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Antisera preparation and epitope mapping of a recombinant protein comprising three peptide fragments of the cystic fibrosis transmembrane conductance regulator

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ABSTRACT

Antibodies targeting a single epitope of the cystic fibrosis transmembrane conductance regulator (CFTR) have been reported to influence the validity of immunological analyses; however, autoimmune mechanisms associated with CFTR epitopes are not well understood. In this study, antiserum raised against a multi-epitope recombinant protein composed of three peptide fragments of CFTR (r-CFTR-3P) was prepared and B cell epitope mapping of the protein was carried out using biosynthetic peptides. The r-*CFTR-3P* gene was cloned into the pSY621 expression plasmid and the protein was expressed in the BL21 strain of *Escherichia coli*. The rabbit r-CFTR-3P antiserum recognized the native CFTR antigen extracted from human sperm and the GST188 fusion peptides CFTR$^{25-36}$, CFTR$^{103-117}$, and CFTR$^{1387-1480}$ spanning different regions of CFTR. Four novel r-CFTR-3P B cell epitopes were identified: ^{29}RQRLEL^{34}, ^{104}RIIASY^{109}, ^{111}PDN^{113}, and ^{1447}VKLF^{1450} of CFTR. Other proteins from various species shared sequence homology with the identified epitopes based on NCBI BLAST alignment. This study provides new tools for detecting CFTR protein and insight into the characteristics of minimal B cell epitopes of CFTR and associated immunological mechanisms.

Introduction

Cystic fibrosis transmembrane conductance regulator (CFTR)[1] is an ion channel that conducts chloride ions across epithelial cell membranes [1]. *CFTR* gene mutations result in the dysregulation of epithelial fluid transport and cystic fibrosis (CF), an autosomal recessive genetic disease affecting the respiratory, digestive, and reproductive systems [2]. Hence, CFTR is a biomarker for the clinical diagnosis of CF [3–7]. To date, nearly 2000 CF-causing mutations have been identified. A variety of commercial CFTR detection kits and CFTR-specific antibodies are available [8]; however, *CFTR* gene polymorphisms or mutations that alter protein structure can influence the validity of immunological methods that detect a specific epitope [9,10].

Certain antibodies as well as autoimmune responses are correlated with CF patient phenotypes [11]. For instance, short CFTR consensus sequences that are homologous to proteins expressed by bacteria or viruses can induce autoimmunity via molecular mimicry [12]. Accordingly, epitope mapping of CFTR is important for understanding autoimmune and immune responses [13], gaining insight into mechanisms of therapeutic antibodies [14,15], and securing and protecting intellectual property [16].

In this study, we cloned a recombinant CFTR protein composed of three peptide fragments (r-CFTR-3P) into the pSY621 expression plasmid, which was expressed in *Escherichia coli* strain BL21 (DE3) cells. We also evaluated antisera raised against CFTR to identify novel B cell epitopes.

Materials and methods

Materials

Plasmids pSY621 [17], pXXGST-1, pXXGST-2 [18], and *E. coli* strain BL21 (DE3) were provided by the Shanghai Institute of Planned Parenthood Research (Shanghai, China). The anti-CFTR monoclonal antibodies (mAbs) MM13-4 (ab77961) and CF3 (ab2784), as well as horseradish peroxidase-conjugated goat anti-mouse IgG + IgM H&L (ab47827) were purchased from

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[1] *Abbreviations used:* CFTR, cystic fibrosis transmembrane conductance regulator; CF, cystic fibrosis; r-CFTR-3P, recombinant CFTR composed of three-peptide fragments.

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Abcam (Cambridge, UK). Anti-CFTR mAb AG1 was obtained from Santa Cruz Biotechnology (Dallas, Texas, USA). Restriction endonucleases (*Bam*HI, *Eco*RI, and *Sal*I) and T4 DNA ligase were purchased from Takara Biotechnology (Otsu, Japan). Ampicillin (Amp), low molecular weight pre-stained protein standards, horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit IgG, *o*-phenylenediamine (OPD), and nitrocellulose membranes were purchased from Shanghai Sangon Biotechnology Co. (Shanghai, China). Lysis buffer, gel, and loading buffer were purchased from Beyotime Institute of Biotechnology (Jiangsu, China).

All DNA sequences were synthesized by Shanghai Generay Biotech Co. (Shanghai, China). Male New Zealand white rabbits were purchased from Sheng Wang Experimental Animal Breeding Co. (Shanghai, China). Complete and incomplete Freund's adjuvant were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Design and synthesis of the r-CFTR-3P gene

Three antigenic peptide fragments of CFTR were selected based on sequences obtained from GenBank (accession No. NM_000492.3) and product inserts from commercially available antibodies. $CFTR^{25-36}$ (P1; RKGYRQRLELSD) was recognized by MM13-4 mAb; $CFTR^{103-117}$ (P2; GRIIASYDPDNKEER), by CF3 mAb; and $CFTR^{1443-1457}$ from $CFTR^{1387-1480}$ (P3; TLKQAFADCTVIL CEHRIEAMLECQQFLVIEENKVRQYDSIQKLLNERSLFRQAISPSDRVKLFP HRNSSKCKSKPQIAALKEETEEEVQDTR), by AG1 mAb. C-terminal arginine and glycine residues and the N-terminal arginine were removed from P2 to maintain the isoelectric point of r-CFTR-3P in the acidic range to generate a truncated P2 peptide. The amino acid sequence of the r-CFTR-3P protein (134 residues) was obtained by splicing P1, P3, P2, and truncated P2 peptide ($CFTR^{105-116}$, IIASYDPDNK) in this order, and a methionine (M) required in the open reading frame was added to the N terminus to obtain the following sequence: MRKGYRQRLELSDTLKQAFADCT VILCEHRIEAMLECQQFLVIEENKVRQYDSIQKLLNERSLFRQAISPSDRVK LFPHRNSSKCKSKPQIAALKEETEEEVQDTRLGRIIASYDPDNKEERIIASY DPDNKEE. The complete gene sequence encoding the r-CFTR-3P peptide was as follows: 5′-ATGAGGAAAGGATACAGACAGCGCCTG GAATTGTCAGACACTCTAAAACAAGCATTTGCTGATTGCACAGTAATTC TCTGTGAACACAGGATAGAAGCAATGCTGGAATGCCAACAATTTTTGG TCATAGAAGAGAACAAAGTGCGGCAGTACGATTCCATCCAGAAACTG CTGAACGAGAGGAGCCTCTTCCGGCAAGCCATCAGCCCCTCCGACAGG GTGAAGCTCTTTCCCCACCGGAACTCAAGCAAGTGCAAGTCTAAGCC CCAGATTGCTGCTCTGAAAGAGGAGACAGAAGAAGAGGTGCAAGATA CAAGGCTTGGAAGAATCATAGCTTCCTATGACCCGGATAACAAGGAG GAACGCATCATAGCTTCCTATGACCCGGATAACAAGGAGGAATAA-3′. An *Eco*RI site at the 5′ end was used for insertion into the pSY621 expression plasmid and the termination codon TAA was linked via the *Sal*I site to the 3′ end. There were no other restriction enzyme sites in the DNA sequence, as confirmed by sequence analysis prior to synthesis.

Construction of plasmids

The r-CFTR-3P expression plasmid (pSY621-r-CFTR-3P) was generated according to standard protocols [19]. The *r-CFTR-3P* gene was removed from the vector by digestion with *Eco*RI and *Sal*I, and inserted downstream of the methionine start codon at the corresponding restriction sites in the prokaryotic expression plasmid pSY621 downstream of the P_RP_L promoter (Fig. 1).

Recombinant pXXGST-1 plasmid was generated as previously described [20,21]. Briefly, complementary DNA fragments (r-CFTR-3P, P1, P2, P3, and a series of 8-mer peptides, P4–P32) fused to GST188 were cloned into the pXXGST-1 plasmid between the *Bam*HI and *Sal*I restriction sites. The pXXGST-1 plasmids were transfected into BL21 cells. DNA fragments were sequenced and the expression of GST188-fusion peptides was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE).

Expression of fusion peptides

BL21 cells transfected with pSY621-r-CFTR-3P and pXXGST-1 GST188-fused oligopeptides were inoculated in 3 ml Luria Bertani (LB) culture medium with 100 μg/ml ampicillin and grown overnight at 30 °C. Cell suspensions were inoculated 1:50 (v/v) in 3 ml of fresh LB medium and cultured with shaking for 2–3 h at 30 °C. When the optical density (OD) of the suspensions at 600 nm reached 0.6–0.8, cells were induced by incubating at 42 °C for 4 h before harvesting.

Purification of r-CFTR-3P proteins

Proteins were purified as previously described [21]. Briefly, induced cells were sonicated in 0.5% (v/v) Triton X-100 at room temperature and inclusion bodies were concentrated by centrifugation at 12,000*g* and 4 °C. After washing with 1 M urea, proteins were solubilized in 50 mM Tris–HCl buffer (pH 9.8) with 8 M urea. Supernatants were mixed with 70 mM β-mercaptoethanol and separated by SDS–PAGE. Protein bands were excised from the gel, electro-eluted, and dialyzed against phosphate-buffered saline (PBS). Eluted proteins were dialyzed against water overnight and freeze-dried to obtain purified proteins.

SDS–PAGE and western blot analysis

The expressed proteins were identified by SDS–PAGE and western blotting as previously described [21]. Cells were harvested and total protein was resolved on 15% acrylamide gels and visualized by Coomassie Blue staining or western blotting. For the latter, proteins were transferred to a nitrocellulose membrane in Tris–glycine buffer with 20% methanol, which was then blocked with 5% (w/v) non-fat milk and incubated with antibodies against MM13-4 (1:3000) or CF3 (1:100) or r-CFTR-3P-specific antiserum (1:200). Membranes were washed and incubated with peroxidase-conjugated goat anti-mouse IgG or IgM (1:10,000), washed, and protein bands were detected with Amersham ECL Plus Western Blotting Detection reagent (GE Healthcare, Buckinghamshire, UK). Densitometry analysis was performed using the Tanon-4100 digital GIS imaging system (Beijing, China).

The specificity of rabbit r-CFTR-3P antiserum for native CFTR antigen was assessed by western blotting as previously described [22]. The protocol was approved by the Medical Ethics Committee, Zhejiang Academy of Medical Sciences, and informed consent was obtained from all semen donors. Spermatozoa (1×10^8 cells/ml) were washed with PBS and total protein was extracted in lysis buffer containing Protease Inhibitor Cocktail (Sigma–Aldrich) and 1 mM fresh phenylmethylsulfonyl fluoride. Proteins were mixed with loading buffer and separated by 10% SDS–PAGE and transferred to Immunoblot-P membranes (Millipore, Billerica, MA, USA), which were blocked with 5% (w/v) non-fat milk in Tris-buffered saline (TBS) and incubated with rabbit r-CFTR-3P antiserum or CF3 mAb (1:500). After washes with TBS containing 0.01% (v/v) Tween-20, membranes were incubated with peroxidase-conjugated goat anti-rabbit or mouse IgG (Invitrogen, Carlsbad, CA, USA). After additional washes, proteins were detected using the SuperSignal West Femto Trial Kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. The molecular weights of the detected proteins were deduced by comparison with a pre-stained protein ladder (Fermentas/Thermo Fisher Scientific, Waltham, MA, USA).