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MHC class II-bound self-peptides can be effectively separated by isoelectric focusing and bind optimally to their MHC class II restriction elements around pH 5·0

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SUMMARY

More than 90% of the major histocompatibility complex (MHC) class II molecules on antigen-presenting cells (APC) have in their binding site a peptide derived from an extracellular protein ingested by the APC or from a protein of the APC itself. These self-peptides can be eluted from affinity-purified MHC class II molecules by acid elution, and have been studied with a variety of techniques. We show here that the self-peptides eluted from the mouse MHC class II molecules A^d, E^d and E^k bind specifically to MHC class II molecules of the allelic type from which they were derived. The pH optimum for binding is around 5·0, i.e. the same optimum at which synthetic peptides representing sequences of foreign antigens bind to MHC class II molecules. This suggests that the physiological compartment where MHC class II molecules bind self-peptides may be very late in the endocytic pathway. The chemical properties of the eluted and labelled MHC class II peptides were studied by isoelectric focusing. This method was able to separate the peptides very efficiently, and enabled a rapid comparison of peptides eluted from different MHC molecules. The ^{125}I-labelled peptides displayed a broad range of isoelectric points with values predominantly below neutral. This suggests that such peptides bind to MHC in a predominantly non-charged state.

INTRODUCTION

Major histocompatibility complex (MHC) class II molecules bind peptides. Each MHC molecule binds one peptide in the binding cleft on the top of the molecule, and more than 90% of the class II molecules isolated from antigen-presenting cells (APC) contain a peptide.[1] Binding to MHC seems to protect effectively the peptides against further proteolytic degradation[2,3] and to enable the MHC molecules to transport the bound peptides to the cell surface and present them to T cells of the helper phenotype. The peptides are derived from proteins from several sources. Some are extracellular proteins endocytosed by the APC, including proteins secreted by the APC. Others are cellular proteins from the APC endocytic pathway and from the membrane proteins on the APC surface.[4] The peptides are created by proteolytic fragmentation in the endocytic pathway and their binding to the cell's MHC class II molecules occurs in an intracellular acid, as yet unidentified, compartment. We and others[5–10] have shown previously that the optimal pH for the association of antigenic peptides and MHC class II is around 5·0, suggesting that the physiological binding may occur very late in the endocytic pathway, possibly in the lysosome itself.[10] There are also ultrastructural data to support this idea.[11,12]

The peptides bound to MHC class II molecules are 10–25 amino acids long.[13] Many seem to be derived from protein segments having the potential to be in an amphipatic, α helical conformation.[14–16] The conformation of MHC class II-bound peptides is not known, although it is likely that they exist in an extended conformation, as do MHC class I-bound peptides.[17,18] The peptides which bind to a distinct MHC class II allele constitute a heterogeneous collection of many, perhaps thousands, of peptides, but, like the peptides which bind to MHC class I molecules,[19–22] each of them contains an allele-specific binding motif enabling them to bind to the particular MHC class II allele.[1,4,23–26] Most of the MHC class II/peptide-binding studies described earlier have been concerned with synthetic peptides derived from well-defined, exogenous, soluble protein antigens. It is clear, however, that most MHC class II molecules have their binding site occupied by peptides derived from proteins of the endocytic pathway, from proteins which are continuously endocytosed by the APC

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Abbreviations: A^k, E^k, A^d and E^d, murine MHC class II molecules of the k and d haplotypes, respectively; APC, antigen-presenting cell(s); HEL(Y46-61), a peptide derived from hen egg lysozyme amino acid positions 46–61; IEF, isoelectric focusing.

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from the extracellular environment, and by peptides derived from the APC's own surface molecules. Since these are not considered as antigens they have been named 'naturally processed peptides'[4,13,20,26] or 'self-peptides'.[1,21,22,27]

It is of considerable interest to learn more about the MHC-bound self-peptides and to develop efficient methods for analysing them. In this paper we have eluted the peptides from MHC class II molecules, radiolabelled them, and studied their capacity for binding directly to MHC class II molecules with a binding assay based on a gel filtration principle.[28] We report that peptides eluted from one allelic form of MHC class II molecule only bind to the same allelic form and that the optimal pH for their binding to the MHC class II molecule is around 5·0. Furthermore, we have analysed the physical properties of ^{125}I-labelled self-peptides by isoelectric focusing (IEF) and found that the range of isoelectric points displayed by these peptides is predominantly below neutral.

MATERIALS AND METHODS

Mice and large-scale production of MHC class II molecules

For the purpose of A^k and E^k production, the AKR-derived B-lymphoma cell line, AKTB-1b, was used.[3,10,28] It was maintained *in vivo* by serial transfer in (AKR × CF1) F_1 hybrid mice. Single-cell suspensions were obtained from AKTB-1b spleen tumours and red cells were removed by osmotic lysis. The BALB/c-derived B lymphoma, A20–1.11, was grown in large quantities in spinner cultures and used for A^d and E^d production.[3,10,28] The tumour cells were detergent lysed at 10^8 cells/ml in phosphate-buffered saline (PBS; pH 7·2) containing 1% Nonidet P(NP)-40, 50 mM iodoacetamide, 10 mM sodium orthovanadate and 1 mM phenyl methyl sulphonyl fluoride. The solution was cleared of debris by centrifugation (Sorvall SA-600, Wilmington, DE; 10,000 *g*, 20 min), and stored frozen at −80° until use.

MHC class II purification

This was done as previously described[3,10,28] using the monoclonal antibodies 14–4-4S (anti-E^k, E^d), MK.D6 (anti-A^d) and 10–3.6.2 (anti-A^k) covalently coupled at 10 mg/ml to cyanogen bromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). Briefly, these affinity matrices were charged with lysates containing the appropriate MHC class II molecules, washed extensively with PBS containing 0·5% NP-40 and 0·1% SDS, equilibrated in PBS containing 1% n-octylglucoside (Sigma, St Louis, MO), and eluted with 0·15 M NaCl containing 50 mM diethyl amine and 1% n-octylglucoside (pH 10·8). The eluate was immediately neutralized with 1/20 volume 2 M Tris (pH 6·8) and concentrated by vacuum dialysis (Sartorius). The protein content was determined using a micro bicinchoninic acid assay (Pierce, Rockford, IL) and checked by SDS–PAGE.

Elution and purification of self-peptides from MHC class II molecules

Five hundred micrograms of affinity-purified MHC class II molecules in concentrations ranging from 300 to 500 μg/ml in PBS was added to a Centricon 10 ultrafiltration device (Amicon, Lausanne, Switzerland; cat. no. 4205; cut-off 10,000 MW), and concentrated about 10 times in a centrifuge at 4000 *g*, 60 min. Two millilitres of PBS was added and the concentration procedure was repeated. This washing of MHC was repeated three times with PBS. One millilitre of double-distilled H_2O containing 0·1% trifluoro acetic acid, pH 2·0, was added to the concentrate and incubated 1 hr at 37°. The sample was concentrated again and the ultrafiltrate containing the peptides eluted from the MHC class II molecules was lyophilized.

Edmann degradation of eluted peptides

Samples of the lyophilized peptides were redissolved in 30% acetic acid and sequenced on an Applied Biosystems sequencer (model 477A; Woolston, U.K.) equipped with an on-line PTH-amino acid analyser (model 120A) according to the protocol supplied by the manufacturer.

Peptide synthesis

The peptide HEL(Y46–61) was synthesized automatically on KA resin (Novasyn, Nottingham, U.K.) using conventional FMOC chemistry on a crystal automatic peptide synthesizer, according to the recommendations of the manufacturer (Novasyn). After completion of the synthesis, protecting groups were removed, and the peptide was cleaved off the resin in the presence of a cocktail of scavengers (81·5% trifluoro acetic acid, 5% thioanisole, 2·5% ethane dithiol, 1% triisopropylsilane, 5% phenol), precipitated with ether, and lyophilized. The natural hen egg lysozyme peptide HEL(46–61) was extended with an amino-terminal tyrosine to ensure effective labelling with ^{125}I. The peptide was purified by reversed-phase high-pressure liquid chromatography (HPLC) (Merck, Darmstadt, Germany) before use, and the amino acid composition of the purified peptide was analysed using amino acid analysis. The sequence of HEL(Y46–61) is, in one-letter code, YNTDGSTDYGILQINSR.

Peptide radiolabelling

About 2 μg peptide was labelled with 1 mCi ^{125}I (Amersham, Birkerød, Denmark) using chloramine T.[29] The labelled peptide was separated from free iodine by Sephadex G10 (Pharmacia) size chromatography and examined by SDS–PAGE and autoradiography. The labelled peptide mixture was stored at −20° in PBS containing 25 mM 2-mercaptoethanol until use.

Peptide–MHC class II binding assay

Peptide–MHC complexes were produced by incubating ^{125}I-labelled peptide with affinity-purified MHC class II molecules (2–10 μM) at room temperature for 3 days.[3,10] This has been shown previously to be the optimal incubation time.[28] The reaction buffer contained 8 mM citrate, 17 mM phosphate, and 0·05% NP-40 (pH 5·0). The binding assays were conducted at room temperature. Peptide–MHC class II complexes were separated (in duplicate) from free peptide by gel filtration using G25 spun columns.[3,10] Seven hundred microlitres of G25 slurry in PBS containing 0·5% NP-40 was centrifuged at 100 *g* for 2 min in a 1-ml column. Fifteen microlitres of the same buffer was applied to the column and the centrifugation repeated. A 15-μl sample was applied to the column and centrifuged at 100 *g* for 2 min. Fifteen microlitres of buffer was applied again and the column was subsequently centrifuged at 100, 400 and 900 *g* for 2 min. The radioactivity of the excluded 'void' volume and of the included volume, respectively were

measured by γ spectrometry (Packard Instruments, Lombard, IL). The fraction of peptide bound to MHC class II relative to the total amount of offered peptide was calculated and corrected by subtracting the fraction of peptide that appeared in the void volume in the absence of MHC.

Isoelectric focusing (IEF) of self-peptides eluted from MHC class II molecules

Self-peptides eluted from MHC class II were analysed by one-dimensional IEF focusing in a pH gradient ranging from 4 to 9. Polyacrylamide gels (16 × 20 cm, T = 8%, C = 5%) containing 8 M urea, 2% NP-40 and 5·5% ampholite (Pharmacia) were casted in a vertical SDS–PAGE apparatus (Protean 2; BioRad, Richmond, CA). A 20-μl sample of radiolabelled self-peptide containing 500,000 c.p.m. dissolved in IEF-sample buffer [9·5 M urea, 2% (v/v), NP-40, 2% (v/v) ampholite, 5% (v/v) β-mercaptoethanol] was added to each well. An overlay of IEF sample buffer (10 μl/well) diluted 1 : 3 in double-distilled H_2O was added and a constant power of 20 W was applied to the gel for 4 hr. The cathode buffer used was 0·1 M NaOH and the anode buffer was 1 M H_3PO_4. These electrode buffers have been used previously by Righetti and Hjertén[30] for the purpose of preparative IEF of peptides. During this time the voltage increased from approximately 400 V to 2500 V. In order to measure the actual pH gradient in the gel, the gel sandwich was then immediately disassembled and a 1·5-cm lane cut out. This lane was cut into 0·5-cm slices and each was incubated for 30 min in 1·5 ml 0·1 M KCl solution. The pH in this solution was then measured and plotted against the distance from the gel top. After disassembly of the gel sandwich, the gel was immediately washed for 10 min in water, transferred to a Whatman filter paper and dried in a BioRad gel-dryer. Rapid handling of the gel was essential to avoid diffusion of the peptides and broadening of the gel bands. The gel bands were developed by autoradiography.

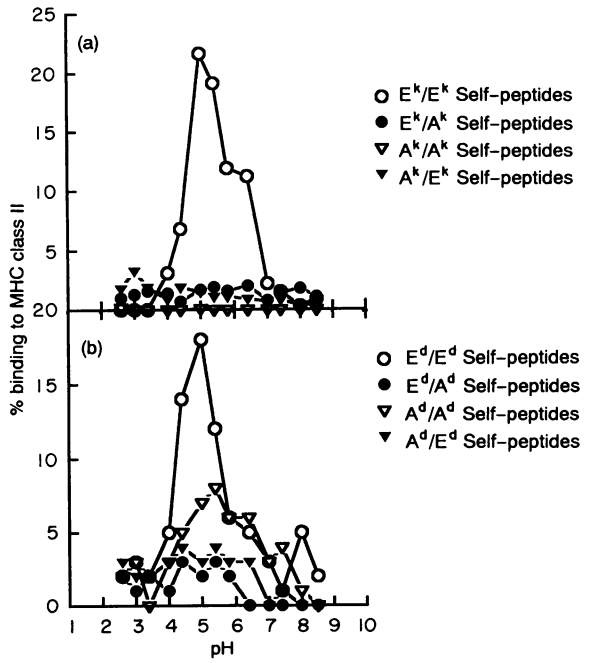

Figure 1. Binding of self-peptides to MHC class II molecules. Peptides were eluted from MHC class II molecules, radiolabelled with ^{125}I and their binding to the different allelic forms of MHC class II molecules was measured using the gel filtration assay. (a) Binding of self-peptides eluted from E^k and A^k to E^k and A^k class II molecules. (b) Binding of self-peptides eluted from E^d and A^d to E^d and A^d class II molecules.

RESULTS

The pH optimum for binding of eluted self-peptides to MHC class II molecules

Figure 1 shows the binding of eluted and labelled self-peptides to each of the four MHC class II molecules at different pH. Peptides eluted from E^k bound effectively to E^k, but not to A^k (Fig. 1a). The pH optimum for the binding was between 4·5 and 6·0. Peptides eluted from A^k failed to bind to A^k. Nor did they bind to E^k (Fig. 1a). Peptides eluted from E^d bound effectively to E^d, but not to A^d (Fig. 1b). The optimal pH for this binding was between 4·0 and 6·0. Finally, peptides eluted from A^d bound to A^d, although not very effectively. These peptides did not bind to E^d. The pH optimum for the binding was between 4·5 and 7·0 (Fig. 1b). The fraction of radiolabelled self-peptides appearing in the void volume of the spun column in the absence of MHC molecules was relatively high (6–15%) compared to the values when using synthetic peptides (usually less than 1%). These background values were all subtracted from the values when MHC molecules were present.

The optimal pH for the binding of HEL(Y46–61) to A^k

As shown above, peptides eluted from A^k molecules failed to bind to A^k. This was unexpected, but the same result was obtained in three out of three experiments using different batches of A^k molecules and of eluted peptides. We therefore measured the binding of the synthetic peptide, HEL(Y46–61) to a new batch of A^k. HEL(Y46–61) uses A^k as its functional restriction element and is known to be an avid binder to A^k.[10] The binding of HEL(Y46–61) and of peptides eluted from A^k to the new batch of A^k were then compared. Figure 2 shows that HEL(Y46–61) bound effectively to A^k with a pH optimum around 5·0. The labelled peptides eluted from A^k again failed to

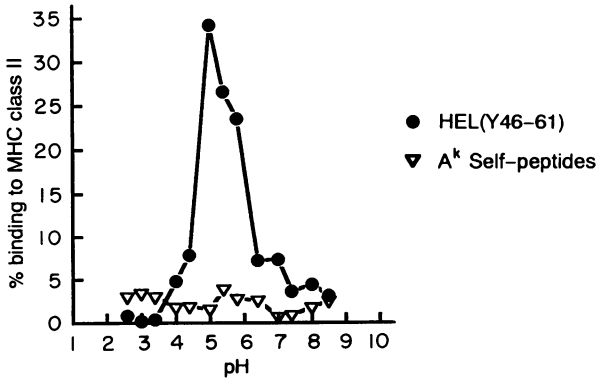

Figure 2. Binding of self-peptides eluted from A^k and of synthetic HEL(Y46–61) peptide to A^k MHC class II molecules. The peptides were radiolabelled with ^{125}I and their binding to A^k was measured with the gel filtration assay.

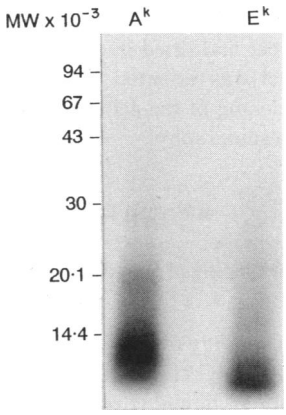

Figure 3. Autoradiograph of a SDS–PAGE of self-peptides eluted from MHC class II with trifluro acetic acid, radiolabelled with ^{125}I and ultrafiltrated on an Amicon filter, Centricon 10.

bind. Thus the A^k molecules of this batch were capable of peptide binding, but the peptides eluted from A^k could not bind to them.

Edmann degradation of acid-eluted peptides from MHC class II

The mixtures of peptides released from A^k and E^k molecules, respectively, were applied directly to the amino acid sequencer to analyse the size of the peptides. The maximum size of the peptides was around 20 amino acid residues, since all PTH amino acids reached a constant base-line level within this interval. There was no preponderance of distinct amino acids at distinct positions in the sequenced peptides that might suggest the occurrence of a common binding motif (data not shown).

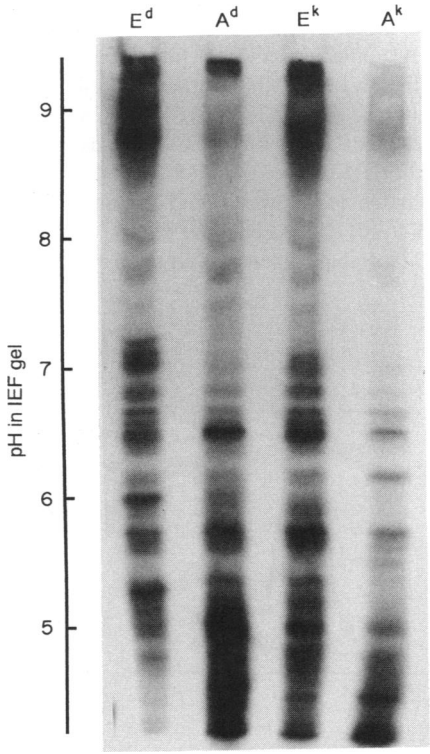

Figure 4. Autoradiograph of IEF gels of peptides eluted from MHC class II molecules and labelled with ^{125}I. E^d, A^d, E^k and A^k indicate the source of the eluted peptides.

SDS–PAGE and autoradiography of labelled peptides

Peptides were acid eluted from affinity-purified MHC class II molecules E^d, A^d, E^k, and A^k at pH 2·0, and were separated from the MHC molecules by ultrafiltration. The purified peptides were labelled with ^{125}I using chloramine T. Figure 3 shows a SDS–PAGE autoradiograph of the labelled peptides. All of the radiolabelled material is of MW lower than 15,000, indicating that the peptides had been successfully separated from the MHC class II molecules. Even when gels were exposed for a very long time, no trace of MHC was seen (data not shown).

Isoelectric points of peptides eluted from MHC class II molecules

The four preparations of labelled peptides were subjected to IEF in a linear pH gradient from 4 to 9. Figure 4 shows autoradiographs of the focusing gels. Fifteen to 30 distinct bands appeared in each lane, indicating that some of the self-peptides had been effectively separated. Nearly all of the labelled peptides had isoelectric points below neutral.

DISCUSSION

We have studied some of the properties of peptides eluted from mouse MHC class II molecules. The peptides were eluted from affinity-purified MHC class II molecules using trifluoro acetic acid and were radiolabelled with ^{125}I for the purpose of tracing. The acid-eluted material migrated in SDS–PAGE with MW below 15,000. The low MW peptide nature was analysed further by Edmann degradation. Each cycle yielded a heterogenous collection of PTH amino acids, but a constant base-line level was reached after approximately 20 cycles (data not shown). Thus, by far, most of the eluted material consisted of peptides of no more than approximately 20 amino acids. This is in agreement with results published by other investigators.[23–25] The molecular weight of some of the eluted peptides, as detected by SDS–PAGE (Fig. 3), seemed to be higher than 2500, which would correspond to a peptide size of about 20 amino acids. Buus *et al.*[1] previously estimated the molecular mass of self-peptides using gel-filtration. Although the peptides were found using this technique to have an average MW of around 3000, many peptides also seemed to be much larger.[1] The reason for the apparent contradiction between these observations and the results obtained with Edmann degradation is not clear. It may be due to aggregation of some peptides.

No signs of anchor positions[21] were observed during the Edmann degradation of the self-peptides. This may be due to the fact that MHC class II-bound peptides, contrary to MHC class I peptides, have ragged ends[23–25] sticking out of the binding grove,[31] which implies that the N-termini are not synchronized during the Edmann degradation process. In this way possible anchor residues will not appear at certain cycle positions.

The MHC class II molecules, A^k and E^k, were purified from AKTB lymphoma cells grown in mice and taken directly from

the spleen. Hence the peptides associated with A^k and E^k must derive from extracellular proteins of the mouse itself ingested by the AKTB lymphoma cells, and from proteins of the endocytic pathway and the cell surface of the AKTB lymphoma cells. In contrast, the MHC class II molecules A^d and E^d were purified from A20–1.11 lymphoma cells grown in culture medium with fetal calf serum. Hence some of the peptides associated with A^d and E^d were also derived from fetal calf serum. In this paper we have used the name self-peptides for those peptides which result from the constitutive ingestion and processing of extracellular proteins, in order to distinguish them from peptides derived from well-defined foreign antigens, in spite of the fact that some of them are derived from the fetal calf serum in the culture medium.

The peptides eluted from one allelic form of MHC class II molecule did, with the exception discussed below, bind to MHC class II molecules of the same allelic form, but not to MHC class II molecules of a different form. Thus, the peptides eluted from E^k bound to E^k but not to A^k, peptides from A^d bound to A^d but not to E^d, and peptides from E^d bound to E^d but not to A^d. Binding of MHC-eluted peptides to the same allelic form of MHC class II molecules has been demonstrated previously indirectly by others using a competitive inhibition binding assay.[1] In this work the binding of the eluted and labelled peptides was measured directly, and at different pH values. Binding was found to be optimal at a pH of around 5·0. Thus, the very low pH optimum for the binding of antigenic synthetic peptides demonstrated by several groups[5–10] holds true also for peptides derived from autologous proteins. This is the first direct demonstration of a low pH optimum for the binding of self-peptides to MHC class II molecules. Our finding supports the concept that the physiological binding of processed peptide to MHC class II molecules occurs at very low pH in a lysosome-like cellular compartment.[10–12] It is tempting to speculate that the MHC class II molecule at low pH may attain a special conformation in the binding site which favours the binding of a peptide.

The fraction of radiolabelled self-peptides appearing in the void volume of the spun column in the absence of MHC molecules was found to be relatively high (6–15%) compared to the values when using the synthetic peptides (usually less than 1%). The reason for that is not clear, since SDS–PAGE of the labelled material followed by autoradiography clearly showed a complete absence of high molecular weight material (Fig. 3) which might pass through the spun column in the void volume. The explanation could be that a significant number of the eluted peptides had an amphipatic structure.[14,15] We have observed that synthetic peptides having a defined amphipatic structure show a tendency to adhere to detergent micelles (S. Mouritsen, unpublished observation). Such micelle–peptide complexes will pass through the spun columns in the void volume.

One of the MHC class II molecules studied here, A^k, failed to bind labelled peptides eluted from A^k. In parallel experiments A^k did efficiently bind the synthetic peptide HEL(Y46–61), for which A^k is the functional restriction element. A possible explanation could be that the ^{125}I-labelling of the eluted peptides destroyed their ability to bind because a tyrosine is one of the anchor residues constituting the binding motif. This, however, is not likely to be the reason for the lack of binding, since the ^{125}I-labelled peptide HEL(46–61), which has only one tyrosine in the middle of the peptide, bound equally well to A^k compared to HEL(Y46–61) (data not shown). Alternatively, among the eluted self-peptides there may exist some dominating tyrosine-free peptides with high affinity for A^k and which outcompete the ^{125}I-labelled self-peptides for binding to A^k.

An intriguing observation was that more than 80% of the peptides which were eluted from each of the four MHC molecules and subsequently labelled with ^{125}I displayed isoelectric points between 4·5 and 6·0. This suggests that these radiolabelled peptides bind to MHC class II molecules in a predominantly non-charged state. Provided that the radiolabelling procedure did not interfere significantly with the pI of the self-peptides, this would mean that peptide back-bone interactions are more important than charge interactions of the peptide side chains in the binding groove of MHC. Convincing evidence for this has been presented recently.[32] An important explanation for the observed low pI range of the labelled self-peptides may also be that iodination of peptides interferes with the charge of the labelled peptides, making them more acidic.[33] This possibility is, however, difficult to study using the technique described here, since short, non-labelled peptides are very difficult to visualize in IEF gels.[34]

The IEF technique used here appears to be quite powerful in resolving the heterogenous collection of self-peptides from a class II molecule into a number of distinct bands. The eluate from each class II molecule yielded between 15 and 30 bands. Each band represented one or more tyrosine-containing self-peptide present in relatively large quantities on the MHC class II molecules of the cell. It was possible to identify clearly self-peptides of one allelic type of MHC class II molecule which were not present on the other types (Fig. 4). Because of its very high resolving power for small peptides,[34] IEF seems to be very suitable for analysis of the peptides eluted from MHC molecules. This can also be achieved very efficiently using electron spray mass spectrometry,[19] but the method described here may be useful as an inexpensive and rapid way to compare MHC-bound peptides from different MHC molecules, or for the comparison of peptides eluted from identical MHC molecules isolated from different cells.

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