

## **Selective Release of Some Invariant Chain-derived Peptides from HLA-DR1 Molecules at Endosomal pH**

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### **Summary**

The predominant peptides bound to major histocompatibility complex class II molecules expressed on human B cells are derived from a relatively limited number of self proteins. To determine whether any of the prebound self peptides might be released in endosomes during recycling, water-soluble HLA-DR1 molecules were incubated with a high affinity synthetic peptide at pH 4.0 and 7.0 at 37°C. The resulting bound peptide repertoire was then acid extracted, and separated by reversed-phase high performance liquid chromatography. Using a combination of mass spectrometry and ultraviolet spectroscopy, prebound self peptides and newly bound synthetic peptide were characterized. Most self peptides bound to HLA-DR1 were not appreciably released during extended exposure to pH 4.0. However, some invariant chain-derived peptides were uniquely released at this pH.

The majority of peptides bound by MHC class II molecules during antigen loading are not derived from ingested and processed foreign proteins, but instead are primarily derived from a finite number of self proteins (1, 2). During progression outward through the exocytic pathway, class II-invariant chain (Ii) complexes are exposed to a decreasing pH gradient reaching a lower limit of 4.5–5.5 (for a review see reference 3). However, the exact organelles (and resulting pH) where Ii is proteolytically removed and peptide binding ensues are not well understood and peptide binding may occur in multiple intracellular compartments depending on the source protein and the site of its proteolysis.

The effect of pH on class II-peptide interaction in vitro is highly dependent on the class II molecule, the peptide, and the method employed for the analysis. In general, the on rate ( $k_{on}$ ) for many peptides is measurably increased as the pH is lowered from 7 to 4 but the off rate ( $k_{off}$ ) is not as dramatically changed (4–10).

Substantial insight into the specificity and affinity of class II-peptide interaction has been obtained by way of in vitro binding assays, but whether particular families of peptides or small amounts of many different individual peptides are exchanged during these peptide binding experiments has remained unknown. To address these questions, water soluble HLA-DR1 molecules purified from human B cells (the use of which avoids the secondary effects due to detergent) have been produced. A combination of reversed-phase chromatography (RPC) and mass spectrometry was used to closely scru-

tinize the influence of acid pH on class II-bound self peptides and peptide exchange.

### **Materials and Methods**

*Purification of Water-soluble HLA-DR1.* HLA-DR1 (DRA1/DRB1\*0101) was immunoaffinity purified from the Epstein-Barr-transformed homozygous human B cell line LG-2 then proteolytically digested with papain as previously described (11), but after digestion the procedure was modified as follows. The papain/HLA-DR mixture was dialyzed for 12 h at 4°C against distilled water (12–14 kD exclusion tubing; GIBCO BRL, Gaithersburg, MD), then sedimented at 40,000 g for 1 h (pelleting a substantial fraction of the total papain and any undigested HLA-DR molecules). The 40,000 g supernatant fraction was adjusted to 50 ml with a final concentration of 2% ampholytes (BioLytes pH 3–10; Bio-Rad Laboratories, Richmond, CA). The sample was then subjected to preparative isoelectric focusing (PIEF; Rotofor, Bio-Rad Laboratories) for 5 h at 4°C using a constant power setting of 12 W. The focused fractions were harvested by vacuum aspiration and an aliquot of each to measure the pH and protein content. Samples containing purified HLA-DR1 were pooled and differentially treated as described below. Using PIEF, and an ampholyte gradient from pH 3–10, water soluble class II molecules were separated from contaminating papain and focused to a region of pH 4.0–5.0 over the course of 5 h at 4°C. PIEF had no detectable effect on the stability of HLA-DR dimers and, in fact, PIEF-purified material crystallized with similar crystal morphology as material purified without the use of PIEF (12, and Brown, J., personal communication).

*Exposure of HLA-DR1 to Acidic pH.* After PIEF, fractions con-

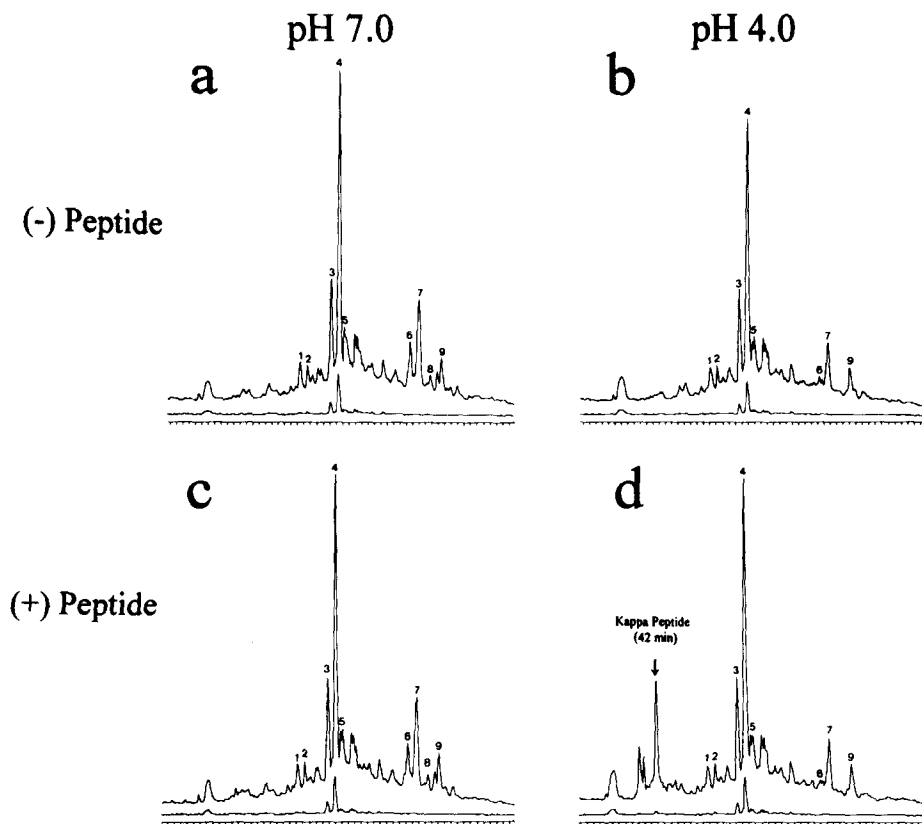
taining HLA-DR1 molecules were placed into 12–14 kD exclusion dialysis tubing and dialyzed against 20 mM 2-(*N*-morpholino) ethanesulfonic acid (MES), pH 5.0, for 48 h at 4°C to remove ampholytes. After dialysis, the sample was divided into two equal fractions and each placed into a 25-kD exclusion vacuum dialysis colloid membrane (Schleicher & Schuell, Inc., Keene, NH). One sample was vacuum dialyzed/concentrated to 1 ml against citrate buffer, pH 4.0, and the other sample was vacuum dialyzed/concentrated to 1 ml against MES, pH 7.0, both at 4°C. Each sample was again divided into two equal fractions and all four samples placed into 1-kD exclusion dialysis tubing (Spectra/Por 1,000; Spectrum Medical Industries, Inc., Houston, TX). To one pH 4.0 and one pH 7.0 sample, a synthetic peptide derived from the human Ig  $\kappa$  chain was added (50  $\mu$ M; residues 188–202; KHKVYA-CEVTHQGLS), but to the two other samples no peptide was added. The two pH 4.0 and the two pH 7.0 samples were placed into vesicles containing prewarmed citrate buffer (pH 4.0) and MES (pH 7.0), respectively, and all samples were maintained at 37°C for 24 h. HLA-DR1 molecules were then repurified by HPLC-size exclusion chromatography (HPLC-SEC) in 20 mM MES, pH 6.5 (BIO-SIL TSK-250; Bio-Rad Laboratories).

**Analysis of HLA-DR Bound Peptide Repertoire.** Peptides were extracted and separated by RPC as previously described (1). To determine a precise retention time, 25 pmol of the  $\kappa$  synthetic peptide 188–202 was subjected to the identical RPC gradient conditions used for the total extracted pools. The peptide repertoire present in each RPC fraction was determined by matrix-assisted laser desorption mass spectrometry (MALDI-MS, Lasermat; Finnigan MAT, San Jose, CA) (2). Briefly, RPC fractions were concentrated to 25  $\mu$ l and applied to autosampler targets using 2  $\mu$ l of sample and 0.4  $\mu$ l of matrix. An internal standard was used to calculate exact mass assignments.

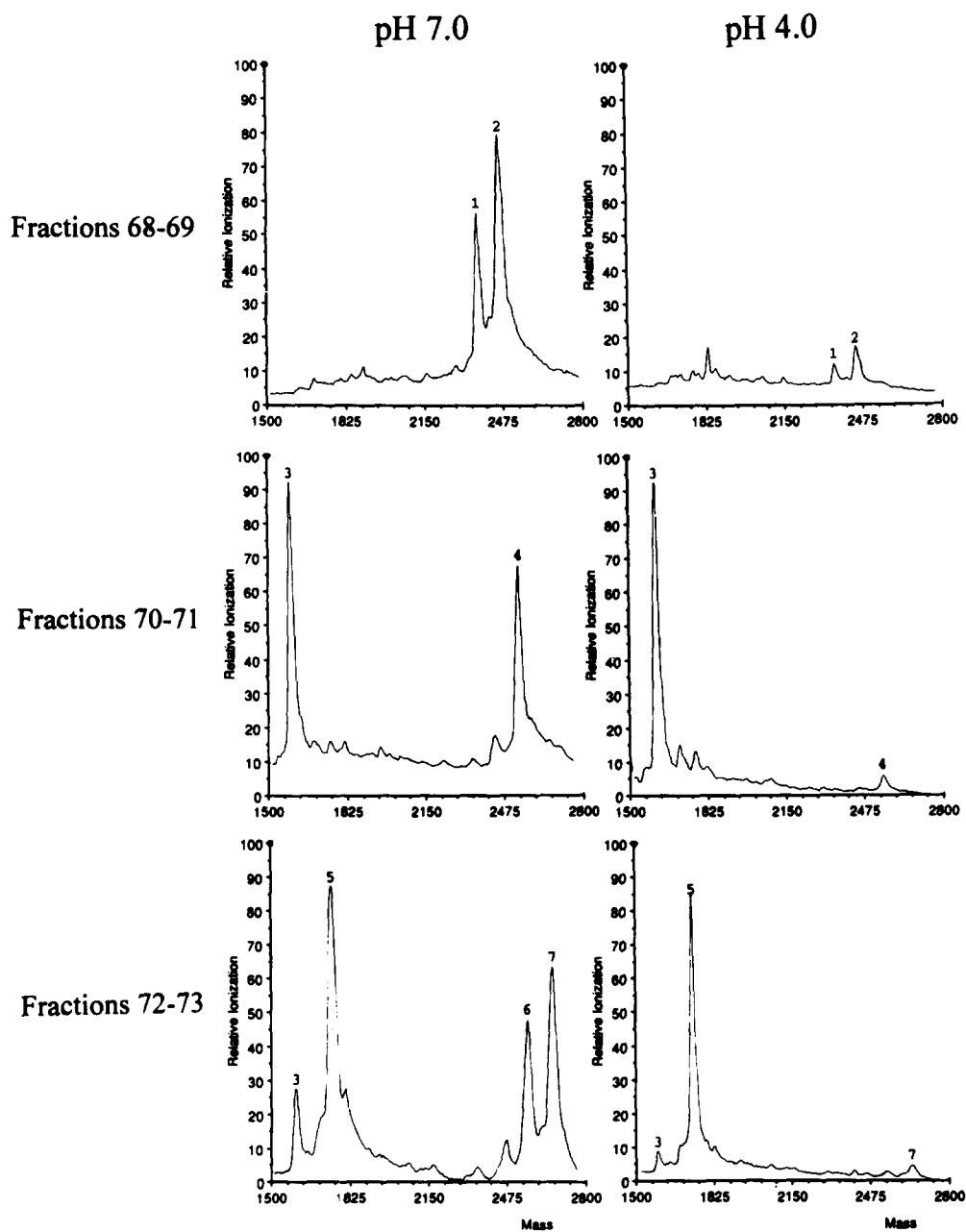
## Results and Discussion

To determine whether any of the predominant peptides in the HLA-DR1 repertoire were preferentially released at pH 4.0, the following experiments were performed. First, the difficulties associated with the presence of detergent were eliminated by digesting HLA-DR1 molecules with papain to remove the transmembrane domain and cytoplasmic tail. Water-soluble class II molecules were then adjusted to pH 4.0 and 7.0 and incubated at 37°C in the presence or absence of synthetic peptide derived from the Ig  $\kappa$  L chain (known to bind HLA-DR1; 2). After 24 h, HLA-DR-peptide complexes were separated from free peptide by HPLC-SEC and the MHC bound peptides acid extracted, then separated by microbore RPC (Fig. 1).

The remarkable similarity between the pH 7.0 and 4.0 chromatograms indicates that in weak acid, little or no release of most prebound peptides occurs and that microbore RPC of these complex peptide mixtures is highly reproducible (Fig. 1, *a* and *c*). Although each individual chromatographic peak represents a mixture of multiple peptides when RPC is used coordinately with MS, the relative amount of each individual peptide present can be determined. Close inspection indicated that all four chromatographic peaks containing the nested set of Ii-derived peptides (Fig. 1, peaks 6–9) were reduced after exposure to acidic pH, but each peak was known to contain multiple peptides (1). In addition, not all the Ii-containing peaks were equally affected (compare Fig. 1, *a* and *b*). To determine if particular Ii-derived peptides were sensitive to acidic pH, comparative MS was performed on these



**Figure 1.** RPC peptide profiles from pH-treated and non-pH-treated HLA-DR1 molecules. Chromatograms are plotted from 30 to 83 min with a full-scale UV absorption at 210 and 277 nm of 125 mAU. (*c*) The predominant peptides in the labeled peaks are as follows: 1. Bovine fetuin 56–74, YKHTLNQIDSVKVVWPRRP; 2. Bovine fetuin 56–73, YKHTLNQIDSVKVVWPRR; 3. Na<sup>+</sup>/K<sup>+</sup> ATPase 199–216, IPADLRISANGCKVDNS; 3. HLA-A2-like 104–117, GSDWRFLRGYHQYA; 3. HLA-A2-like 105–117, SDWRFLRGYHQYA; 4. Transferrin receptor 680–696, RVEYHFLSPYVSPKESP; 4. HLA-A2-like 103–117, VGSDWRFLRGYHQYA; 4. HLA-A2-like 103–116, VGSDWRFLRGYHQY; 5. HLA-A2-like 103–120, VGSDWRFLRGYHQYAYDG; and 6–9. 11 Ii peptides spanning 97–121, LPKPPKPVSKMRMATPLLMQALPMG.



Peak Number	Peptide Sequence	Length	Observed Mass (daltons)	Calculated Mass (daltons)	pH Sensitivity
1	<u>K</u> PKPVSKMRMATPELLMQALP	21	2335.2	2334.9	+
2	<u>PK</u> PKPVSKMRMATPELLMQALP	22	2433.2	2432.0	+
3	KMRMATPELLMQALP	14	1601.0	1601.0	-
4	<u>L</u> PKPKPVSKMRMATPELLMQALP	23	2545.2	2545.2	+
5	KMRMATPELLMQALP <u>M</u>	15	1732.7	1732.2	-
6	<u>PK</u> PKPVSKMRMATPELLMQALP <u>M</u>	23	2562.1	2563.2	+
7	<u>L</u> PKPKPVSKMRMATPELLMQALP <u>M</u>	24	2675.3	2676.4	+

**Figure 2.** MALDI-MS analysis of li-derived peptides. Fractions 68-89, 70-71, and 72-73 were pooled and peptides present in each sample identified by MALDI-MS as described in Materials and Methods. The previously identified li-derived peptides (1) are numbered in each spectra and listed in the table at the bottom.

fractions (Fig. 2). It is interesting to note that all the Ii-derived peptides that contained residues 99–105 were markedly released by exposure to acidic pH, whereas those that lacked this region were not. Little or no release of these Ii-derived peptides was observed if the experiment was performed at room temperature (data not shown). Using HLA-DR3-Ii peptide complexes purified from the cell line T2 which is defective in antigen presentation, release of the Ii-derived peptides was obtained at low pH, enabling the HLA-DR3 molecule to bind exogenous peptide (13). It is interesting to note that in these studies all the prebound peptides were derived from Ii and all contained residues 99–105 in addition to the core residues (13, 14). The cellular origin of these complexes containing the long Ii peptides, i.e., intracellular or cell surface, is presently unknown. It seems possible that the 99–105 region of full-length Ii is also involved in the acid-induced dissociation of Ii-class II complexes in endosomes.

Treatment at pH 4 was also performed in the presence of a high affinity exogenous peptide derived from Ig  $\kappa$  chain (2). A unique peak was seen in the pH 4/peptide (+) profile at 42 min (Fig. 1, *d*), not found in the pH 4/peptide (–) profile (Fig. 1 *b*). Comparison of this peak to the RPC chromatographic analysis of the purified synthetic  $\kappa$  peptide eluted under the same conditions, confirmed that both had identical retention times and spectral ratios (210, 277, and 292 nm). Although exact quantitation was not possible, the amount of  $\kappa$  bound was approximately equal to the sum of Ii peptide that had been released. A small unique peak (unlabeled) was also seen in the control pH 7/peptide (+) sample at 42 min (Fig. 1 *c*). From these data it is evident that binding of the  $\kappa$  peptide to HLA-DR1 molecules was enhanced at pH 4. These observations have two possible explanations: (a) the affinity of the  $\kappa$  peptide is substantially increased at acidic pH, or (b) the increase in the available binding sites at acidic pH (largely attributable to the release of the long-Ii peptides) resulted in the higher levels of  $\kappa$  peptide binding. Additionally, it should be noted that reduction of peaks 6 and 8 after exposure to acidic pH was not enhanced or “catalyzed” by the presence of a large excess of exogenously added peptide (15).

To verify that the unique peak at 42 min in the pH treated/peptide (+) RPC profile indeed represented bound  $\kappa$  peptide, MALDI-MS was performed on fractions spanning this time region in both the peptide (–) and peptide (+) profiles. A unique mass (1,700  $m/z$ ) corresponding to the  $\kappa$  peptide was found only in the peptide (+) and not in the peptide (–) sample (data not shown). In addition, a new mass of 3,398  $m/z$  was also found in significant quantities in fraction 42. Because the  $\kappa$  peptide contains an internal cysteine (position 7 of the 15 residue peptide), a disulfide-linked dipeptide was suspected. When the RPC fractions were reduced with 2  $\beta$ -ME (2-ME) before MS a mass of 1,778  $m/z$ , corre-

sponding to the reduced peptide with a 2-ME adduct was detected concomitant with a dramatic reduction in the amount of the 3,398  $m/z$  peak supporting the presence of disulfide-linked  $\kappa$  peptides.

Whether MHC class II molecules, like many others, are constitutively recycled is unclear. Some studies would predict up to 60 recycling events occur during the lifetime of a class II molecule (16–19) whereas other reports detect no recycling (20–23). Because most studies have employed either pharmacologic strategies to block normal membrane trafficking and/or antibody tagging of surface receptors, cross-comparison of the data is difficult. Recently, L cell transfectants expressing MHC class II molecules and cytoplasmic tail deletion mutants of Ii have demonstrated that surface-expressed class II-Ii complexes can reenter endosomes, remove bound Ii and class II-peptide complexes return to the cell surface (24). However, the ability of surface class II molecules to reenter the endocytic pathway and then return to the cell surface may well be dependent on the species and/or cell type used. In cases where surface-expressed class II molecules do reenter the endocytic pathway, a fundamental obstacle of productive recycling is the slow dissociation of prebound peptides. The data presented here clearly demonstrate that exposure to pH 4 alone does not increase the release of most prebound peptides from class II complexes and is in agreement with data obtained by others. However, this pH does enhance the release of some Ii-derived peptides. In cases where a substantial fraction of the class II population contains Ii peptides (HLA-DR1 for example) this fraction might provide a means by which class II molecules reentering the endocytic pathway from the cell surface could exchange prebound Ii-derived peptides with peptides derived from other source proteins. However, the kinetic basis for such an exchange system has yet to be demonstrated. Moreover, the selective chemical release of Ii peptides at pH 4 *in vitro* examined here does not exclude the possibility that conditions or proteins present in endosomes *in vivo* might facilitate the release of Ii or other peptides bound to MHC class II molecules.

MHC-peptide binding studies have reached a new technological level. Not only can the amount of newly bound peptide be determined but the influence, if any, that the exogenously added peptide had on the preexisting peptide-class II complexes can be determined (compare references 15 and 25). For example, in the present study, most of the  $\kappa$  peptide binding was attributed to the replacement of prebound long-Ii peptides. In other MHC allotypes, with their distinct prebound peptide repertoires, other prominent self peptides may be specifically replaced. In fact, the outcome of these types of processes may help to explain why different class II allotypes bind different amounts of exogenously added peptide at equilibrium.

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