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A novel synthetic bipartite carrier protein for developing glycotope-based vaccines

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ABSTRACT

Development of successful vaccines against glycotopes remains a major challenge. In the current studies, we have successfully developed a novel carrier protein for glycotopes based on the concept of antigen clustering and specific stimulation of T helper cells to mount strong antibody response to glycotopes. The bipartite carrier protein consists of a tandem repeat of a cysteine-rich peptide for docking of clustered glycotopes to effectively activate B cells and an Fc domain for antigen delivery to antigen presenting cells (APCs). To demonstrate its utility, we conjugated the tumor-specific monosaccharide antigen Tn to this novel carrier protein and successfully developed a Tn vaccine against cancer in animal models. The Tn vaccine effectively elicited high-titer IgG1 antibodies against Tn in immunized mice, and effectively suppressed the development of prostate cancer in Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mice. Our results suggest that this novel bipartite carrier protein could be effectively used for developing anti-glycotope vaccines such as the anticancer Tn vaccine.

1. Introduction

Among the cell surface tumor markers, tumor-associated carbohydrate antigens (TACAs) have spurred intense research into exploiting TACAs for the development of anticancer vaccines [1–6]. Some mucin-related carbohydrate epitopes, such as Tn (GalNAcα1-O-Ser/Thr), STn (NeuAcα2-6GalNAcα1-O-Ser/Thr) and TF (Galβ1-3GalNAcα/β-O-Ser/Thr), are highly overexpressed in carcinomas such as breast, prostate, and ovarian cancers [5,7,8], and have been targeted for cancer vaccine development [2,4,9,10].

Glycotopes are haptens which need to be conjugated to carrier proteins for inducing high affinity IgG antibodies. Glycoptope-carrier protein conjugates, through the MHC-II pathway in antigen presenting cells (APCs), can activate type II T helper cells that aid class switching and affinity selection of stimulated B cells leading to secretion of high affinity IgG antibodies [11–13]. Consequently, designing effective carrier proteins for glycotopes is an essential step for developing successful glycotope-based vaccines. Indeed, carrier proteins such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) and tetanus toxoid (TT) have been used for developing TACA-conjugated vaccines [3,14–16]. However, these carrier proteins still present several problems for developing TACA vaccines [10]. For example, the conjugation reaction is difficult to control and the immune response against unconjugated linker leads to epitope suppression [17]. Furthermore, the carrier proteins, which are often huge in size, contain multiple B cell epitopes which can readily induce antibody response against the carrier proteins themselves.

In the current studies, we report a novel synthetic bipartite carrier protein ((Mr = 34 kDa) which contains the IgG Fc domain (referred to as IFD) for aiding uptake/presentation by APCs and a tandem repeated cysteine-rich peptide (referred to as the antigen clustering domain or ACD) for glycotope docking. It has been demonstrated that carrier proteins containing the immunoglobulin (Ig) Fc domain (IFD) facilitate uptake through receptor-mediated endocytosis [13,18–21] and hence antigen presentation through the MHC II pathway in APCs [22–24]. It has also been demonstrated

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that carrier proteins containing antigen clustering domain (ACD) are highly effective in inducing strong B cell activation [12,25–27]. Indeed, we show in the current studies, such a bipartite carrier protein is highly effective in inducing high affinity IgG1 antibody against the glycotope Tn. Our results could suggest the general applicability of using this bipartite carrier protein for developing glycotope-based vaccines.

2. Materials and methods

2.1. Materials

The animal protocol was approved by Academia Sinica Institutional Animal Care and Utilization Committee. Human prostate cancer specimens for staining with anti-Tn antibody were retrieved from the archives of the Department of Pathology, Tri-Service General Hospital (Taipei, Taiwan). Samples were fully encoded to protect patient privacy and were used through a protocol approved by the Institutional Review Board of Tri-Service General Hospital National Defense Medical Center.

Female New Zealand White rabbits and female BALB/c mice were acquired from the animal center at National Taiwan University.

2.2. Construction of the plasmid encoding the bipartite carrier protein

The DNA fragments consisting of tandem repeats of the 30-mer DNA encoding the cysteine-rich peptide (Pro-Cys-Cys-Gly-Cys-Cys-Gly-Cys-Gly-Cys) was generated by template-repeat polymerase chain reaction (TR-PCR) [26]. The seven repeats-containing DNA fragment was excised from the plasmid DNA and subcloned into different expression vectors, which express mFc (GenBank ID: EF392839), rFc (GenBank ID: K00752) or PEIa (GenBank ID: K01397.1). The following plasmids were created: prFc(Cys_{42})$Histag_2$, pmFc(Cys_{42})$Histag_2$, and pPEIa(Cys_{42})$Histag_2$. The plasmid DNA encoding a single repeat was cloned into the GST expression vector to create pGST(Cys_6).

2.3. Expression and purification of the bipartite carrier proteins

Plasmids were transformed individually into the *Escherichia coli* strain BL21(DE3)*pLysS*, and cultured in LB medium containing ampicillin (50 μg/ml) at 37 °C. After induction, the bacteria were harvested and resuspended in the binding buffer (20 mM sodium phosphate, pH 7.9, and 8 M Urea) containing 1 mM DTT. After sonication and centrifugation, supernatants were applied to the nickel column (Pharmacia). The column was washed with the binding buffer containing 0.2 mM Tris(2-carboxyethyl) phosphine (TCEP) and 100 mM imidazole. Recombinant proteins were then eluted with the binding buffer containing 0.2 mM TCEP and 500 mM imidazole. Plasmid GST(Cys_6) was transformed into the *E. coli* strain TOP10. The transformant was cultured in LB under the same conditions as described above. The bacteria were harvested and resuspended in PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mg/ml lysozyme. GST(Cys_6) was purified by glutathione-agarose affinity column (Pharmacia), and eluted with 50 mM Tris–HCl containing 10 mM glutathione, pH 8.0.

2.4. Preparation of glycotope-carrier protein conjugates

Glycotope, are the carbohydrate epitopes, which are normally the carbohydrate part of a glycoprotein or glycolipid, such as Tn, STn, sialic acid and GM3. The synthesis and purification of Tn, STn, sialic acid and GM3 were described in previously studies [28–30]. Tn was conjugated to rFc(Cys_{42})$Histag_2$, mFc(Cys_{42})$Histag_2$ or PEIa(Cys_{42})$Histag_2$ at a glycotope/carrier protein weight ratio of 5 to 1 (STn was used as example to find an optimal condition for maximum conjugation) (Supplementary Fig. 1). Conjugation was performed in elution buffer (20 mM sodium phosphate, pH 7.9, 8 M urea, 500 mM imidazole, and 0.2 mM TCEP). After 48 h, conjugates were refolded against PBS containing 0.2 mM TCEP. GST(Cys_6) was dialyzed against PBS containing 0.2 mM TCEP. Different glycotopes and Linker (N-Succinimidyl-6-Maleimidocaproate) were conjugated to GST(Cys_6) at 4 °C for 48 h.

2.5. Immunization of mice and rabbits with Tn-carrier protein conjugates

Six- to eight-week-old female BALB/c mice and ten-week-old TRMAP mice (Jackson Laboratory) were immunized subcutaneously with 10 μg of mFc(Cys_{42}-Tn)$Histag_2$ in complete Freund's adjuvant, followed by immunizing with the same dose of conjugates in incomplete Freund's adjuvant three times at biweekly intervals. For immunization of rabbits, sixteen-week-old female New Zealand White rabbits were subcutaneously injected with 100 μg of rFc(Cys_{42}-Tn)$Histag_2$ following the same schedule described above.

2.6. Immunocompetition assay

Approximately 5×10^5–1×10^6 HeLa cells (purchased from ATCC) were cultured on coverslips in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Invitrogen) at 37 °C in 5% CO_2. Cells were fixed, permeabilized and stained with rabbit anti-Tn serum (1:1000 dilution) which was pre-incubated with different amount of Tn antigen (0, 1, 10, 50, and 100 μg) for 30 min at 37 °C. Coverslips were then incubated for 1 h at 37 °C. Bound antibodies were visualized following treatment with the goat anti-rabbit Texas Red (1:500 dilution) and incubation at 37 °C for 30 min. Nuclei were stained with DAPI (Thermo Scientific) (1:5000 dilution) for 10 min at room temperature.

2.7. Quartz crystal microbalance (QCM) analysis

The 9-MHz gold chips were available from ANT Tech (Taipei, Taiwan). The binding experiments were performed using an ANTQ300 instrument equipped with a flow-injection system. For K_d measurement, PEIa(Cys_{42}-Tn)$Histag_2$ was coated on the chip surface, and serial dilutions of purified anti-Tn IgG antibody ranging from 0.0625 μM to 2 μM were injected onto the coated chip. PBS was used to wash out the unbound molecules at a flow rate of 40 μl/min.

2.8. Immunohistochemical (IHC) staining of cancer tissues with anti-Tn antibody

Tissue sections were dewaxed in xylene and rehydrated in alcohol. Antigen retrieval was carried out by incubating tissue sections in 0.01 mM citrate buffer (pH 6.0) at 95 °C for 40 min. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide for 30 min. Sections were then incubated with 5% normal horse serum in PBS for 30 min at room temperature in order to block nonspecific antibody reaction. After washing with TBS plus 0.1% Tween 20, slides were incubated for 40 min at 4 °C with anti-Tn antiserum. Sections were then rinsed in TBS plus 0.1% Tween 20 and incubated for 10 min at room temperature with HRP polymer conjugated secondary antibody (SUPERPICTURE POLYMER KIT, Zymed Laboratories, Inc.). Subsequently, sections were stained with DAB chromogen/substrate, counterstained with Mayer's hematoxylin, dehydrated, and then mounted.