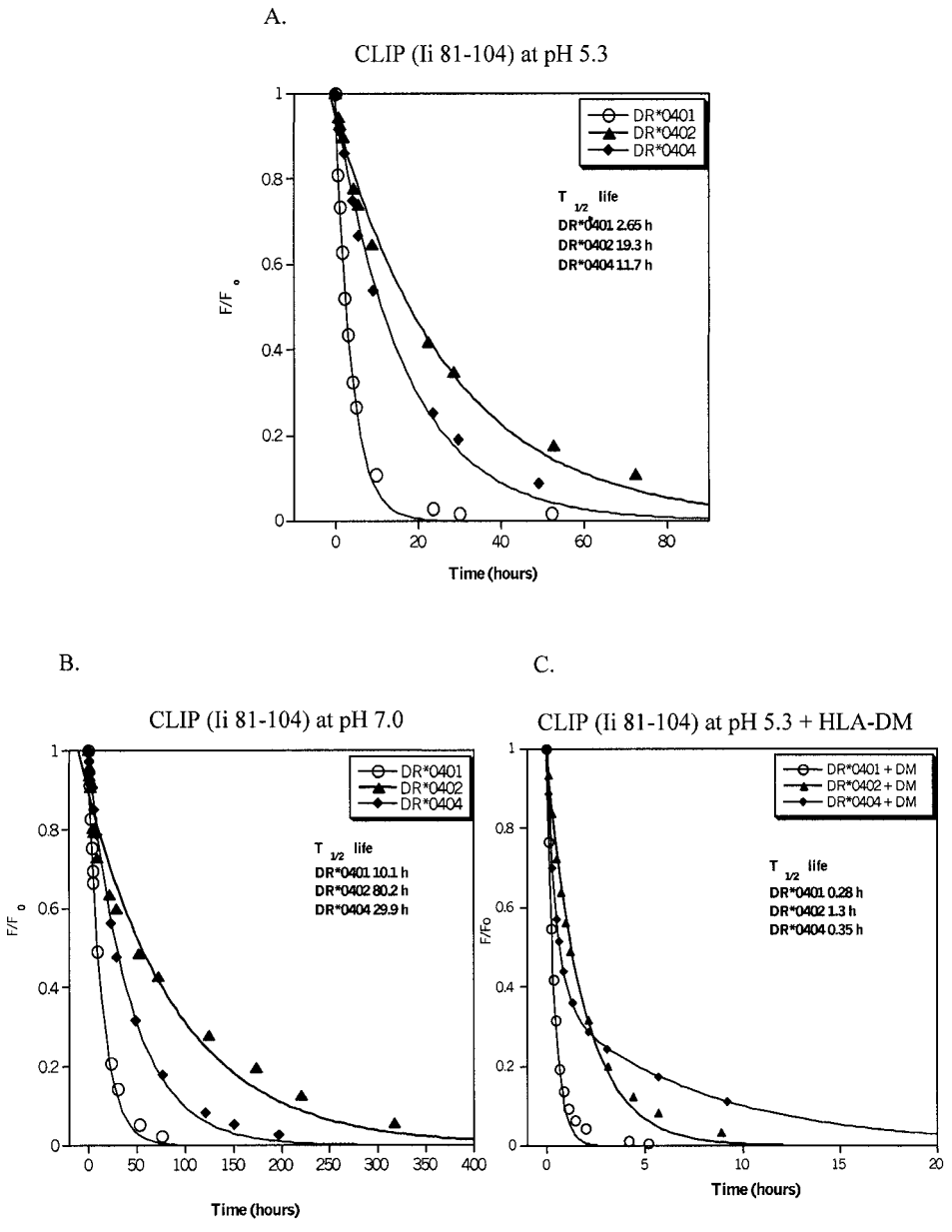

FIGURE 4. In vitro dissociation of DR4-CLIP complexes. *A*, DR4-CLIP complexes were formed overnight at 37°C, and kinetics of dissociation was measured by high performance size-exclusion chromatography at pH 5.3. *B*, Dissociation kinetics of DR4-CLIP complexes at pH 7. *C*, Dissociation kinetics of DR4-CLIP complexes measured at pH 5.3 in the presence of HLA-DM. The circles represent dissociation of DR*0401-CLIP, triangles represent DR*0402-CLIP, and diamonds represent DR*0404-CLIP complexes. All dissociation profiles have been fitted to a single exponential decay curve, except for DR*0402-CLIP at pH 7 and DR*0404-CLIP in the presence of HLA-DM. Each experiment was done at least twice. The summary of the pooled data is shown in Table II.

DR*0404-CLIP complex showed biphasic dissociation kinetics in the presence of HLA-DM, with ~65% of the complex dissociating with a $t_{1/2}$ of 0.34 h. This biphasic dissociation raised the possibility of two isomeric DR*0404-CLIP complexes that may be differentially susceptible to catalysis by HLA-DM. The existence of two kinetic isomers has been reported for other MHC class II-peptide complexes (41, 52).

Ii association with the three DR4 alleles is comparable

Several lines of evidence showed that the DR*0402-CLIP complex is relatively stable. We wondered whether this stable CLIP binding would result in increased association with Ii and enhanced assembly of DR*0402 $\alpha\beta$ heterodimers, compared with DR*0401 and DR*0404. Allelic variations in Ii dependency of MHC class II subunit assembly have been reported for murine class II molecules (15).

To assess DR/Ii interaction, we performed a pulse-chase experiment using DR4-expressing 5.2.4 cells. At 5–25 min postsynthesis, class II molecules were immunoprecipitated with an anti-DR dimer Ab and analyzed by SDS-PAGE. At this early stage in MHC class II biosynthesis, most of the class II molecules are in the ER, being assembled to form nonameric MHC-Ii complexes. We observed comparable kinetics and levels of DR dimer assembly for all three alleles, as detected by reactivity with the DR-specific Ab, ISCR3, in the metabolically labeled cells (Fig. 6*A*). Similar relative amounts of Ii (33 kDa) and DR molecules were precipitated as calculated by densitometry (DRα plus Ii:DRβ ratios for DR*0401 = 1.2, for DR*0402 = 1.3, and for DR*0404 = 1.1; see *Materials and Methods*). Similar results were obtained with a different anti-DR Ab (DA6.147), indicating that the comparable association of the DR4 alleles was not a result of Ab bias. These results argue that all three DR*04 alleles assemble with full-length Ii with similar efficiency.

As another assay of the efficiency of dimer assembly, we evaluated the kinetics of egress of DR molecules from the ER. ER export is reflected in the sensitivity of newly synthesized molecules to endonuclease H (endo H) digestion: Endo H cleaves high mannose sugars added in the ER, but not the complex glycans generated by Golgi processing. DRβ has one glycan and DRα has

Table II. *Dissociation of human Ii 81–104 CLIP peptide from soluble DR4 molecules*

DR4 Allele	$t_{1/2}$ (h)[a]
Dissociation at pH 5.3	
DR*0401	2.48 ± 0.09
DR*0402	18.44 ± 1.60
DR*0404	11.26 ± 0.76
Dissociation at pH 7.0	
DR*0401	8.91 ± 0.56
DR*0402	82.61 ± 5.8[b]
DR*0404	28.05 ± 0.92
Dissociation in the presence of HLA-DM[c]	
DR*0401	0.24 ± 0.02
DR*0402	1.15 ± 0.06
DR*0404	0.36 ± 0.03[d]

[a] The half-life is given in hours ± 2 × SE, derived from two or more independent experiments represented in Fig. 4.

[b] The kinetics of this complex are best described by a double exponential. The reported value corresponds to the majority of the complex (73.8 ± 2.3%). A minor component (26.2%) is also present with a half-life of 3.1 ± 0.6 h.

[c] Dissociation of the DR-CLIP complexes in the presence of HLA-DM was enhanced by 9.5- to 17.5-fold for the different alleles.

[d] The kinetics of this complex are best described by a double exponential. The reported value corresponds to the majority of the complex (62.4 ± 1.6%). A minor component (38%) is also present with a half-life of 5.4 ± 0.4 h.

two, of which only one is converted to a complex glycan (53). Endo H digestion patterns of the metabolically labeled DR molecules, immunoprecipitated with anti-DR Ab L243, implied very similar kinetics of assembly and transport for the different alleles (Fig. 6*B*). CLIP fragments were first detected for all three DR4 alleles at 3 h of chase (Fig. 6*B*), also arguing for comparable transport kinetics of the three alleles. Thus, it appears that Ii interaction with the three alleles is comparable, although in vitro and cellular data (see below) indicate that CLIP interaction with the three alleles differs. This apparent inconsistency may be explained by the fact that Ii interaction with MHC class II molecules is not limited to the groove-binding segment of CLIP/Ii. Other regions of the Ii, including a region that lies just amino terminal to the groove-binding segment, and a region within the carboxyl-terminal portion of the molecule, mediate binding to class II dimers and may compensate for reduced CLIP affinity to a particular allele (54–56).

*DR*0401-, DR*0404-, and DR*0405-CLIP complexes are less stable than DR*0402- and DR*0403-CLIP in vivo*

In vitro kinetic data showed that the DR-CLIP complexes formed by SE^+ alleles DR*0401 and DR*0404 were less stable than complexes formed by the SE- DR*0402 allele. To determine whether these differences were consequential in the cellular environment, we performed a pulse-chase experiment in which the DR4-expressing 5.2.4 cells were pulsed for 30 min and chased from 1 to 5 days. DR molecules and associated peptides were immunoprecipitated with an anti-DR Ab L243 and analyzed by SDS-PAGE.

The differences in the CLIP peptide association were apparent by 1 day of chase and persisted over 3–4 days of chase. At that time, the CLIP peptide was still strongly associated with the DR*0402, but considerably less so with DR*0401 and DR*0404 (Fig. 7*A*). Densitometric analysis confirmed that the CLIP:DRα ratios for DR*0402 were greater than for the other two alleles (by day 3, the differences in stability were most apparent with the ratio <0.05 for DR*0401 and DR*0404 and $= 0.2$ for DR*0402; see *Materials and Methods*). The abundance of labeled CLIP-DR complexes suggested an ~4-fold enhancement of stability of the CLIP-DR*0402 complex, whereas the differences in in vitro $t_{1/2}$ of dissociation of the complexes were more modest. This finding is most likely due to transport of a large proportion of CLIP-DR*0402 complexes to the cell surface, where the neutral pH further stabilizes these complexes. We observed a similar difference when we compared CLIP dissociation from the RA-associated allele DR*0405 and the RA-nonassociated allele DR*0403 in the absence of HLA-DM. CLIP dissociated spontaneously from DR*0405 in DM-null cells, while DR*0403 formed complexes with CLIP peptide that were long lived (Fig. 7*B*).

Only a small amount of CLIP peptide coprecipitated with DR*0404 molecules. However, this small proportion persisted through day 4, suggesting the presence of a minor but stable DR*0404-CLIP complex isomer. This finding is consistent with the in vitro biphasic dissociation curve of the DR*0404-CLIP complex in the presence of HLA-DM (Fig. 4, Table II). We speculate that a small fraction of DR*0404-CLIP complexes is fairly stable, and these show reduced susceptibility to HLA-DM catalysis.

Of note, the DRα and DRβ levels were not very different between the three DR4 alleles after 3–5 days, even though CLIP was barely associated with DR*0401 and DR*0404 molecules (Fig. 7*A*). These data suggest that either these molecules are bound to other peptides or the empty DR molecules are not degraded rapidly.

Discussion

In this study, we evaluated several characteristics of CLIP-DR complexes: their steady state abundance at the cell surface, their SDS stability, and their kinetic stability as measured in vitro and in vivo. By the first two criteria, the DR-CLIP complexes of RA-associated DR*0401, DR*0404, and DR*0405 alleles are less stable than the DR-CLIP complexes of the RA-nonassociated alleles DR*0402 and DR*0403. Of the three alleles studied both in vitro and in cells, DR*0401 forms the least stable complexes with CLIP, in both the presence and absence of HLA-DM. The in vitro differences, although modest, correspond to more substantial differences observed in B cell lines.

The structural bases of these allelic differences can be determined because of the limited sequence variation between these DR4 subtypes. The five DR4 alleles differ at the SE (DRβ 67–74) and at DRβ 86 (Table I). The DRβ 86 V/G dimorphism influences side chain specificity at pocket 1; glycine at this position allows for binding of large aromatic residues, whereas valine limits the size of this pocket, resulting in a preference for aliphatic side chains (8). Thus, the P1 Met of CLIP peptide binds better to the pocket 1 of DR*0404, DR*0403, and DR*0402 (DRβ 86 V) as compared with the pocket 1 of DR*0401 and DR*0405 (DRβ 86 G). However, the difference between DR*0402 and DR*0404 (like the difference between DR*0403 and DR*0404) maps exclusively to the SE region, which is the only site of sequence variation in each pair of molecules. The SE influences the P4 pocket and P7 pocket of the peptide-binding groove (see Table I).

Analyses of binding of CLIP peptide variants corroborate the influence of the SE region on CLIP interaction. Replacing the P7 leucine with alanine in CLIP resulted in a peptide (L97A CLIP) with identical dissociation rates from DR*0402 and DR*0404 at pH 7. This finding suggests that the interaction of the P7 leucine of CLIP with the pocket 7 containing DRβ 67 isoleucine of DR*0402 provides more stable binding than interaction with the pocket 7 containing DRβ 67 leucine of SE^+ alleles, DR*0401 and DR*0404. We also found that the T95A L97A CLIP variant dissociated with identical $t_{1/2}$ from DR*0402 and DR*0404 at both pH 5.3 and pH 7. Under these conditions, we speculate that the threonine side chain is polarized and more likely to form electrostatic interactions or hydrogen bonds with the negatively charged

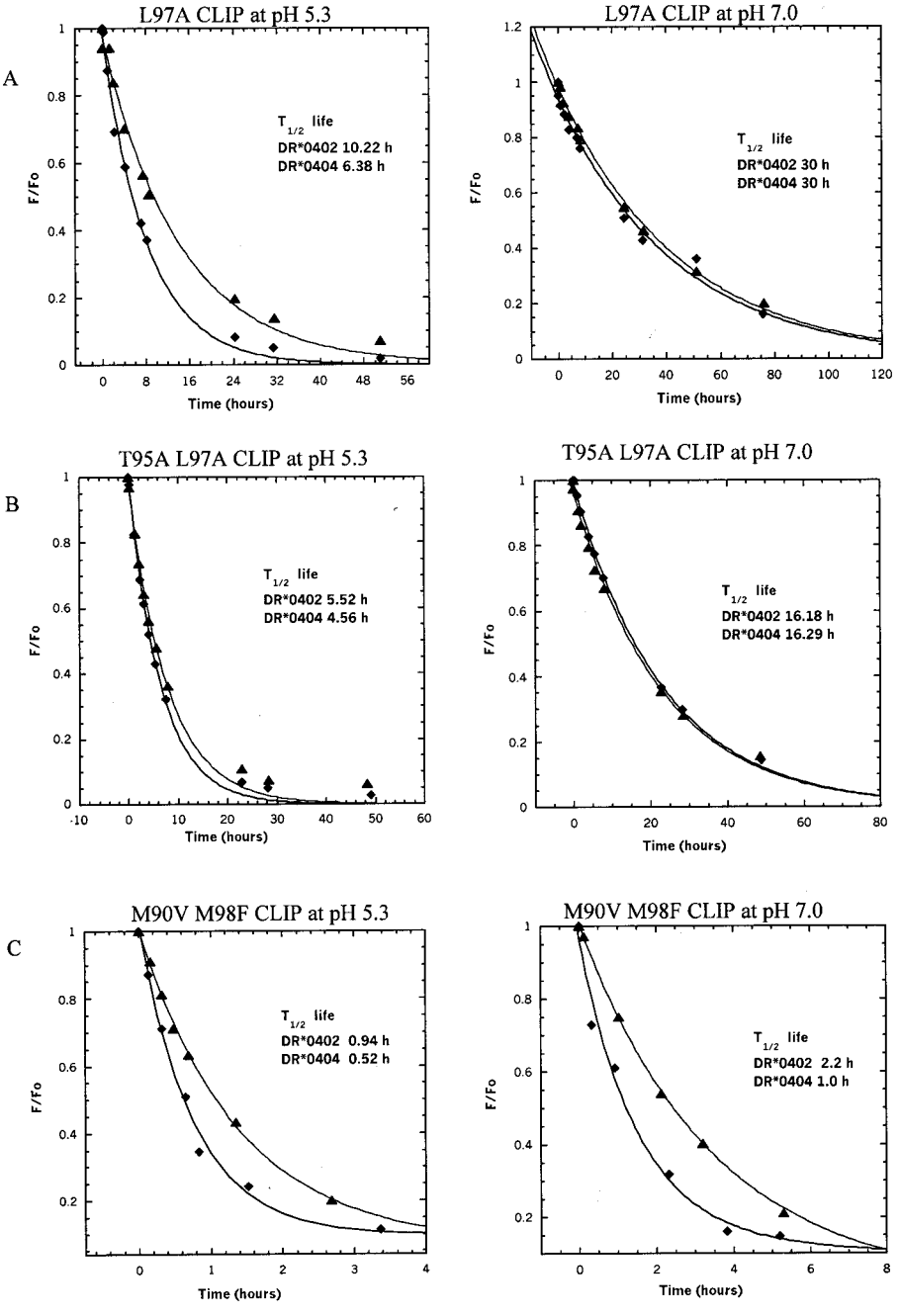

FIGURE 5. In vitro dissociation of CLIP peptide variants from soluble DR*0402 and DR*0404 molecules. *A*, Dissociation of L97A CLIP at pH 5.3 and at pH 7. *B*, Dissociation of T95A L97A CLIP at pH 5.3 and at pH 7. *C*, Dissociation of M90V M98F CLIP at pH 5.3 and at pH 7. The triangles represent DR*0402-CLIP, and diamonds represent DR*0404-CLIP complexes.

residues of DR*0402 as compared with the positively charged residues of DR*0404. The murine CLIP peptide variant M90V-M98F dissociated 2-fold faster from DR*0404 than from DR*0402, suggesting that the SE residues influence the interaction with CLIP peptide residues in their immediate proximity and not as much on the other peptide residues. Together these results imply that the SE region influences interactions with the P5 and P7 CLIP residues to mediate reduced DR-CLIP complex stability.

Peptide elution studies from class II molecules have suggested that each HLA-DR pocket can be characterized by a pocket profile, a quantitative representation of all naturally occurring peptide residues that interact with a given HLA-DR pocket. Using HLA-ligand databases and peptide affinity measurements based on IC_{50} values, Sturniolo et al. (55) have determined pocket profiles. Our data with CLIP variants are in agreement with the predicted pocket 7 preferences of DR4 alleles: DR*0402 interacts more favorably with leucine relative to DR*0404 at this pocket. Furthermore, Sturniolo et al. (55) have shown that each pocket profile is nearly independent of the rest of the peptide-binding groove. Thus, two different alleles with identical residues lining a given pocket are likely to have the same residue preference at that pocket. We have shown that SE^+ alleles with either DRβ71 lysine (DR*0401) or arginine (DR*0404 and DR*0405) result in reduced stability of the DR-CLIP complex. The SE motif would be expected to reduce CLIP interaction in other RA-associated alleles (DRB1*0408, *1402, *0101, and *0102). However, in the DR*0101 allele, which differs from the DR4 alleles at several other residues, other groove residues most likely offset this reduced CLIP interaction, resulting in moderate CLIP affinity, despite the SE residues (17).

It is striking that several MHC class II molecules linked with autoimmune diseases form class II-CLIP complexes with low stability. In addition to the RA-associated DR alleles described in this

Table III. *Dissociation of CLIP peptide variants from DR*0402 and DR*0404*[a]

DR4 allele	pH 5.3	pH 7.0
hCLIP L97A LPKPPKPVSKMRMATP**A**LMQALPM		
DR*0402	9.52 ± 0.58	28.9 ± 1.73
DR*0404	5.93 ± 0.37	27.0 ± 2.34
hCLIP T95A L97A LPKPPKPVSKMRMA**A**P**A**LMQALPM		
DR*0402	4.94 ± 0.35	14.43 ± 0.9
DR*0404	4.47 ± 0.19	15.45 ± 0.54
mCLIP M90V M98F KPVSQ**V**RMATPLL**F**R		
DR*0402	1.12 ± 0.05	2.4 ± 0.09
DR*0404	0.62 ± 0.06	1.38 ± 0.29

[a] The $t_{1/2}$ is given in hours ± 2 × SE, derived from two independent experiments represented in Fig. 5. All experiments except the mCLIP M90V M98F dissociation at pH 7 were done twice. Bold letters indicate altered residues from wild-type CLIP peptide; underlined resides correspond to the core binding motif.

work, low class II-CLIP complex stability at endosomal pH has been demonstrated for I-A^{g7}, a murine class II molecule associated with type I diabetes in nonobese diabetic mice (57). Another class II complex, DQa*0501/DQb*0301, is associated with juvenile dermatomyositis and binds weakly to the CLIP peptide (58). The DR*1501 allele associated with multiple sclerosis has also been demonstrated to have low affinity for CLIP (59). The DRB3*0101 (DRw52) allele that is associated with autoimmune hepatitis and with Graves disease also has very low affinity for CLIP (17, 60–62). Interestingly, the RA-associated allele DR*0101, which has moderate CLIP affinity, is the SE$^+$ allele with the weakest association with RA. DR1 association with RA has a relative risk of 1 (an absolute risk of 1 in 80) as compared with a relative risk of 6 for DR*0401 (absolute risk of 1 in 35), 5 for DR*0404 (absolute risk of 1 in 20), and 100 for DR*0401/*0404 (absolute risk of 1 in 7) (43). Thus, the relative risk for individuals carrying the DR4 alleles is approximately five times higher than that of individuals not carrying these alleles, while the DR*0101 allele does not confer risk on its own. Furthermore, presence of DR4-positive RA-associated alleles DRB1*0401 or *0404 is consistently correlated with severe disease, whereas DR*0101 is associated with milder or rheumatoid factor-negative disease (43).

The accumulating evidence of low class II-CLIP complex stability among autoimmune disease-associated alleles implies that this may be an important property contributing to disease pathogenesis. In vitro kinetic experiments indicate that following release of CLIP, class II molecules remain in a peptide-receptive or active state for various lengths of time, depending on the allele (40). For class II alleles with high affinity for CLIP, the generation of peptide-receptive molecules will be tightly linked to arrival in HLA-DM-containing compartments, where CLIP release can be catalyzed. There, DM-mediated peptide editing for stable class II/peptide complexes will also occur. In contrast, low stability of class II-CLIP complexes will favor spontaneous CLIP release, with the possibility that peptide loading will occur in cells or compartments lacking DM. Examples of sites with low DM levels and available Ag include early endosomal compartments, where processing of certain Ags takes place (63). The amount of HLA-DM also is generally low on the cell surface. Moreover, even for cells with detectable surface DM, such as immature dendritic cells, DM activity is not optimal at the neutral pH of the extracellular space (64, 65). Thus, peptide exchange at the cell surface may be to a large extent DM independent, and class II-CLIP complexes of low stability that reach the cell surface will most likely be preferentially susceptible to peptide exchange. Indeed, we have previously shown that DR*0401 B cells lacking HLA-DM bind and present exogenous peptides more effectively than DR*0402 cells without

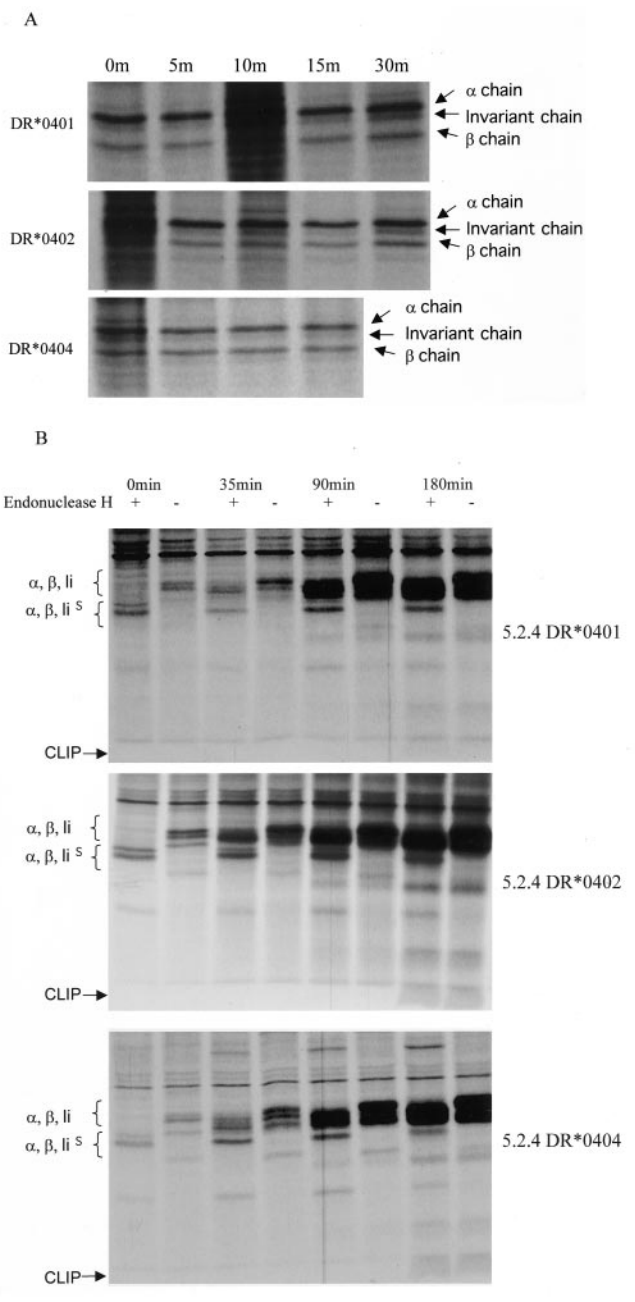

FIGURE 6. Association of Ii with DR4 in vivo. *A*, 5.2.4 cells expressing DR*0401, DR*0402, or DR*0404 were pulsed for 5 min, chased for the times shown, and immunoprecipitated with anti-DR Ab ISCR3 and resolved by SDS-PAGE. Arrows indicate the positions of the α- and β-chains and of Ii. Equal counts of each cell lysate were loaded for all time points. *B*, DR4-expressing 5.2.4 cells were pulse labeled with [^{35}S]Met/Cys for 20 min. Class II molecules were immunoprecipitated with anti-DR Ab L243 after the indicated periods of chase, and the precipitates were treated with Endo H (or mock treated) and analyzed by SDS-PAGE. Ab L243 was used because it detects more mature forms of DR molecules, unlike mAbs ISCR3 and DA6.147, which recognize the earlier biosynthetic forms of DR. Arrows indicate the positions of the α- and β-chains and of Ii; s indicates Endo H-sensitive bands. Equal counts of each cell lysate were loaded for all time points, and all cell lines showed comparable amounts of precipitated class II molecules at the same time points.

HLA-DM, more than either allele in the presence of DM (22). These considerations raise the possibility that low CLIP affinity may predispose to presentation of self peptides using pathways that are unavailable to alleles that are more tightly regulated due to

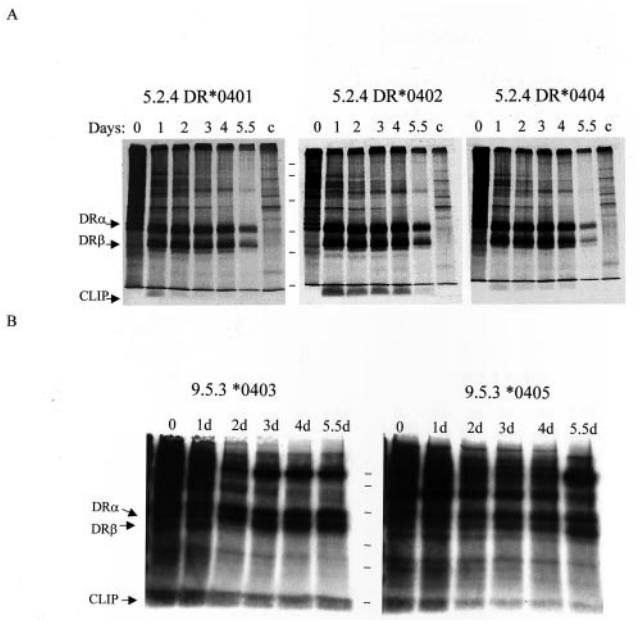

FIGURE 7. Persistence of DR4-CLIP complexes in vivo. DR4-expressing 5.2.4 cells (*A*) and 9.5.3 cells (*B*) were pulse labeled with [^{35}S]Met/Cys for 30 min and 2 h, respectively. Class II molecules were immunoprecipitated with anti-DR Ab L243 (*A*) or DR4-specific Ab NFLD.D1 (*B*) after the indicated periods of chase (days) and analyzed by SDS-PAGE. Arrows indicate the positions of the α- and β-chains and of CLIP peptide, just below the dye front. Equal amounts of radioactively labeled material were loaded for all time points.

high CLIP affinity. Consistent with this possibility, DR*0401 cells lacking HLA-DM have been shown to present peptides derived from non-Ii endogenous Ags to T cells (66). Other models can also be envisioned in which reduced CLIP affinity may influence thymic selection events. Medullary thymic epithelial cells that are involved in negative selection have been shown to present more CLIP peptides as compared with cortical thymic epithelial cells (54). The medullary cells present slightly different CLIP peptide variants (85–104 and 85–105) that may be important in negative selection and establishing tolerance. Therefore, lower CLIP on the surface of the medullary thymic epithelial cells could contribute to defective negative selection. In another scenario, low CLIP affinity may result in generation of empty molecules whose shortened $t_{1/2}$ results in lower class II levels at the cell surface. In addition, there is some evidence that free CLIP reduces the surface abundance of class II molecules, at least when administered exogenously (67). A model in which reduced levels of expression of class II on thymic APCs influence the development of autoimmunity has been proposed (68).

Several lines of evidence lend support to the notion that the low CLIP affinity of certain SE$^+$ alleles may contribute to the pathogenesis of RA in particular. Louis-Plence et al. (69) have reported that HLA-DM transcripts and protein levels are reduced in peripheral B cells of RA patients as compared with patients with inflammatory arthritis. This decreased expression is unrelated to HLA-DM promoter or allelic polymorphism and does not affect HLA-DR genes. The result is a decrease in the DM:DR ratio in B cells of RA patients. Under such conditions of relative DM deficiency, expression of class II alleles with low affinity for CLIP could result in presentation of non-CLIP self peptides with possible autoimmune consequences. Additionally, in RA, the synovial joint environment is proteolytic and mildly acidic and may generate extracellular peptides (70, 71). These self peptides may be presented when readily exchangeable DR-CLIP complexes are found at the surface of synovial cells and could provoke an autoimmune T cell response. Finally, it is intriguing that cathepsin S knockout mice have been reported to be less susceptible to collagen-induced arthritis (72), an animal model of RA. In these mice, more stable class II-p10 Ii peptide complexes are present instead of class II-CLIP complexes (72).

Although many disease-associated alleles form relatively less stable class II-CLIP complexes, DR*0402, which forms a stable DR-CLIP complex, is also associated with an autoimmune disease, pemphigus vulgaris (73). Thus, it is clear that the presence of an unstable class II-CLIP complex is not a requirement for the breakdown of self-tolerance. However, it seems likely that the explanations for the numerous HLA-disease associations will vary, at least in the details. It will be of interest to learn whether the mechanistic basis for HLA-DR*0402 disease association with pemphigus differs from the mechanism of HLA-DR association with RA, as would be predicted from the findings reported in this work.

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