

## ФУНКЦИОНАЛНА ХАРАКТЕРИСТИКА НА РАЗТВОРИМИ РЕКОМБИНАНТНИ МНС КЛАС II ПРОТЕИНИ

Цветелина Бацалова, Балик Джамбазов

Катедра «Биология на развитието», ПУ «П. Хилендарски», 4000 Пловдив.

\*Автор за кореспонденция: E-mail: balik@uni-plovdiv.bg

## FUNCTIONAL CHARACTERISTIC OF SOLUBLE RECOMBINANT MHC CLASS II PROTEINS

Tsvetelina Batsalova, Balik Dzhambazov

Department of Developmental Biology, Plovdiv University, 4000 Plovdiv,

Bulgaria; \*Corresponding author: E-mail: balik@uni-plovdiv.bg

### Abstract

We have produced soluble recombinant HLA-DR4 molecules in a *Drosophila melanogaster* expression system. After affinity purification with L243 antibody-coupled column, the protein was loaded with antigenic peptide from collagen type II (CII) and the formed complexes were further purified. In this report, we demonstrate that our DR4/CII peptide complexes are functional both *in vitro* and *in vivo*. The MHC II/CII complexes were able to specifically stimulate DR4-restricted T cell hybridomas *in vitro* and also to initiate immunological tolerance to collagen type II *in vivo*.

### Introduction

The major histocompatibility class II (MHC II) molecules specifically control the adaptive immune system by presentation of antigenic peptides to CD4<sup>+</sup> T lymphocytes (1). These glycoproteins are heterodimers composed of 30-34 kDa  $\alpha$ -polypeptide and 28-30 kDa  $\beta$ -chain noncovalently associated with each other. Each chain contains two distinct extracellular domains ( $\alpha$ 1 $\alpha$ 2 and  $\beta$ 1 $\beta$ 2), a transmembrane hydrophobic domain and a short intracellular C-terminal domain. The  $\alpha$ 1 and  $\beta$ 1 domains form the peptide-binding pocket of the molecules as shown by crystallographic studies (2). Interestingly, similarities in the sequence of the antigen-binding groove in some HLA haplotypes (most commonly DR4) confer susceptibility to rheumatoid arthritis (RA), a chronic disabling autoimmune disease (3).

In order to study the interaction between MHC II/CII peptide and the T cell receptor (TCR) of pathogenic lymphocytes that occurs during arthritis development, we have generated complexes of recombinant HLA-DR4 molecules and glycosylated CII peptide 259-273. The chosen peptide is the immunodominant T cell epitope from collagen type II and it is a key player in inducing disease in mice from the B10.Q strain with symptoms similar to human rheumatoid arthritis (4). Furthermore, specific response against this epitope was reported also in humanized animal models as well as in RA patients (5,6).

In this study, we describe the process of DR4/CII peptide complexes generation as well as their functional characterization. We show that the produced compounds are functional both *in*

*vitro* and *in vivo* indicating their possible use in experiments, aiming to visualize and track antigen-specific T cells and also in developing a new therapy against rheumatoid arthritis.

## **Materials and Methods**

### ***Antigens***

The following CII 259-273 peptides were used: K264 (unmodified rat CII 259-273 with lysine at position 264; GIAGFKGEQGPKEP), GalOK264 (CII 259-273 with  $\beta$ -D-galactopyranosyl-5-hydroxy-L-lysine at position 264). The synthesis and purification of these peptides were previously described (7).

### ***Preparation of DR4/peptide complexes***

After purification, the DR4 protein was incubated with GalOK264 peptide or unmodified CII 259-273 peptide for 4 days at 4°C. The formed complexes were purified by anion-exchange HPLC (resource Q column) using an ÄKTA explorer 100 Air system (Amersham Biosciences) and Unicorn V4.00 software. The separation was performed by gradient elution with 1M NaCl in 30 mM Tris buffer. The protein fractions were examined by ELISA. The samples positive for DR4 protein content were pooled and purified further by gel filtration and Superdex 200 column. Protein fractions with correct molecular weight were pooled and stored at 4°C until use.

### ***T cell hybridoma test***

MDR-1.1 DR4-restricted and GalOK264-specific T cell hybridoma line was used. Hybridoma cells ( $5 \times 10^4$ ) were incubated with increasing concentrations of DR4/GalOK264 complexes ranging from 7.81 to 500  $\mu\text{g/ml}$ . The protein was diluted in sterile PBS (Gibco) and coated overnight onto 96-well sterile plates (Nunc) at 4°C. The plates were then washed with sterile PBS and hybridomas were added in triplicates. After 48h incubation, culture supernatant was collected and evaluated for IL-2 content by sandwich ELISA. Costar plates were coated with 5  $\mu\text{g/ml}$  anti-mouse IL-2 antibody (clone Jes6IA12) for 2h at room temperature. Plates were washed, blocked with 2% non-fat milk for 1h and after subsequent washing culture supernatant was added in triplicates and incubated for 2h at room temperature. Supernatant with known IL-2 concentration was added to all plates as a positive control and standard. After washing, specifically bound IL-2 molecules were detected by addition of 1  $\mu\text{g/ml}$  biotinylated anti-mouse IL-2 antibody (clone 5H4). Plates were developed using  $\text{Eu}^{3+}$ -labeled streptavidin and DELFIA system. IL-2 specific fluorescence was detected on a Wallac multi-label reader (Perkin Elmer).

### ***Mice***

Mice expressing transgenic HLA-DR4 and human CD4, but not murine MHC class II molecules were used in the *in vivo* experiments. The original founders of transgenic animals were provided by Prof. L. Fugger (8). These mice were backcrossed for 10 generations to the B10.Q background.

### ***DR4/CII peptide treatment and T cell assays***

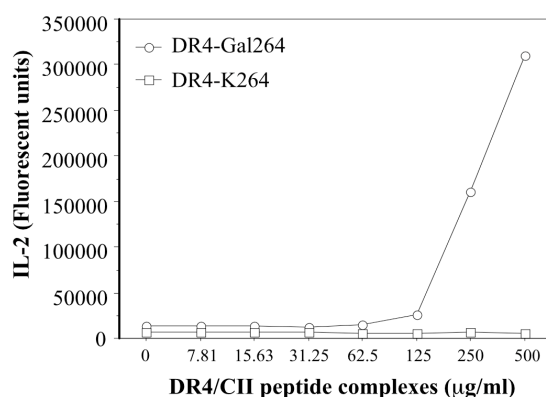
Mice were injected intravenously with either 100  $\mu\text{g}$  of DR4/GalOK264 or PBS in a total volume of 200  $\mu\text{l}$ . After 3 days, the animals received intradermal injection of 100  $\mu\text{g}$  rat CII mixed with Complete Freund's Adjuvant (CFA). Five days after the CII injection, mice were sacrificed and draining inguinal lymph nodes were harvested. Single cell suspensions were derived from the organs by passing through 40  $\mu\text{m}$  nylon strainer (BD Labware). Cells were plated *in vitro* in triplicates and restimulated with GalOK264 peptides (5  $\mu\text{g/ml}$ ) and CII protein (50  $\mu\text{g/ml}$ ). Following 96h incubation cell-free supernatant was collected. Levels of secreted IL-2 were measured by sandwich ELISA as described above. IFN- $\gamma$  production was also evaluated by sandwich ELISA. The method included the same steps as the IL-2-specific ELISA. 5  $\mu\text{g/ml}$  anti-mouse IFN- $\gamma$  clone R46 was used as a capture antibody. As a detection antibody was used biotinylated anti-mouse IFN- $\gamma$  antibody (clone AN18). Culture supernatant from Con-A stimulated spleen cells with known concentration of IFN- $\gamma$  was added to each plate as a positive control and standard.

### Results and Discussion

Empty DR4 molecules purified by affinity chromatography were incubated with GalOK264 peptides. Addition of peptide results in stabilization of the MHC II protein by forming of stable complex (9). We further purified these compounds by ion-exchange and gel-filtration chromatography. In order to characterize the functionality of DR4/CII peptide complexes *in vitro*, we used DR4-restricted mDR-1.1 T-cell hybridoma, which is specific for GalOK264 peptide.

Incubation of increasing concentrations of DR4/GalOK264 and mDR-1.1 cells gave rise to a dose-dependent stimulation of the hybridoma line. To confirm that the reaction is peptide specific and there's no cross-reactivity, we performed criss-cross hybridoma test. mDR-1.1 line was incubated with DR4

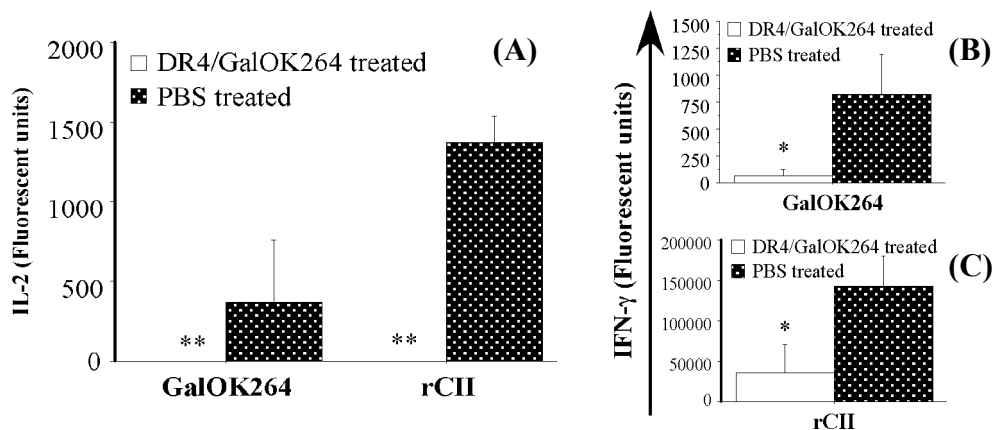
protein in complex with CII peptide 259-273 that differed from GalOK264 only by the lack of posttranslational modification at position 264 (K264). We observed no cross-reactivity, because DR4/K264 complexes were not able to induce IL-2 secretion by mDR-1.1 cells (Fig. 1).



**Figure 1.** DR4/GalOK264 complexes stimulate specifically mDR-1.1 T-cell hybridoma. IL-2 secretion from the cells was measured by ELISA.

These data demonstrate that the produced DR4/GalOK264 complexes are functional and could stimulate T cell hybridomas in MHC-restricted and peptide-specific way.

After proving the functional properties of DR4/CII peptide complexes by *in vitro* assays we extended our studies with *in vivo* experiments. Our aim was to see whether DR4/CII peptide complexes are functional in the *in vivo* situation, if they could present the CII antigen to T cells and induce immunological tolerance. Thus, we injected i.v. DR4-transgenic mice from the B10.Q strain with 100 µg of DR4/GalOK264 complex. The experiment included also a control group injected only with PBS. Each group consisted of 3 mice. Three days after the i.v. treatment animals were given a standard injection of 100 µg CII mixed with CFA, which normally induce immune response against CII. Mice were sacrificed 5 days later, draining lymph nodes were harvested and single cell lymphocyte suspensions were restimulated *in vitro* with GalOK264 peptide and native CII molecule. In order to assess the magnitude of T cell response to collagen protein and the collagen peptide, we took supernatant after 96h of incubation and measured the levels of IFN- $\gamma$  and IL-2. As shown in Figure 2, lymphocytes from mice treated with DR4/GalOK264 complexes secreted significantly less IFN- $\gamma$  and even no IL-2 in response to both GalOK264 peptide and CII compared to mice treated only with PBS. These data clearly suggest that DR4/GalOK264 complexes efficiently presented the CII peptide *in vivo*. Such presentation in the absence of costimulatory molecules most likely resulted in partial stimulation of CII-specific T cells that subsequently ended up in T cell anergy and peripheral tolerance to CII.



**Figure 2.** T-cell response in treated mice. (A) Secretion of IL-2 by T cells following restimulation with GalOK264 peptide and CII protein; (B) levels of IFN- $\gamma$  in the culture supernatant after *in vitro* restimulation of the lymphocytes. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

### Conclusion

We have successfully produced complexes of recombinant HLA-DR4 molecules and GalOK264 peptide from collagen type II. In this report, we demonstrate that these complexes are functional and could efficiently present the CII peptide both *in vitro* and *in vivo*. Most importantly, treatment with DR4/GalOK264 complexes was able to induce immunological tolerance to CII in humanized DR4-transgenic mice. It was shown before that such process could inhibit both T and B cell pathogenic responses and could suppress inflammation and collagen-induced arthritis (9). Thus, our data represents a new intriguing possibility to treat rheumatoid arthritis.

### References

- 1 Germain R.N., Margulies D.H. 1993. The biochemistry and cell biology of antigen processing and presentation. *Annu Rev Immunol* 11:403-50.
- 2 Stern L.J., Brown J.H., Jardetzky T.S., Gorga J.C., Urban R.G., Strominger J.L., Wiley D.C. 1994. Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. *Nature* 368:215-21.
- 3 Gregersen P.K., Silver J., Winchester R.J. 1987. The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to RA. *Arthritis Rheum* 30:1205-13.
- 4 Michaelsson E., Andersson M., Engstrom A., Holmdahl R. 1992. Identification of an immunodominant type-II collagen peptide recognized by T cells in H-2q mice: self tolerance at the level of determinant selection. *Eur J Immunol* 22:1819-25.
- 5 von Delwig A., Altmann D.M., Isaacs J.D., Harding C.V., Holmdahl R., McKie N., Robinson J.H. 2006. The impact of glycosylation on HLA-DR1-restricted T cell recognition of type II collagen in a mouse model. *Arthritis Rheum* 54:482-91.
- 6 Backlund J., Carlsen S., Hoger T., Holm B., Fugger L., Kihlberg J., Burkhardt H., Holmdahl R. 2002. Predominant selection of T cells specific for the glycosylated collagen type II epitope (263-270) in humanized transgenic mice and in rheumatoid arthritis. *Proc Natl Acad Sci U S A* 99:9960-5.
- 7 Broddefalk J., Forsgren M., Sethson I., Kihlberg J. 1999. Preparation of a Diglycosylated Hydroxylysine Building Block Used in Solid-Phase Synthesis of a Glycopeptide from Type II Collagen. *J Org Chem* 64:8948-8953.
- 8 Fugger L., Michie S.A., Rulifson I., Lock C.B., McDevitt G.S. 1994. Expression of HLA-DR4 and human CD4 transgenes in mice determines the variable region beta-chain T-cell repertoire and mediates an HLA-DR-restricted immune response. *Proc Natl Acad Sci U S A* 91:6151-5.
- 9 Dzhambazov B., Nandakumar K.S., Kihlberg J., Fugger L., Holmdahl R., Vestberg M. 2006. Therapeutic vaccination of active arthritis with a glycosylated collagen type II peptide in complex with MHC class II molecules. *J Immunol* 176:1525-33.