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Interactions of the human class II major histocompatibility complex protein HLA-DR4 with a peptide ligand demonstrated by affinity capillary electrophoresis

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Abstract

The interactions of empty recombinant major histocompatibility complex (MHC) class II molecules (*DRA1*0101/DRB1*0401*) with a known peptide ligand [the HA(307–319) fragment of influenza virus hemagglutinin] were studied by capillary electrophoresis. Using an alkaline buffer system with the addition of non-ionic or zwitterionic detergent and high sensitivity laser-induced fluorescence detection, both slowly and rapidly equilibrating binding could be demonstrated. This was accomplished using a pre-equilibration approach as well as migration shift experiments where receptor molecules were added to the electrophoresis buffer. This system may be useful for the study of both peptide binding to MHC molecules and screening for inhibition or amplification of binding by other ligands as well as for the study of the interactions of T-cell receptors with MHC–peptide complexes. © 1997 Elsevier Science B.V.

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1. Introduction

Major histocompatibility complex (MHC) molecules of both class I and class II are highly polymorphic membrane glycoproteins that present antigenic peptides for T-lymphocytes [1]. The class I molecules are structurally different from class II molecules, are found on most nucleated cells and seem to bind peptides derived from endogenous proteins. Class II molecules have a more restricted cellular distribution and predominantly present peptide fragments derived from exogenous proteins [2,3].

The binding of peptides by MHC molecules and the subsequent interactions between the peptide–MHC complexes and specific T-cell receptors are of central importance for the regulation of immune responses in health and disease and have, therefore, been studied intensively by a variety of techniques [4,5]. It appears that even though the binding of peptides to MHC molecules may be of medium to high affinity (dissociation constants in the μM–nM range), binding to a given MHC molecule is possible for a quite diverse array of peptides with structural constraints posed only on a few anchor residues [5–7]. However, once formed, the peptide–MHC complex is very stable [8]. This stability means that the majority (>80%) of MHC molecules purified from natural sources are already occupied by tightly

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bound peptide ligands acquired in vivo [5,8]. This complicates the execution and interpretation of binding assays using purified MHC molecules from natural sources and has led to the establishment of in vitro expression systems for the production of apparently empty MHC molecules [9].

Another potential problem associated with MHC–peptide interaction studies is that the quite slow association rates necessitate incubations for extended time periods (10 h or more) at 25–37°C [5–10]. The techniques traditionally used to separate complexed- from free peptide, such as spin ultrafiltration or gel filtration [10], HPLC gel filtration [9], solid-phase immunocapture assays [9] or flow cytometry of cell-bound ligand [6], do not necessarily account for the peptide ligand integrity after such incubations. Furthermore, these techniques rely, in many cases, on secondary reagents and labelled molecules.

We are therefore exploring the potential of capillary electrophoresis for use in the characterization of the binding interactions of a *Drosophila* cell-expressed full length human class II molecule, HLA-DR4 (rheumatoid arthritis-associated genotype [11]: *HLA DRB1*0401*) with a well-characterized MHC class II molecule ligand [4], the influenza virus hemagglutinin peptide fragment comprising residues 307–319 [HA(307–319)].

Electrophoresis has been quite widely applied for the characterization of various interacting molecules (for reviews, see e.g. [12,13]). The method uses different migrations of free analyte compared to complexed analyte to quantitate and characterize binding interactions [12–15]. The approach obviously requires that the migration velocity of the analyte is influenced by binding to its receptor molecule. However, with the high dependency of analyte mobility on charge/mass ratios in free-solution capillary electrophoresis (CE) [14,16,17], this was expected to occur in the present study where a small peptide is binding to an approximately 40-times larger receptor molecule.

We present here the initial results on MHC-peptide binding in a system where CE is combined with laser-induced fluorescence (LIF) detection to get sufficient sensitivity to enable the characterization of high-affinity binding interactions.

2. Experimental

2.1. Reagents and materials

All chemicals were of analytical grade from Sigma (St. Louis, MO, USA), except where otherwise stated. CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate} came from Serva (Heidelberg, Germany) and HPLC-grade water was from Merck (Darmstadt, Germany). N-glycosidase F and Complete protease inhibitor tablets were from Boehringer (Mannheim, Germany). Protein G Sepharose 4 Fast Flow was from Pharmacia Biotech (Uppsala, Sweden). Fluorescein-5-thiosemicarbazide was obtained from Molecular Probes (Eugene, OR, USA). Synthetic peptides, HA(307–319) (H-PKYVKQNTLKLAT-OH), corresponding to influenza virus hemagglutinin residues 307–319, and N-terminally fluoresceinylated HA(307–319) (FL-HA(307–319)) were made by Schafer-N (Copenhagen, Denmark). Peptide masses were verified by mass spectrometry on a Voyager Elite MALDI instrument (Perseptive Biosystems), with delayed ion extraction using 3-hydroxypicolinic acid as a matrix [18]. The expected and observed molecular masses agreed to within 1.5 u. FL-HA(307–319) was subjected to an additional reversed-phase high-performance liquid chromatographic (HPLC) C_{18} purification step before being used in binding assays. The purified component was quantitated after being dried down and resolubilized in HPLC-grade water by means of an HPLC-derived standard curve ($r^2 > 0.99$). The monoclonal LB3.1 IgG_{2b} antibody, specific for the α-chain of native DRαβ heterodimers [19], was coupled to CNBr-activated Sepharose (Pharmacia, Lund, Sweden) according to the instructions of the manufacturer.

2.2. Production and isolation of HLA-DR4 molecules

The human MHC class II full-length HLA-DR4 molecule was produced in *Drosophila melanogaster* Schneider S2 cells, as will be described elsewhere. Briefly, the cells were stably transfected with cDNAs of *HLA-DRA1*0101* (α-chain) and *HLA-DRB1*0401* (β-chain) cloned into two separate

expression vectors controlled by a Cu^{2+}-inducible promoter [20]. The recombinant HLA-DR4 was purified after detergent lysis of the *Drosophila* cells by immunoaffinity chromatography on a Sepharose-coupled monoclonal antibody, LB3.1. The final HLA-DR4 protein concentration, as estimated by a BCA protein assay from Pierce (Rockford, IL, USA), was 1–2 mg/ml in phosphate buffered saline (PBS), pH 7.4, containing 1% (v/v) β-octyl glucoside and 0.1% (w/v) NaN_3. The peptide binding of the purified MHC molecules was ascertained in an inhibition assay, essentially as described in Ref. [5].

2.3. Carbohydrate-specific HLA-DR4 fluoresceinylation

HLA-DR4 molecules, produced as described above, were labelled with fluorescein on their carbohydrate moieties using fluorescein-5-thiosemicarbazide after periodate oxidation [21]. A 10-μl volume of HLA-DR4 (1.87 mg/ml) was diluted with 100 μl of PBS, pH 7.4, containing 1% (v/v) CHAPS (PBS–CHAPS) and was then applied to a 200-μl LB3.1–Sepharose column. After washing with 5 ml of PBS–CHAPS, 100 μl of 18 m*M* freshly prepared $NaIO_4$ was mixed with the column material and incubated in the dark at room temperature for 30 min. The column was then washed as before and 100 μl of freshly prepared 10 m*M* fluorescein-thiosemicarbazide in dimethylsulfoxide was mixed with the column material and incubated at room temperature for 1 h. After washing with 5 ml of PBS–CHAPS, the column was eluted with 50 m*M* sodium phosphate–NaOH, pH 11.5, containing 1% (v/v) CHAPS. Fractions of 300 μl were collected into 30 μl of 2 *M* Tris–HCl, pH 6.0, to maintain the pH at 7–8. The fractions were tested for HLA-DR-positive material by dot-blotting using the LB3.1 antibody (9 mg/ml) diluted 1:4000. LB3.1-reactive fractions were pooled and spin-concentrated with simultaneous buffer change to PBS–CHAPS using M_r cut-off 30 000 ultrafiltration devices (Millipore, Bedford, MA, USA).

2.4. Capillary electrophoresis (CE) experiments

A Beckman P/ACE 2050 instrument equipped with a laser-induced fluorescence detector was used. Fluorescence was induced by an argon-laser using a band pass filter at 480 nm and a 520-nm emission filter. A detector gain setting of ten was employed. Uncoated fused-silica capillaries of 57 or 49 cm lengths (detector windows at 50 and 42 cm, respectively) with inner diameters of 50 μm were used for separations at 15–20 kV with isotonic borate, pH 9.1 (45 m*M* sodium tetraborate, 20 m*M* boric acid, 12 m*M* NaCl) or 0.1 *M* phosphate, pH 7.4 (91.5 m*M* Na_2HPO_4, 8.5 m*M* NaH_2PO_4) both containing 1% (v/v) β-octyl glucoside or 1% (v/v) CHAPS, as electrophoresis buffers. The capillary cooling fluid was kept at 25°C.

Binding experiments (details are given in the figure texts) were performed in two ways. In preincubation experiments, peptide and MHC molecules were mixed in the presence of a protease inhibitor cocktail (Complete, Boehringer), dissolved at the concentration recommended by the manufacturer. The components of the mixture were subsequently analyzed by CE during incubation in the sample carousel of the CE instrument. Without sample cooling, the incubation conditions corresponded to incubation temperatures of 25–30°C. In migration shift experiments, various amounts of MHC molecules were added to the electrophoresis buffer and ensuing changes in the time of peak appearance and in the peak shape of the electrophoresed FL-HA(307–319) were analyzed.

3. Results and discussion

To optimize buffer and analysis conditions, it was necessary to characterize the migration behavior of both peptide and MHC molecules in CE. To minimize sample consumption and to measure high affinity binding, LIF detection using fluoresceinylated molecules was employed. The HA(307–319) peptide was fluoresceinylated during synthesis. The recombinant DR4 molecule was labelled with fluorescein on its carbohydrate moiety [21,22], to avoid modifying the protein backbone and influencing the peptide binding characteristics. N-linked carbohydrate of murine MHC class II molecules has previously been reported not to be essential for

peptide binding [23]. In experiments that are not shown, the recombinant DR4 molecule was demonstrated to contain N-linked carbohydrate by migration shifts in sodium dodecyl sulfate–polyacrylamide gel electrophoresis after N-glycosidase F treatment. Initial attempts at using galactose oxidase for carbohydrate-specific oxidation [22], prior to the reaction with the fluoresceinylated thiosemicarbazide did not result in labelling of the DR4-molecules. This may be due to under-glycosylation in the *Drosophila* cell system, analogous with the results in other insect cell expression systems [24].

Using the sodium periodate oxidation procedure, described in Section 2, however, an analyzable molecule that was detectable as a single component by UV detection at 280 nm (results not shown) and by LIF detection was obtained (Fig. 1). The component was specifically recognized by the anti-DR antibody, LB3.1, as shown in Fig. 1. This antibody is specific for the α-chain of native HLA-DR class II heterodimers [9,19] and, thus, the results shown in Fig. 1 indicate that the fluoresceinylated DR4 molecules were native and intact.

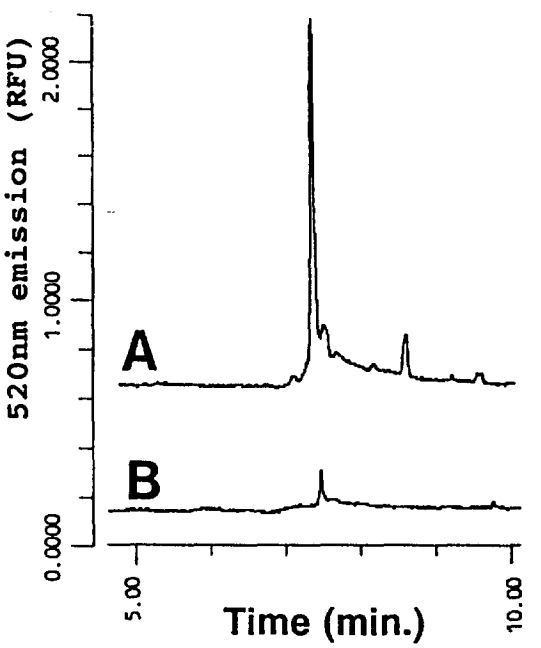

Fig. 1. Fluoresceinylated DR4 (FL-DR4) analyzed in CE with LIF detection. Immunochemical identification of product as a DR4-derivative, based on specific immunoabsorption. Aliquots (5 μl) of carbohydrate-fluoresceinylated DR4 (see Section 2) were mixed with (A) a 5-μl suspension of Protein G Sepharose (1:1 in PBS, pH 7.4, containing 1% β-octylglucoside) or (B) a 5-μl suspension of an anti-DR antibody (LB3.1) immobilized on Sepharose (1:1 suspension as above). Electrophoresis was performed at 20 kV in 0.1 *M* phosphate, pH 7.4, containing 1% β-octylglucoside.

Using an isotonic borate buffer, pH 9.08, containing 1% of the zwitterionic detergent, CHAPS, the fluoresceinylated DR4-molecule (FL-DR4) was clearly separated by CE from the fluorescein–peptide FL-HA(307–319) (Fig. 2A). Since a slow association phase followed by the formation of a stable complex was expected for the interaction [2,8,9], a mixture of peptide and DR4 was incubated and the fate of the components followed over time by repeated injections (Fig. 2A). As can be seen, there is a gradual increase in the area of the peak corresponding to FL-DR4, with a simultaneous decrease in the FL-HA(307–319) peak. This may indicate that the peptide binds to the MHC molecule and that the MHC–peptide complex has a migration corresponding to the migration of empty MHC molecules, i.e. that the charge/mass ratio of the complex is not very different from that of the empty MHC molecules themselves under the analysis conditions. Thus, the peptide was chosen as the analyte to characterize binding through migration changes. The experiment illustrates how interactions may be followed over time for a small volume incubation mixture (12 μl) and Fig. 2B is a graph of the development of the FL-DR4 peak position area as a function of incubation time. The data suggest that the association phase is slow and that an equilibrium under the specific conditions of the experiment is not established before approximately 10 h. This time is shorter than the time (48 h) required for maximum binding to murine class II MHC molecules purified from the natural source [8], but it corresponds quite well with the result of another study of the binding of HA(307–319) using HLA-DR1 molecules expressed in an insect cell system [9] and suggests that the MHC species are also expressed as empty molecules in the *Drosophila* cells. Attempts at reversing the binding after formation of stable complexes by adding an excess (>1000-fold over FL-HA(307–319)) of unlabelled HA(307–319) to the incubated mixture did not lead to discernable changes in distributions of peak areas over 24 h (results not shown). Even

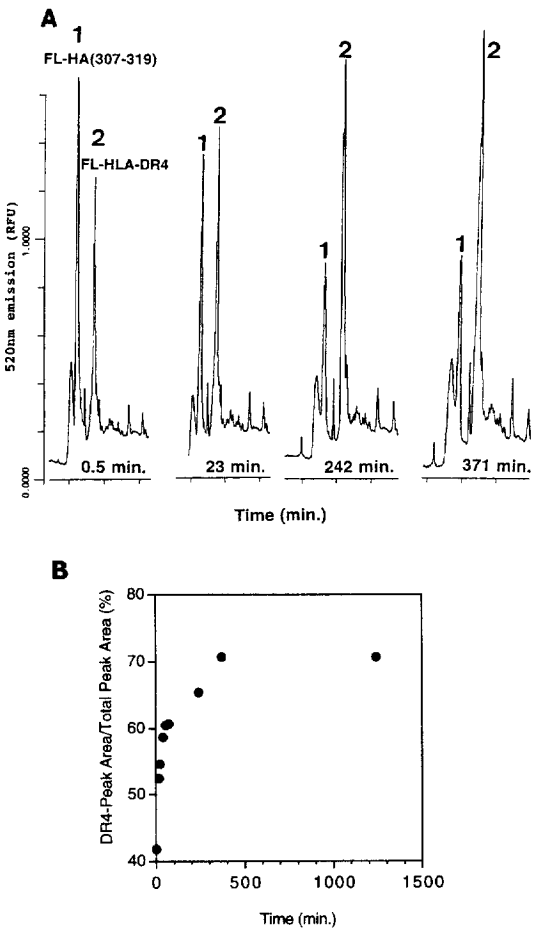

Fig. 2. Time course study of the binding of fluoresceinylated HA(307–319) peptide (1) to carbohydrate-fluoresceinylated HLA-DR4 (2). (A) CE with LIF detection. Repeated 10-s injections at times indicated after start of incubation of a sample consisting of 2 μl of 0.45 n*M* FL-HA dissolved in the presence of a protease inhibitor cocktail and 10 μl of FL-DR4 in PBS, pH 7.4, containing 1% β-octylglucoside. Electrophoresis was at 15 kV in isotonic borate, pH 9.08, containing 1% CHAPS. (B) Graph of peak areas from a series of time course experiments as shown in (A). Area of the FL-HLA-DR4 peak as a percentage of the total (FL-HA+FL-HLA-DR4) peak area as a function of incubation time.

though detergent may influence binding [4], its presence was necessary to keep the full length molecules in solution during incubation and only the mild non-ionic β-octyl glucoside was used for this purpose [2]. Furthermore, since the MHC molecules are normally anchored in a cell membrane, the detergent may actually induce a more physiological state of the MHC molecules than in the absence of detergent.

An estimate of the association rate constant for the interaction of HA(307–319) is, in principle, obtainable from the initial rate of peptide binding seen in the plot of Fig. 2B. However, in these experiments, the exact concentration of fluoresceinylated DR4 molecules was not known, but if a 50% labelling efficiency and 50% recovery through the whole labelling procedure are assumed, a rough estimate of 22 μmol of FL-HA(307–319) bound per mole of DR4 per hour is obtained, corresponding to an association rate of approximately 80 M^{-1} s^{-1}. This may be compared to the value of 12 M^{-1} s^{-1} estimated by other methods for the binding of this peptide to an insect cell-expressed DR1 molecule [9]. A more accurate estimate in the present study requires a knowledge of the precise DR4 concentration in the FL-DR4 preparation.

The possibility exists that the observed alterations of the electropherograms were caused by degradation of the FL-HA(307–319) peptide, e.g. induced by contaminating proteolytic activity in the FL-DR4 preparation. However, several points seem to argue against this: a protease inhibitor cocktail (inhibitory towards a broad spectrum of serine, thiol and metalloproteases, with at least 95% activity remaining after 1 h incubation, according to the manufacturer) was included in the incubation mixture, the alterations in peak areas leveled out after a defined time period (Fig. 2B), twice affinity-purified DR4 molecules were employed and, if proteolytic activity was the cause of the changes, the activity only resulted in cleavage products with one migration time, i.e. corresponding to only one cleavage site. Thus, proteolysis artifacts seem unlikely but cannot be completely excluded. Peptide ligand degradation, however, can be followed much easier using CE-based binding assays than in traditional approaches.

Since lower affinity peptide–MHC interactions may precede the formation of stable peptide–MHC complexes [2], we examined the possibility of interactions with fast on-and-off rates that would escape detection by the pre-equilibration approach. This was done with migration shift experiments where the MHC molecules were added to the electrophoresis buffer in which the FL-HA(307–319) was analyzed

(Fig. 3). As can be seen, the addition of the DR4 molecule in the 0.2–1 μ*M* concentration range had effects on both the FL-HA(307–319) peak shape and on the peak appearance time relative to an internal marker (a non-reactive impurity in the peptide preparation marked with a dot in the figure). The experiment clearly is indicative of lower affinity (μ*M* dissociation constants) interactions taking place between the peptide and the DR4 molecule. The broadening of the peak with increasing DR4 concentration suggests that the dissociation rate is too slow to be compatible with a dynamic equilibrium throughout the electrophoresis, i.e., not all molecules spend equal time in complexed and free form [25,26]. Analysis of the peak shape changes that take place as a function of DR4 concentration may make it possible to estimate rate constants for the interaction, as has been shown in other affinity CE applications [27]. More experiments are needed to get precise estimates of equilibrium and rate constants in the present study but the results do appear to confirm the existence of lower affinity interactions as a prelude to the stronger complex formation that is demonstrated by the preincubation experiments. These weaker interactions are not easily revealed with traditional binding assays, such as gel filtration, filter assays or solid-phase capture assays, and the present type of assay may therefore prove helpful for the elucidation of the biological relevance of these weak binding interactions.

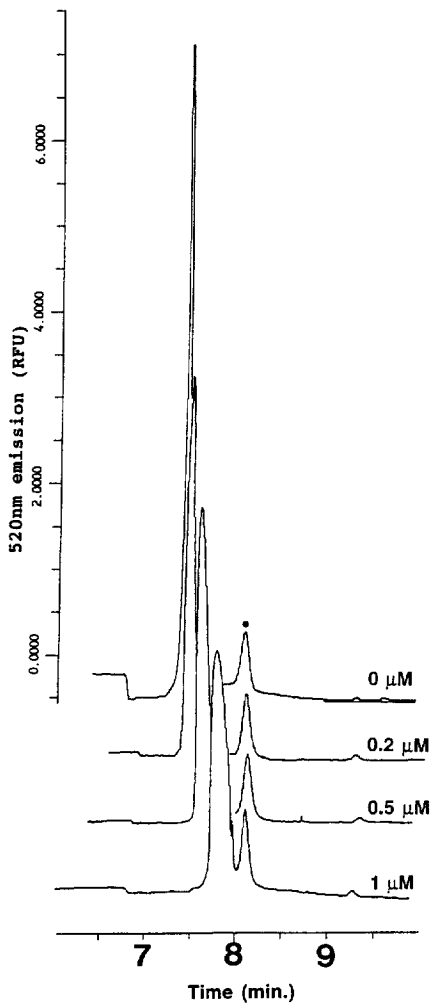

Fig. 3. Affinity CE analysis of FL-HA(307–319) electrophoresed in the presence of different amounts of unlabelled DR4 added to the electrophoresis buffer. Injection for 2 s of 4.5 n*M* FL-HA diluted in electrophoresis buffer (isotonic borate, pH 9.1, containing 1% β-octylglucoside) and CE with LIF detection at 15 kV after a 0.5-min pre-rinse with electrophoresis buffer containing the stated approximate concentrations of affinity purified unlabelled DR4 molecules. The symbol ● marks an impurity in the FL-HA(307–319) preparation that serves as an internal marker.

4. Conclusions

The demonstration of binding of the FL-HA(307–319) peptide to recombinant full-length human DR4 by CE was accomplished in an alkaline buffer system containing zwitterionic or non-ionic detergent. Using two different approaches for binding studies in CE, i.e., pre-equilibration experiments and experiments utilizing ligand addition to the electrophoresis buffer, there were indications of both a lower affinity interaction (fast rate constants and a dissociation constant in the μ*M* range) and a higher affinity binding, resulting in the formation of stable complexes.

Even though the results are preliminary and peptide proteolysis cannot be completely ruled out as a cause of some of the changes in the electropherograms, it is demonstrated that CE may be useful for the analysis of full length detergent-solubilized empty MHC molecules interacting with peptides. The assay is a direct binding assay using reactants in solution, consumes little sample and allows for multiple analyses from small volumes. Furthermore,

the integrity of the peptide ligand is monitored as a part of the assay itself. This work constitutes a basis for the estimation of equilibrium binding constants for the interactions and may represent a convenient way to quickly screen for substances that block or enhance binding, by co-incubation experiments with the labelled tracer peptide described here. Also, the approach may ultimately be extendable to binding studies of MHC–peptide complexes with T-cell receptors. This is presently technically difficult and involves immobilization of reactants [28–30] but is of fundamental importance for understanding the regulation of the immune response.

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