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Identification and characterization of CD4$^+$ T-cell epitopes on GapC protein of *Streptococcus dysgalactiae*

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ABSTRACT

The GapC protein is highly conserved surface dehydrogenase among *Streptococcus dysgalactiae* (*S. dysgalactiae*) and is shown to be involved in bacterial virulence. Immunization of GapC protein can induce specific CD4$^+$ T-cell immune responses and protect against *S. dysgalactiae* infection. However, there are no studies to identify immunodominant CD4$^+$ T-cell epitopes on GapC protein. In this study, *in silico* MHC affinity measurement method was firstly used to predict potential CD4$^+$ T-cell epitopes on GapC protein. Six predictive 15-mer peptides were synthesized and two novel GapC CD4$^+$ T-cell epitopes, GapC$_{63-77}$ and GapC$_{96-110}$, were for the first time identified using CD4$^+$ T-cells obtained from GapC-immunized BALB/c (H-2^d) and C57BL/6 (H-2^b) mice spleen based on cell proliferation and cytokines response. The results showed that peptides containing 63–77 and 96–110 induced significant antigen-specific CD4$^+$ T-cells proliferation response *in vivo*. At the same time, high levels of IFN-γ and IL-17A, as well as moderate levels of IL-10 and IL-4 were detected in CD4$^+$ T-cells isolated from both GapC and peptide-immunized mice *in vivo*, suggesting that GapC$_{63-77}$ and GapC$_{96-110}$ preferentially elicited polarized Th1/Th17-type responses. The characterization of GapC CD4$^+$ T-cell epitopes not only helps us understand its protective immunity, but also contributes to design effective T-cell epitope-based vaccine against *S. dysgalactiae* infection.

1. Introduction

As one of important *streptococcal* species, *Streptococcus dysgalactiae* (*S. dysgalactiae*) is a significant pathogen associated with bovine mastitis in lactating and nonlactating dairy cows, causing a severe inflammatory response of the mammary gland, which results in major economic losses to the dairy industry [1,2]. *S. dysgalactiae* is particularly problematic due to the fact that this so-called environmental *streptococcus* is ubiquitous in the dairy environment [3]. In the intramammary area, *S. dysgalactiae* can attach to and internalize into mammary epithelial cells, multiply and colonize the tissue [4–6]. *S. dysgalactiae* produces a number of surface proteins that have been associated with its virulence properties, which specifically interact with host proteins and are assumed to play important roles in eliciting host immune reactivity [7,8].

Nowadays, several *S. dysgalactiae* surface proteins have been developed as recombinant vaccine components, and their partial protections against the *streptococcus* infection have been achieved [9]. One of these proteins is the GapC protein, which was first identified in Group A streptococci (GAS). It possesses activity of the Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [10]. The GAPDH is a glycolytic enzyme involved in bacterial energy generation that is essential for growth. In several pathogenic bacteria, GAPDH has been described as a protein associated with virulence due to its ability to bind several host proteins or to confer resistance against reactive oxygen species produced by host phagocytic cells [11]. Especially, the GapC in different *S. dysgalactiae* species shares considerable homology at the DNA and amino acid levels [10], suggesting that GapC protein might be a good immunodominant

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antigen. GapC protein functions as the immunodominant protein and is responsible for eliciting *S. dysgalactiae* antibodies. At the same time, BALB/c mice vaccinated with the recombinant GapC induced a strong $CD4^+$ T-cells response [9]. Therefore, there is emerging evidence that $CD4^+$ T-cells contribute to the immunity that protects against *Streptococcus*-associated disease [12,13]. GapC protein possibly contains $CD4^+$ T-cell epitopes, some of which may elicit protective responses. However, the protection effects of $CD4^+$ T-cells induced by GapC are still not clear nor there is a report on the identification of $CD4^+$ T-cell epitopes from GapC protein.

Our previous study suggested that the fragment of 1–150 amino acids located at the N-terminus of *S. dysgalactiae* GapC protein could induce same immune response as the full-length GapC protein [9]. Thus in this study, the truncated GapC protein, which we named $GapC_{1-150}$, was used as the immunodominant fragment. Using *in silico* MHC affinity measurement method plus proliferation and cytokine release assays, we have identified and validated 2 epitopes of $GapC_{1-150}$. We also demonstrated that both epitopes induced significant $CD4^+$ T-cells proliferation, high levels of IFN-γ and IL-17A, as well as moderate levels of IL-10 and IL-4. These data not only provides us better understanding of GapC cellular immunity, but also highlights the possibility of developing the epitope-based vaccine against *S. dysgalactiae* infection.

2. Materials and methods

2.1. Prediction of GapC $CD4^+$ T-cell epitopes

SYFPEITHI (http://www.syfpeithi.de/Scripts/MHCServer.dll) [14], MHCPred (http://www.ddg-pharmfac.net/mhcpred/MHCPred/) [15], IEDB (http://www.iedb.org/) [16] and ProPred (http://www.imtech.res.in/raghava/propred/) [17] external software were used to predict peptide binding affinities to mouse I-A and I-E as well as HLA-DR, -DP and -DQ [18–20]. Peptide-binding motifs for each MHC class II molecule can be validated by three or more algorithms. For each method, peptides were tested and ranked by their scores on IC_{50} for better binders. Peptides were classified into binders (IC_{50} < 500 nM) and non-binders ($IC_{50} \geq$ 500 nM), as practical cutoffs. Meanwhile, the sequence of $GapC_{1-150}$ was used to predict the protein structure as well as β-turn (t) using PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/) [21] and COUDES (http://bioserv.rpbs.jussieu.fr/Coudes/index.html) [22] software. Finally, the amino acid sequences of epitope were aligned with relevant *S. dysgalactiae* strains.

2.2. Peptide synthesis

According to binding affinities to MHC II molecule and incorporation with high conserved amino acid and secondary structure, six 15-mer peptides of *S. dysgalactiae* $GapC_{1-150}$ was synthesized by the solid phase synthesis method (Table 1). The synthesized peptides were acetylated at the N-terminus and ended with a COOH-terminal (Hang zhou, China). These peptides (>95% purity) were dissolved in a mixture (v/v) of 75% dimethyl sulfoxide and 25% water to a concentration of 70 mM, and then divided into small aliquots and stored at −80 °C.

2.3. Mice immunization

Specific-pathogen-free female BALB/c and C57BL/6 mice (6–8 weeks old, weighting 18–20 g) were purchased from the Changchun Institute of Biological Products (Changchun, China). Animal experiments were approved by the Animal Ethics Committee of HeiLongJiang BaYi Agricultural University. The mice were supplied with water and food adlibitum according to the Regulations for the Administration of Affairs Concerning Experimental Animals. In brief, each group (10 mice) were administered with 100 μg of purified recombinant $GapC_{1-150}$ in a volume of 0.1 ml PBS emulsified in equal volume Complete Freund's adjuvant (CFA, Sigma) by intramuscular route in the four limbs and boosted 2 weeks later with the same protein in incomplete Freund's adjuvant (IFA, Sigma). At the same time, mice were administered with 100 μg synthetic peptide in IFA according to the above procedure. PBS immunization was treated as negative control.

2.4. Preparation of antigen presenting cells (APCs) and $CD4^+$ T-Cells

Mice were humanely sacrificed and spleens were harvested. After treatment with erythrocyte lysing buffer (0.83% NH_4Cl_2), splenocytes were washed two times with RPMI-1640 and incubated for 24 h at 37 °C in 5% CO_2, resuspended to 1×10^8 cells/ml in RPMI-1640 complete medium (RPMI-1640, 10% FBS, 100 U penicillin/ml, 100 U streptomycin/ml) with the addition of 10 μg/ml mitomycin-C (Sigma, USA). Then these cells were incubated for 2 h at 37 °C in 5% CO_2 followed by washing with RPMI-1640 three times. The supernatant was discarded and the pelleted cells (APCs) were resuspended in RPMI 1640 complete medium. In order to obtain $CD4^+$ T-cells, the $GapC_{1-150}$ or synthetic peptide-immunized mice were humanely sacrificed to collect spleen. Individual cell suspensions of spleen were prepared by aseptically removing tissues and passage through a sterile wire screen. Isolated $CD4^+$ T-cells were further separated by OctoMACS™ (Miltenyi Biotec) using negative selection.

2.5. $CD4^+$ T-cells proliferation analysis

For the analysis of specific $CD4^+$ T-cells proliferation, 100 μl $CD4^+$ T-cells (5×10^5 cells per well) and mitomycin C-treated feeder cells (10^5 cells per well) were seeded into 96-well flat bottom culture plates (Falcon, Montreal Canada) in complete medium containing 1 μg of $GapC_{1-150}$ or synthetic peptide at 37 °C in 5% CO_2. The 5 μg/ml ConA (Simga, USA) was used as positive control [23]. After 2 days of *ex vivo* antigen stimulation, the cell proliferation was measured using cell counting kit (CCK)-8 according to the manufacturer's instruction. Briefly, 20 μl of CCK solution was added to the culture medium and incubated for additional 2 h. The absorbance was determined at 450 nm wavelength using ELISA Reader (Bio-Rad, CA, USA).

Table 1
Prediction outcomes of *Streptococcus dysgalactiae* $GapC_{1-150}$.

Synthetic peptides	Sequence	Theoretical Mr	Actual Mr	Purity (%)
$GapC_{30-44}$	TRINDLTDPNMPAHL	1707.93	1706.54	96.45
$GapC_{41-55}$	PAHLLKYDTTQGRFD	1761.96	1762.23	98.23
$GapC_{52-66}$	GRFDGTVEVKEGGFE	1626.74	1626.34	97.42
$GapC_{63-77}$	GGFEVNGQFVKVSAE	1567.72	1567.52	95.28
$GapC_{74-88}$	VSAEREPANIDWATD	1673.76	1673.29	97.46
$GapC_{96-110}$	ATGFFASKEKAEQHI	1663.85	1663.16	96.54