Journal of Chromatography, 548 (1991) 343–350
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2193

Non-radioactive detection of MHC class II–peptide antigen complexes in the sub-picomole range by high-performance size-exclusion chromatography with fluorescence detection

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ABSTRACT

In order to avoid chemical or structural modification of T-cell epitopes by labelling, a high-performance size-exclusion chromatographic fluorescence binding assay was developed, based on the intrinsic Trp fluorescence of major histocompatibility complex (MHC) proteins. The increase in Trp fluorescence intensity of the isolated human MHC product HLA-DR 1 on complex formation with unlabelled influenza matrix peptide[18–29] (IM[18–29]) was examined. Binding of IM[18–29] to the heterodimeric form of HLA-DR 1 (K_d = 4.8 m*M*) and to the disassembled α-and β-subunits (K_d = 9.2 m*M*) could be demonstrated. In addition, the assay showed the peptide-induced formation of a dimeric conformer of HLA-DR 1, the nature of which is still undefined. Detection of HLA-DR 1 subunit–peptide complexes was possible in amounts of 25 ng in 10 μl (80 fmol/μl). The technique proved to be reproducible and less time consuming than common methods that need fluorescence or radioactive labelling.

INTRODUCTION

Human lymphocyte antigens DR (HLA-DR) are products of the human major histocompatibility complex (MHC) class II and consist of two non-covalently linked glycoprotein subunits: α (34 kilodalton) and β (29 kilodalton) [1]. The N-terminal domains of each subunit consitute the antigen binding site. According to the hypothetical model of class II proteins [2], deduced from the X-ray crystallographic analysis of the class I molecule HLA-A2, the binding groove is characterized by two lateral α-helices lying on a floor formed by β-pleated sheets [3,4]. Polymorphic positions, which explain the peptide binding specifities of different HLA subtypes, accumulate significantly in this region. Non-immunogenic self-peptides, originating from the endogenous degradation of self-proteins [5,6], and immunogenic foreign peptides are assumed to compete for the same binding site [7]. However, only the latter are succesfully presented to a corresponding T-cell receptor leading to an immune response [8]. Self-peptides co-eluting and co-crystallizing with HLA isolates [2,9], are the reason why only a small fraction (1–15%) of the HLA molecules is available for binding exogenous peptides. Previous binding studies using immunogenic peptides and solubilized or native MHC molecules in cellular systems have shown this [5,10].

0021-9673/91/$03.50

In order to work out the rules that make HLA proteins potent peptide receptors with a very broad binding specificity, short synthetic peptides are systematically tested for their HLA affinities [11–13]. Influenza matrix peptide[18–29] (IM[18–29]) is one of the T-cell epitopes of influenza matrix protein. IM[18–29] is HLA-DR 1-restricted, direct binding to living Epstein–Barr virus transformed B-cell lines being shown with the radiolabelled peptide [10,14]. In addition to radioiodination of peptides, often extra-tyrosylation, as in the case above, or coupling of bulky fluorophores is necessary to obtain sufficient signal intensities. Our idea was to establish a binding assay without modifying the sequence of the T-cell epitopes. Leaving IM[18–29] and the purified HLA-DR 1 molecule in their natural state, we co-incubated them and investigated the change in intrinsic Trp fluorescence on formation of the bimolecular complexes. The amount and the nature of the so-formed HLA-DR 1–IM[18–29] complexes were analysed by high-performance six-exclusion chromatography (HPSEC) using fluorescence detection.

EXPERIMENTAL

Chemicals and reagents

High-performance liquid chromatographic (HPLC)-grade water and 3-[3-(cholamidopropyl)dimethylamino]-1-propanesulphonate (CHAPS) were obtained from E. Merck (Darmstadt, Germany). All buffers were filtered through 0.45-μm filters (Millipore, Eschborn, Germany) and degassed with helium.

Chromatography

The chromatographic system consisted of an L 6200 HPLC intelligent pump (Merck–Hitachi, Darmstadt, Germany) connected to a Merck–Hitachi F 1050 spectrofluorimeter operating at an excitation wavelength of 285 nm and an emission wavelength of 335 nm. The integrator was a Model D2500 from Merck–Hitachi. Separation was achieved on a Superose 12 HR 10/30 column (Pharmacia, Freiburg, Germany) using 0.1 *M* sodium phosphate buffer (pH 7.0) containing 0.025% (w/v) CHAPS (HPSEC buffer). The column was operated at a flow-rate of 0.6 ml/min and a pressure of 200 p.s.i. For peptide purification, the HPLC system was connected to an L-4000 UV detector (Merck–Hitachi).

Peptide synthesis and purification

Peptides were synthesized by continuous-flow solid-phase peptide synthesis using a MilliGen 9050 peptide synthesizer based on Fmoc strategy. All peptides were purified by HPLC using a Nucleosil C_{18} column (250 × 10 mm I.D., 7 μm particle size) (Macherey, Nagel & Co., Düren, Germany). The mobile phase contained (A) 0.1% triffluoroacetic acid (TFA) and (B) acetonitrile–0.1% TFA (80:20, v/v) and elution was performed by a gradient starting from 20% B to 100% B in 30 min. The flow-rate was 2.5 ml/min. All separations were monitored at 214 nm. After lyophilization, peptides were analysed by amino acid analysis and by sequencing using an Applied Biosystems (Foster City, CA, USA) 477 A sequencer.

Preparation of biological samples

HLA-DR 1 was purified by immunoaffinity chromatography essentially as de-

scribed by Gorga *et al.* [15] using the Epstein–Barr virus-transformed B-cell line WT-100 (BIS), which is homozygous at the DR locus. Change of buffer and detergent was carried out by ultrafiltration through membranes of Microsep microconcentrators, size exclusion 100 kilodalton (Filtron, Karlstein, Germany). The new buffer contained 100 m*M* sodium phosphate (pH 8.0) and 0.025% (w/v) CHAPS and was used to adjust the final protein concentration to 0.2 μg/μl. Protein content was evaluated by the Bradford protein assay. Purified HLA-DR 1 was kept in a stock solution at 4°C for 1 month prior to use in the HPSEC assay.

The integrity of all HLA-DR 1 samples was routinely tested by sodium dodecyl sulphate polyacrylamide gel electrophoresis, (SDS-PAGE) immunoblotting and HPSEC analysis.

Binding assay

Aliquots of 2 μl of the stock solution of HLA-DR 1 were co-incubated with various doses of synthetic influenza matrix peptide [18–29], using a peptide stock solution of 10 μg/μl. The stock solution was prepared by dissolving the lyophilized peptide in HPSEC buffer. The peptide concentrations ranged from 0.1 to 5 m*M*, the final assay volume being adjusted to 40 μl with HPSEC buffer. The samples were incubated for 50–120 h at room temperature. Aliquots of 10 μl were used for HPSEC analysis.

Release assay

After a 120-h incubation at 20-μl aliquot of the binding assay was adjusted to pH 2.0 with 1 *M* HCl. After 10 min the sample was readjusted to pH 7.0 with 2 *M* NaOH and analysed by HPSEC.

Scatchard plot analysis

The dissociation constant, K_d, in the binding assays was calculated using the following Scatchard equation [16]:

F_{335}/c(HLA-DR 1)c(peptide) = $1/K_d - F_{335}/c$(HLA-DR 1) K_d where F_{335} is the measured fluorescence (proportional to the concentration of bound peptide), c(HLA-DR 1) and c(peptide) are the concentrations of HLA-DR 1 and IM[18–29], respectively. K_d can be obtained from the slope of the Scatchard plot of F_{335}/c(HLA-DR 1)c(peptide) *vs.* F_{335}/c(HLA-DR 1).

RESULTS AND DISCUSSION

Purified heterodimeric HLA-DR 1 partially dissociates into the α- and β-subunits in dilutions $\leqslant$0.2 mg/ml. The extent of dissociation of the isolates used in our study was calculated by comparing the peak areas of the dimer (a) and the monomer peaks (b) in the HPSEC analysis (Fig. 1). Detecting Trp fluorescence, the relationship was dimers : monomers = 22:78. The same result was obtained with UV detection (not shown). The resolution in SEC is not high enough to distinghuish the subunits, the latter therefore co-eluting with peak b. Peak a disappears quantitatively in favour of peak b on acidification of the sample (see Fig. 4). This was assumed to happen because under these conditions self-peptides can be released from the MHC molecule, destabilizing the heterodimer [7]. In contrast, fresh samples in dilutions $>$0.5 mg/ml

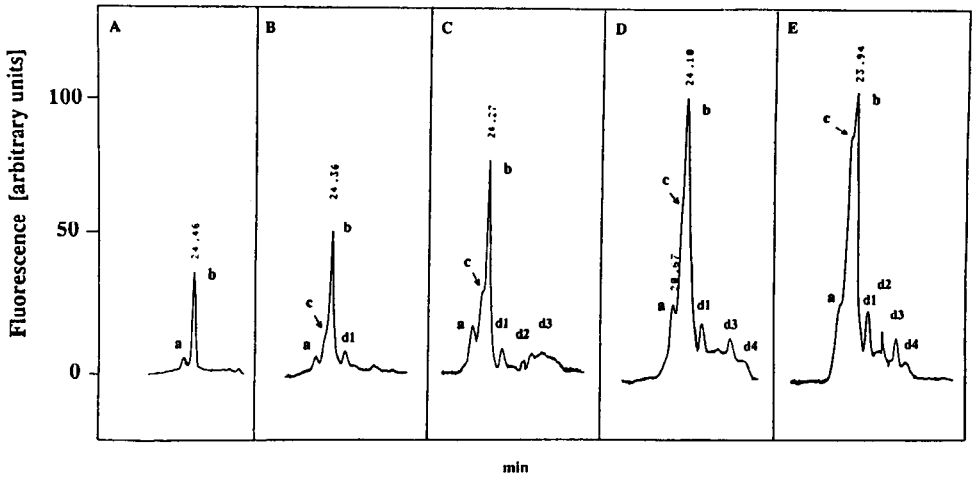

Fig. 1. HPSEC of purified HLA-DR 1 (250 n*M*) after 120-h co-incubation with various doses of influenza matrix peptide[18–29]. Fluorescence detection, attenuation 32. Peptide concentrations: (A) 0; (B) 2; (C) 3; (D) 4; (E) 5 m*M*. Peaks: a = HLA-DR 1 heterodimers (floppy conformer); b = HLA-DR 1 subunits; c = HLA-DR 1 dimers (compact conformer); d_1–d_4 = endogenous peptides.

show a predominant peak a, representing 90% of the total fluorescence (not shown). Hence it is certain that it is the subunits eluting with peak b.

The basis for the peptide-binding assay is the fact that two Trp residues, Trp-9 and Trp-61 of the β-subunit, being part of the peptide-binding groove of HLA-DR 1, increase their fluorescence intensities on interaction with a peptide [17]. Using this principle, the peptide-binding assay reveals four significant tendencies, three of which have never been reported in comparable chromatographic assays with MHC class II molecules (Fig. 1): (1) peak a, denoting the heterodimer, increases with increasing peptide concentration, reaching its maximum at 4 m*M* IM[18–29]; (2) peak b, denoting the HLA-DR 1 subunits, shows the same characteristics; (3) addition of peptide leads to the appearance of a new species (peak c), located between the heterodimer and the monomer peaks, with an apparent molecular weight of 50–55 kilodalton; peak c, in contrast to peaks and peak b, is still growing at the highest peptide concentration tested; (4) fluorescent low-molecular-weight material elutes (peaks d_1–d_4), the maxima at 4 m*M* IM[18–29] coinciding with the maximum of peaks a and b, respectively, albeit added IM[18–29] shows no fluorescence signal.

The results confirm the data we obtained with FITC- and biotin-labelled influenza matrix peptide [17] and two observations made previously with isolated MHC class II molecules analysed by SDS-PAGE: under non-reducing conditions bimolecular complexes of T-cell epitopes and the α- or β-subunit are stable [18,19]. The second fact was the appearance of a 55 kilodalton band originating from HLA-DR heterodimers [15,18]. This band was ascribed to a compact dimeric conformer which is distinct from the floppy conformer, with a band of 64 kilodalton. Peak c in our binding assay (Fig. 1) seems to be the chromatographic equivalent of the compact conformer, its formation being induced by IM[18–29]. As this requires a trimolecular association, this process is bound to proceed more slowly than the formation of the

bimolecular complexes. This explains the result of the binding kinetics shown in Fig. 2: the intensity of the monomer peak has nearly doubled after a 50-h incubation, whereas the signal of the compact conformer cannot be identified. We have to postulate that IM[18–29] is capable of cross-linking disassembled α- and β-subunits. We cannot rule out the formation of $\alpha\alpha$- and/or $\beta\beta$-homodimers under the influence of connecting peptides. Interestingly, peptides functioning as chaperonins *in vivo* have already been documented with MHC class I molecules [20]. It seems that the rules for building up a quaternary structure are the same for both class I and class II molecules.

The dissociation constants characterizing the affinity of IM[18–29] to the monomeric and dimeric HLA-DR 1 were calculated according to Scatchard (Fig. 3). The slopes of the Scatchard plots indicate that binding to the heterodimer ($K_d = 4.8$ mM) is greater than that to the subunits ($K_d = 8.5$ mM). The data can be explained by the fact that the heterodimer possesses all the elements constituting the peptide-binding groove whereas each individual monomer does not. A compariso of K_d(heterodimer) = 4.9 mM with the few values available for other T-cell epitopes, ranging from 2 to 10 μM, suggests that IM[18–29] belongs to a class of low-affinity peptides. K_d calculations with the original influenze matrix peptide [17–31] have not been done. Data of Ceppellini *et al.* suggest that IM[17–29] has a high affinity to HLA-DR 1 [10]. The lack of N-terminal serine might be the reason for the high K_d value of IM[28–29]. Another reason could be that binding of exogenous peptides to HLA molecules is a two-step phenomenon: using low peptide-concentrations ($\leqslant 1$ mM) only binding to

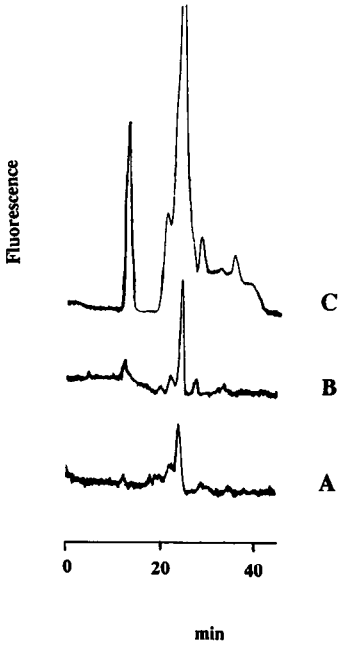

Fig. 2. HPSEC in a kinetic study using HLA-DR 1 (250 nM) and IM[18–29] (3 mM). Incubation time: (A) 0; (B) 50; (C) 120 h.

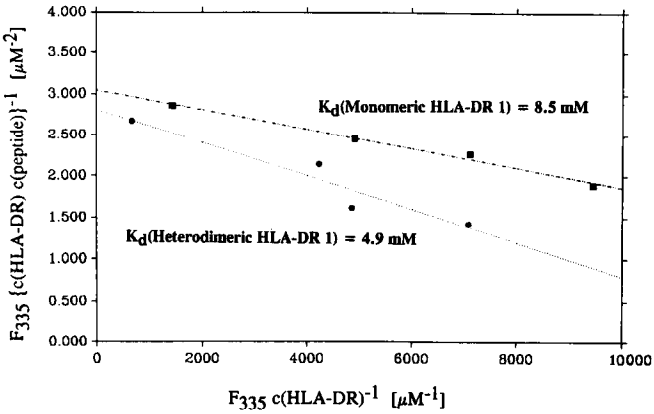

Fig. 3. Scatchard plot. The date originate from the HPSEC binding assay using changes in the intrinsic Trp fluorescence on binding IM[18–29]. K_d(monomeric HLA-DR) = 8.5 mM; K_d(heterodimeric HLA-DR 1) = 4.9 mM.

the fraction of empty HLA molecules is detectable, proceeding with $K_d \approx 2$–10 μM. Co-incubation with a high peptide excess (> 1 mM) leads to competitive displacement of endogenous peptides with the remaining 90% of HLA molecules, either dimeric or monomeric, proceeding with $K_d \approx 5$–10 mM. Evidence for the accuracy of the displacement idea is given by the low-molecular-weight fraction (peaks d_1–d_4), eluting with increasing proportions of bound IM[18–29] (Fig. 1). The fluorescence of the low-molecular-weight material must be ascribed to Trp-containing endogenous peptides, as there is no further increase on addition of IM[18–29] at a concentration of 5 mM. Additional proof of the relevance of the endogenous peptide fraction (EPF) is given by the fact that readdition of EPF to released HLA-DR 1 restores the initial fluorescence intensity [21].

In order to test whether bound IM[18–29] can be released from HLA-DR 1 under the same conditions as endogenous peptides, we acidified HLA-DR 1-IM[18–29] complexes after a 125h-incubation. The result is depicted in Fig. 4: pH 2.0 treatment for 10 min leads to a decline of peaks a, b and c to the starting intensities (*cf.* Fig. 1D), leaving constant the total intensities of peaks d_1 and d_2. Obviously, the bi- and trimolecular complexes dissociated nearly completely. Because no extra fluorescence appeared in the low-molecular-weight range, probably all self-peptides had been previously displaced by IM[18–29]. The increase in the signal of the void volume (800 kilodalton) could be explained by acid-induced additional high-molecular-weight micelles containing trapped HLA-DR 1-IM[18–29] complexes that are not able to dissociate because of dense packing. The formation of high-molecular-weight micelles is also found with high protein and peptide concentrations (*cf.* Figs. 1 and 4), but can be neglected as the relationship of HLA-DR 1 species is unbiased (not shown).

Considering the sensitivity of this peptide-binding assay, in standard measurements 2.5 pmol of purified HLA-DR 1 per HPSEC run were sufficient to obtain the signal shown above, using a signal attenuation of 32. With a signal attenuation of 4, we were able to detect 800 fmol of HLA-DR 1–peptide complexes, in significant

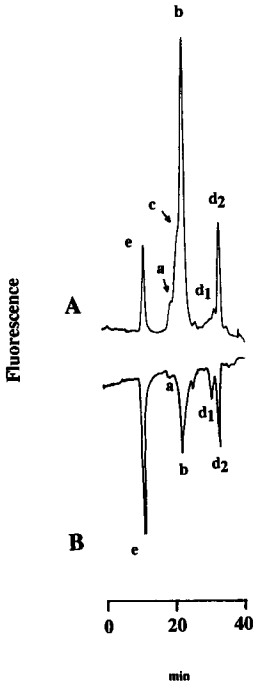

Fig. 4. HPSEC of purified HLA-DR 1 (200 m*M*) after 120-h incubation with IM[18–29] (4 m*M*). Fluorescence detection, attenuation 32. (A) Before acid treatment; (B) after acid release (pH 2.0, 10 min) of endogenous peptides. Peaks: a = HLA-DR 1 heterodimers (floppy conformer); b = HLA-DR 1 subunits; c = HLA-DR 1 dimers (compact conformer); d_1–d_4 = endogenous peptides; e = oligomeric HLA-DR 1.

contrast to the background fluorescence (not shown). Therefore, the assay has a sensitivity comparable to those of standard assays using fluorescence or radioactively labelled peptides. Advantages of the Trp fluorescence assay are that there is no need to label the peptides and the HPSEC system requires less than 1 h per analytical run. In addition, for the first time diferent molecular weight species of HLA-DR–peptide complexes could be distinghuised by a chromatographic system.

ACKNOWLEDGEMENTS

We thank M. Stöhr for the synthesis and purification of the peptides. We are grateful to I. Bohlinger and H. Max for helpful discussions. This work was supported by the Sonderforschungsbereich 120.

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