



Research paper

Protection of IgE-mediated allergic sensitization by active immunization with IgE loops constrained in GFP protein scaffold

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Abstract

Green fluorescent protein (GFP) exhibits a rigid central β -barrel, formed by eleven β -strands with floppy loops spanning between the stands. Herein, we evaluate whether the rigid β -barrel may serve as a scaffold that can constrain the loops of a foreign protein, and thus its antigenicity. The spanning loops, site 6 of GFP, were engineered with RE cloning sites for inserting oligonucleotides corresponding to Fc ϵ RI-binding sequence of human IgE. In a high-throughput format, shortened oligonucleotides encoding eight amino acid residues of the receptor-binding regions were inserted into site 6 of GFP by PCR, followed by enabling sequences for *in vitro* transcription and translation at the 5' end. Antigenized C2-3 linker (C2-3L) was shown by immuno-blots with polyclonal anti-IgE under native gel electrophoresis and transfer. Recombinant antigenized GFP was expressed and purified to homogeneity by metal affinity column, followed by Sephacryl S-200 high resolution gel filtration. Hyperimmune sera from mice immunized with C2-3L antigenized GFP contain anti-IgE reactive with JW8 murine/human chimeric IgE. Further, elevated serum anti-C2-3L and affinity pure antibodies effectively inhibits binding of JW8 IgE to recombinant Fc ϵ RI α , and desensitizes JW8 to rat RBL-2H3 transfected with human Fc ϵ RI α . This observation raised the possibility that active IgE vaccine may be employed in raising active protective anti-IgE in allergic patients as an alternative to passive immunization with MAb-E25 anti-IgE. Taken together, GFP appears suitable protein scaffold for spanning/constraining the C2-3L of human IgE as active vaccine; and this technique may be generally employed for eliciting antibodies to specific B-cell epitopes of other proteins.

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Keywords: GFP; Protein scaffold; Constrained B-cell epitope; Human IgE; Protective anti-IgE

IgE plays a key role in type I-mediated allergic inflammation. Control of levels of IgE can be achieved

by administering monoclonal anti-IgE, MAb-E25, which neutralizes circulating IgE. The regimen requires repetitive injections of MAb up to 300 to 400 mg per injection on a weekly base. It will be important to delineate B-cell epitopes that are directly involved binding to high affinity IgE receptors (Fc ϵ RI). These B-cell epitopes, when appropriately presented are likely to serve as active vaccines that induce antibodies to prevent IgE-mediated allergic inflammation. It was recently

Abbreviations: GFP, green fluorescent protein; IgE, immunoglobulin E; Fc ϵ RI α , Type I high affinity IgE Fc receptor alpha subunit.

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elucidated that four IgE loops around the CH ϵ 2 and CH ϵ 3 domains, i.e., BC, DE, FG and C2-3 linker (C2-3L) loops, are intimately involved in binding to Fc ϵ RI as shown by co-crystal of IgE and Fc ϵ RI receptor (Garman et al., 2000). It follows that induction of endogenous anti-IgE antibodies by active immunization to receptor-binding IgE B-cell epitopes may protect against IgE-mediated immediate hypersensitivity.

Previously it was shown that a foreign B-cell antigenic loop appropriately constrained in a protein scaffold may exhibit its antigenic conformation similar to that presented in the native loop region of the parent molecule, as pioneered by Zanetti and colleagues (Billetta et al., 1991; Abedi et al., 1998; Geyer et al., 1999). The scaffold proteins employed for this purpose are reported in the immunoglobulin gene families such as complementarity-determining region (CDR2/3) of immunoglobulin (Billetta et al., 1991; Lanza et al., 1993), loop regions of fibronectin type III domain (Koide et al., 1998), and CTLA-4 (Nuttall et al., 1999) as well as non-immunoglobulin gene family such as protease inhibitors (Christmann et al., 1999) and lipocalin-related molecules such as β -barrel containing thioredoxin (Geyer and Brent, 2000; Skerra, 2000).

Green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* encoded by 238 amino acids, (Ormo et al., 1996; Tsien, 1998) exhibits a β -barrel structure

formed by 11 β -strands that serve to constrain the eleven native loops exposed to the aqueous phase. Native loops or peptide library replacing the loops, constrained by the β -barrel, are exposed to the aqueous phase. Indeed, members selected from the aptameric peptide library presented by GFP were shown to block enzymes regulating signal transduction and cellular growth (Caponigro et al., 1998; Geyer et al., 1999).

Inserted peptides into Loop 6 (site 6) of GFP are known to exhibit constrained conformation similar to those of peptide loops present in the parent molecules (Skerra, 2000). Herein, we test whether such “antigenized” GFP may therefore serve as an active vaccine in eliciting neutralizing antibodies, which prevent IgE binding to Fc ϵ RI α . Herein, we showed that antibodies elicited to C2–C3L antigenized GFP bind native IgE molecules; furthermore this epitope-specific antibody blocks IgE binding to recombinant Fc ϵ RI α on solid phase as well as Fc ϵ RI α -transfected mast cells (Nechansky et al., 2001).

1. Materials and methods

1.1. Preparation of antigenized GFP vectors

The general design of the antigenized vector was tested in Fig. 1. The 5'MCS following the initiation

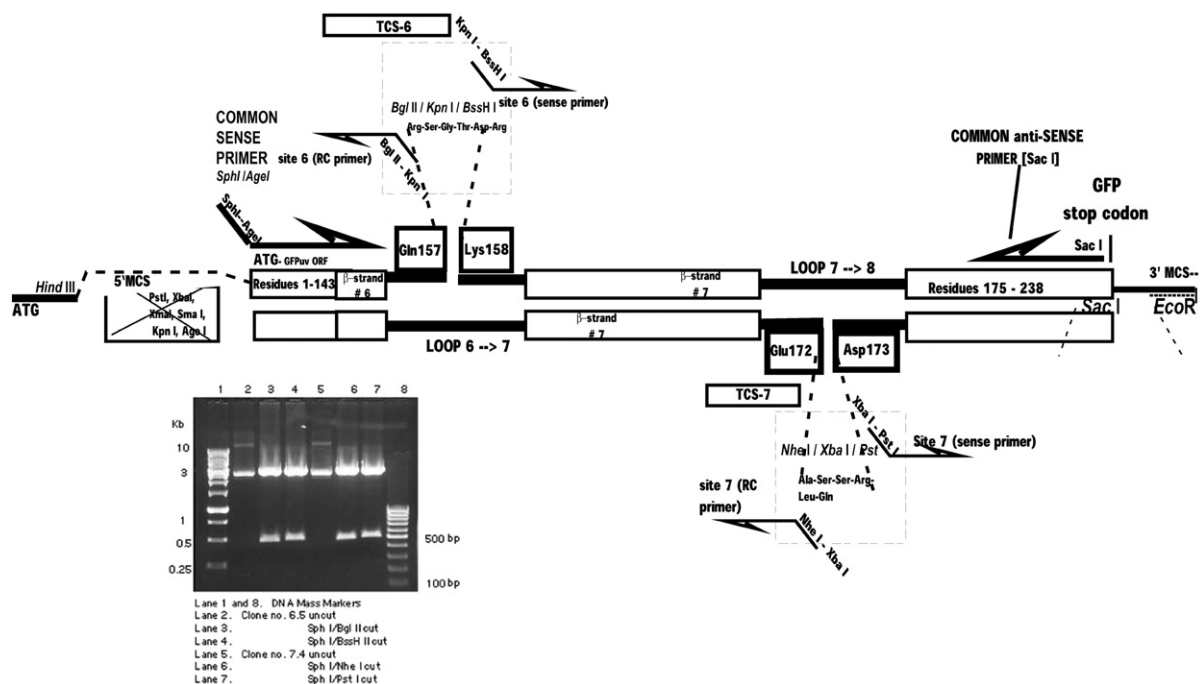


Fig. 1. Strategy of preparing site 6 and site 7 GFP scaffold vector. Site 6 (Gln 157/Lys 158) or site 7 (Glu 172/Asp 173) of GFP was inserted with unique RE cloning sites with the primer pairs described in Materials and methods. Diagnostic cut of site 6 and site 7 GFP scaffold vector. Site 6 scaffold vector, 6.5 was digested with Sph I along with either Bgl II or BssH I. Site 7 scaffold vector 7.4 was digested with Sph I along with Nhe I or Pst I.

codon was shortened to 7 residual amino acids with six out of the eight restriction enzyme sites therefore deleted from the 5'MCS of GFP_{UV} vector (Clontech, Palo Alto, CA). This permits engineering of RE sites in the loop regions of interest, i.e., site 6 at Gln 157/Lys 158 in loop 6 (flanked by Ala 155 and Ile 161) and site 7 at Glu 172/Asp 173 in loop 7 (flanked by Ile 171 and Val 176) of the two surface-exposed, constrained loops. A three-step PCR strategy and design-primer sets for the vector were illustrated in Fig. 1. Primers with restriction enzyme (RE) sites, flanked by the GFP sequences were used to amplify half GFP molecules. GFP scaffolds with modified site 6 or 7 were then assembled by ligating two pieces of the amplified PCR products, and amplified by another round of PCR to yield the scaffolding cloning vector. Thus primers pairs containing the three unique efficient end-cutter RE sites, were engineered into site 6 (flanked by Gln 157 and Lys 158) or site 7 (flanked by Glu 172 and Asp173), i.e., Bgl II/Kpn I/BssH I for the site 6, and Nhe I/Xba I/Pst I for the site 7, respectively. The sequences of the primer pairs were as follow: site 6 antisense: TGA GGTAC (Bgl II) AGATCT (Kpn I) TTGTTTGTCTGCCGTGATGTATAC; site 6 sense: TGA GGTACC (KpnI) GCGCGC (BssH I) AA-GAATGGAATCAAACGTAACCTTC; site 7 antisense: TGA TCTAGA (XbaI) GCTAGC (Nhe I) TTCAATGTTGTGGCGAATTTTGAAG; site 7 sense: TGA TCTAGA (Xba I) CTGCAG (Pst I) GATG-GATTCGTTCAACTAGCAG; common antisense: TGATGATGATGATGATAGAGCTC (Sac I) ATC-CATGCCATGTG; common sense: TGATGATGA (G C A T G C (Sph I) A C C G G T (Age I) AGAAAAAATGAGTAAAGG.

Fig. 1 inset showed diagnostic cuts of non-antigenized site 6 and site 7 expression vector constructs. Double digest with Sph I/Bgl II, or Sph I/BssH I yielding a small .5 kb fragment and a large 3 kb fragment of site 6 construct, while double digest of site 7 construct with Sph I/Nhe I and Sph I/Pst I yielding a similar small fragment and a large 3 kb fragment.

1.2. Purification of GFP

Immobilized metal affinity chromatography (IMAC). IMAC was purchased from Biorad (Hercules, CA). IMAC resins containing iminodiacetic acid (IDA) is charged with Ni²⁺ with 60 µm for flow rate. A clarified of *E. coli* lysate containing C2-3L GFP from DH5α, or GFP_{UV} from JM109 of 100 ml culture was exchanged in buffer 50 mM sodium phosphate, pH 8.0, 0.3 M NaCl containing 5 mM imidazole, the concentrated sample in 1 ml was loaded to a 2 ml column pre-equilibrated with

the exchange buffer. The lysate was flowed through the column and was then washed with the same buffer containing 10 mM imidazole. The sample was then eluted with the same buffer containing 250 mM imidazole. The flow rate was 2 ml/min, and absorbance was monitored at A₂₈₀. Protein eluted from the column was collected in 1 ml fractions.

Bulk preparation of recombinant protein. Half to 1 l of DH5α from LB culture containing the recombinant protein were centrifuged at 5000 rpm in a Sorvall GSA rotor for 15 min at 4 °C. For each gram of pellet, 2 ml of cell lysis buffer with 4 µl of 100 mM PMSF and 80 µl of 10 mg/ml lysozyme. The re-suspension was stirred by addition of Triton 100 at a final 1%; the viscosity was further relieved by 20 µl of 1 mg/ml DNase per gram starting material for 30 min, and finally the lysate with greenish soluble lysates, an indication without inclusion bodies was clarified by 1 h centrifugation at 20,000 rpm in an SS34 rotor. The lysate was dialyzed against buffer containing 50mM PB with 0.15M NaCl, and kept within 15 ml volume by PEG concentration. The protein yield of half a liter culture was approximately 1200 mg in the dialysate (80 mg/ml).

Protein purification was performed in two steps. Samples were exchanged in PBS with 5 mM imidazole and The IMAC adsorption was performed with 5 ml each batch in five successive runs of 1 ml each on a 10 ml of IMAC column in a upscale enrichment of recombinant GFP, constituting a significant constituent in fractions monitored by OD₅₉₅ in the visible spectrum, represented 17 fold enrichment as estimated from the bulk of materials measured by OD₂₈₀ (not shown). The enriched fractions were pooled and concentrated in 1 ml PB. GFP enriched fraction was then further fractionated onto a pre-packed Sephacryl S-200 HR (dimension: 26×60, Pharmacia, Bridgewater, NJ) with bed volume of approximately 300 ml at the flow rate of 0.2 ml/min at 4 °C with sample volume of in 0.5 ml in a column of bed volume of 250 ml packed slurry, and fractions were collected at 2.5 ml per tube for one bed volume, and the UV₂₈₀ was monitored. Peak material was collected and concentrated by PEG, and run on Lammeli SDS-PAGE and pure material of 28 to 30 kDa was identified as a single band. Throughout the purification, antigenized GFP or control GFP enriched fraction was sighted by eye or visualized by illuminating tubes with a hand UV lamp at 365 nm.

1.3. Design and expression of the expression cassette

A complete expression cassette of antigenized GFP was generated using pIVEX 2.3 vector (Roche

Applied Science, Indianapolis, IN) as a template. The two primers used are gfp-f, gatcgagatctcgatcccgcaaat (forward25), and gfp-r, gaacctgcagagcaaaaaccctcaaga (reverse29). This 1 kb PCR fragment contains, in sequence from 5', a T7 promoter, ribosomal binding site (RBS), wide type GFP, a linker, His-tag and T7 terminator, which constitutes a complete *in vitro* expression cassette. PCR1 consists of 5' regulatory sequence and upstream of wide type GFP from loop 6, which was produced using pIVEX 2.3 control GFP DNA as a template with forward primer, gfp-f and the reverse primer, gfp#6-r, gtctgccatgatgtatac. PCR2 fragment incorporating antigenized foreign loop sequences, i.e., IgE loop sequences in replacing native loop 6, was produced using the same reverse primer, gfp-r, and different forward primers. The four forward primers are gfp#6-f-bc8, aattgtatcatcatggcagacGTGGTGGACCTGG CACCCAGCAAG ggaatcaaagtaactcaaaat, gfp#6-f-de8, aattgtatcatcatggcagacAAGCAGCGCAATGGCACGT-TAACCggaatcaaagtaactcaaaat, gfp#6-f-fg8, aattgtatcatcatggcagacCACCCCCACCTGCCAGGGCCCTC ggaatcaaagtaactcaaaat, and gfp#6-f-ch2-3L8, aattgtatcatcatggcagacGATTCCAACCCGAGAGGGGT-GAGCggaatcaaagtaactcaaaat. The underlined sequence is complementary to the primer, gfp#6-r. DNA sequences of IgE inserts were determined with reverse sequencing primer Rd 4 688r and used.

IgE epitope antigenized GFP chimeric proteins were expressed *in vitro* in a transcription-translation system based on *E. coli* lysates for 6 h at 30 °C (Roche, Rapid Translation System RTS 100 *E. coli* HY Kit). Samples were left overnight at 4 °C to allow for efficient folding of newly synthesized protein molecules. Translated protein was precipitated first by acetone to remove the interfering material in the lysates, followed by resubilization in native gel sample treatment buffer containing 1% NP40 without SDS and 2-ME, and also in the absence of heating during sample solubilization in 1% NP-40 alone. The translated proteins were separated by native gel electrophoresis, followed by transferring to nitrocellulose in diluted methanol carrier also in the absence of 0.1% SDS. To diminish background, blots were incubated and blocked with goat IgG (2 mg/ml) followed by detection with anti-GFP antibody (GFP Monoclonal Antibody, purified, mouse clone B34, Covance, Cat. No. MMS-118P, 1/3000, and rat anti-mouse κ , 1/1000). In parallel, duplicate blots were probed with anti-human IgE antibodies of a 1/1 mixture of two commercial sources of antibodies: goat anti-human IgE–HRP conjugate (Bethyl, Cat. No. A80-108P) and goat anti-human IgE–HRP conjugated (Caltag Laboratories, Cat. No. H15707).

1.4. Preparation of antibodies against antigenized GFP

For larger scale of protein production, the above PCR fragments of antigenized GFP (C2-3L and BC loop) were digested with Nco I and Sma I, and the large fragments were cloned into similar double digest of pIVEX 2.3. DH5 α was then transformed with annealed antigenized GFP/pIVEX 2.3. Following IPTG induction, the transformed bacteria were solubilized by 1% Triton, 10 mg/ml lysozyme and antigenized GFP was purified by the IMAC column. BALB/c mice were primed with 10 μ g of antigenized GFP in 2 mg alum for 10 days, boosted three times at the intervals of 14 days, and sera were collected 10 days after the last boost. The purification procedure involved a clarification of 50% ammonia sulfate cut, protein G purification (Pierce), followed by purifying on NP-specific chimeric mouse/human JW8 IgE (clone provided by Dr. Neuberger at MRC) coupled on Sepharose 4B, and assayed by human IgE (PS). Anti-immunoglobulin rheumatoid factor activity was removed by adsorption on mouse and human IgG coupled to Sepharose 4B. Each stage of enrichment was tested by ELISA for immune reactivity on JW8 coated 96-well, followed by HRP–MAb rat anti-mouse IgG and substrate. Up to 5% or more of affinity protein G fraction are specific for human IgE.

1.5. Preparation of recombinant soluble huFc ϵ RI α

The total RNA was isolated from KU812 cells (obtained from Mike Robertson, TSRI). RT-PCR performed with appropriate primers and cloned into pGEM (Promega). A full-length receptor comprising the signal sequence, homology domain D1 and D2 and membrane anchor was cloned. The first strand cDNA was reverse transcribed with both oligo d(T) and random primers. Full length Fc ϵ RI α cDNA was amplified using forward and reverse primer pairs, gaagaattcgaa-gatggctcctgcatgg, and gtagtagcaattgctgatgctgga. Secreted (sR: transmembrane domain truncated, amino acids from 26 to 204) form was constructed using baculo-transfer vector pAcSG2. Another series was made in pAcGP67 with GP67 insect signal sequence (sRA), was used for production of single soluble receptor with HA tag for the convenience of purification.

Recombinant monomeric and dimeric receptors were produced in sf9 insect cells. Receptor proteins in supernatants and cell extracts were pooled. Receptors were fractionated around 22% NaCl gradient (10 mM to 1 M) on Mono-Q column. Alternatively, monomeric receptor can be enriched by anti-HA column by acidic elution. Receptor-based ELISA 96-well were coated overnight

with 10 $\mu\text{g/ml}$ NP-BSA, washed, blocked, and added with NP-specific chimeric mouse/human JW8 IgE for 2 h at r. t. Plates were washed, followed by addition of dimeric and monomeric receptor at 0.1, 1 and 10 $\mu\text{g/ml}$ for 1 h at r. t. HRP conjugate of rabbit anti-HA was then added at 1/3000, followed by substrate.

1.6. ELISA assays

Recombinant huFc ϵ RI α , prepared from the insect sf9 cells expressing single or doubly tandem receptor construct, was used for coating the plates at 10 $\mu\text{g/ml}$ with similar results. JW8 IgE was titrated on receptor-coated plate from 200 ng to 2.4 ng/ml, washed and detected by the HRP–anti-IgE (PharMingen). To perform the inhibition assay, 50 μl of pooled sera were

added to 50 μl of JW8 IgE (200 ng/ml) at the final serum dilutions from 1/2 to 1/8 to 100 ng/ml JW8 IgE. The mixture were incubated for 30 min at r.t., followed by addition to receptor-bound 96-well plate for 1 h at r. t., washed and the presence of receptor-bound IgE on solid phase was detected by HRP–MAB anti-human IgE.

To determine anti-IgE titers, 96-wells were prepared by coating with NP-BSA at 1 $\mu\text{g/ml}$ for 1 h at r. t. followed by anti-NP chimeric murine/human JW8 IgE at 1 $\mu\text{g/ml}$ overnight. As standard, MAB anti-human IgE (PharMingen) was added from 100 ng to 1.6 ng at two fold dilutions, followed by HRP–rat anti-mouse IgG (HRP–RaAMG) and substrate. The titers of the antisera were determined by adding sera from 1000 to 500,000 dilutions to the above IgE-coated plate, followed by HRP–RaAMG, and OD value two fold above the background

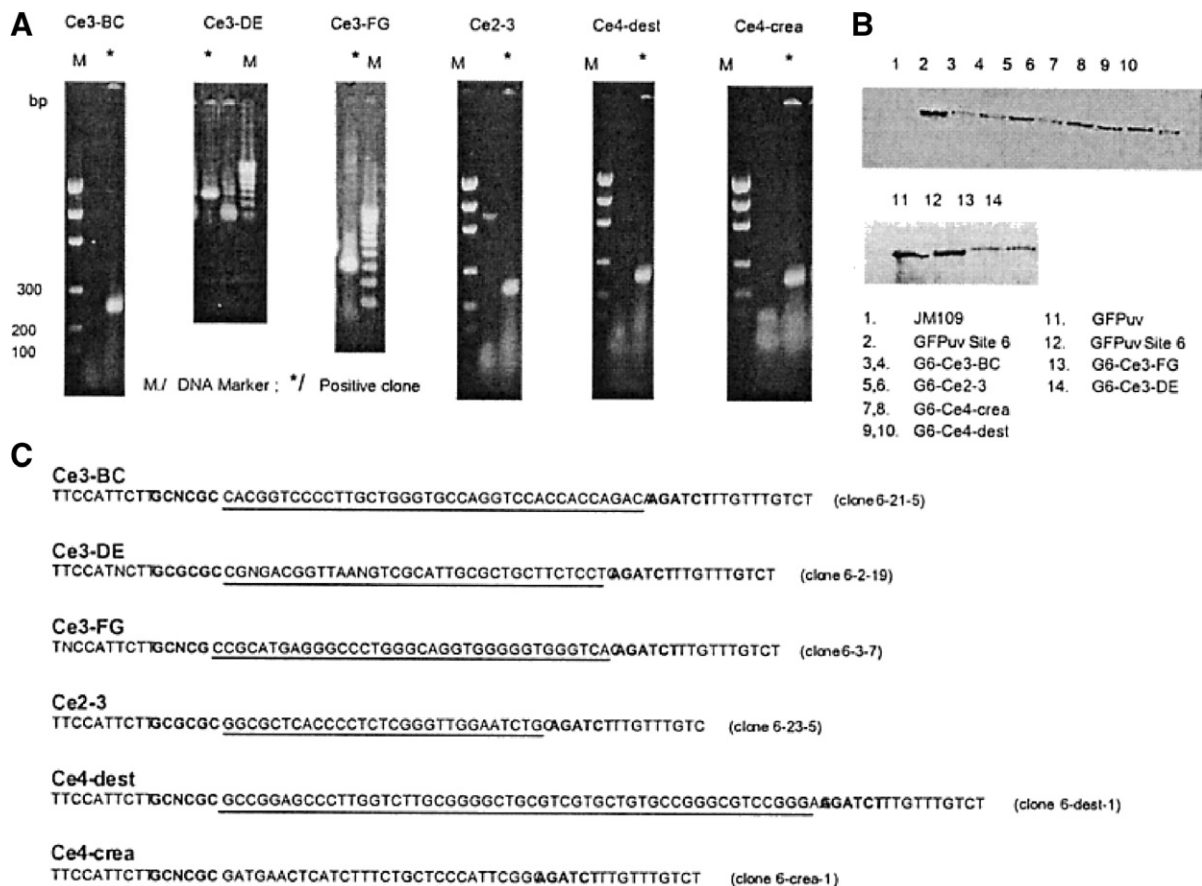


Fig. 2. Site 6 antigenized GFP constructs Six sets of 16-mer oligonucleotides corresponding to Fc ϵ RI α binding sites and non-binding site controls were prepared with Bgl II and Kpn I at 5' and 3' ends. The digests of oligonucleotides and scaffold vector were annealed. Diagnostic cut with Bgl II and Sac I yielded fragments of 250 bp denoted by the star symbol (*) Panel A). Lysates prepared from the transformed cells were blotted by polyclonal rabbit anti-GFP, and expressed antigenized GFP was shown as 25–26 kDa bands (Panel B). Inserted IgE loop sequences at site 6 were verified by DNA sequencing (Panel C).

was considered a positive titer, and the concentrations of anti-IgE calculated from the standard curve.

1.7. Mast cell degranulation

Mast cell line, 2H3 stably transfected with huFcεRIα were pre-treated for 24 h with dexamethasone (10 μM final concentration) to upregulate human FcεRIα expression. Mast cells were then sensitized for 30 min with 1 μg/ml IgE or 1 μg/ml IgE pre-incubated for 1 h with anti-C2-3L from 10 to 100 μg/ml, washed and then challenged with 1 μg/ml NP-BSA. β-hexosaminidase release was then determined. The hexosaminidase release was triggered by NIP₂₃ BSA (obtained from Dr. Garnett Kelsoe, Duke University) added to the cells at the concentrations 1 μg/ml for 45 min at 37 °C. β-hexosaminidase release was calculated as a percentage of the secreted versus total available cell hexosaminidase. The secreted (supernatants) and total (cell lysates) enzyme contents were estimated as OD₄₀₅ after incubation with substrate *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide for 90 min at 37 °C.

2. Results

2.1. Expression of antigenized GFP

Site 6 GFP vector scaffold was inserted with oligonucleotides of human IgE sequences corresponding to FcεRIα binding BC loop, FG loop, DE loop and C2-3 linker loop (C2-3L), respectively (Garman et al., 2000). Sequences from CHε4 predicted as B-cell epitopes (Mac vector Inc., Cary, NC), were employed as control. Fig. 2A showed diagnostic cuts of site 6 antigenized GFP vectors containing the oligonucleotide inserts following digestion of Bgl II and Sac I.

Expression of antigenized GFP protein was then determined by western blots with anti-GFP antibodies. Recombinant native GFP and antigenized GFP from bacterial extracts were electrophoresed and immunoblotted, and detected by anti-GFP antibodies (Fig. 2B). Antigenized GFP proteins migrated as a specific band from 27 to 29 kDa (lanes 2–10; 13–14) in contrast to control bacteria transformed with original vector or site 6 modified vector (lanes 11 and 12), while this material was not found in mock-transformed JM109 (lane 1). The presence of the oligonucleotide inserts in antigenized GFP constructs was also confirmed by DNA sequencing (Fig. 2C). A parallel set of site 7 oligonucleotides antigenized constructs was likewise prepared, inserts sequenced, and recombinant fusion protein migrated as a 27–29 kDa band (not shown).

2.2. Construction of *in vitro* expression cassette for displaying human IgE epitopes using GFP as a scaffold molecule

The feasibility of expressing a soluble antigenized GFP above prompts development of an *in vitro* expression platform for studying the immunogenicity of the inserted sequences. Fig. 3 depicts a strategy to bypass the requirement of steps of molecular cloning, and *in vivo* bacterial transformation and expression. Four different receptor-binding epitopes of human IgE were inserted and replaced the original site 6 residues in GFP. The immunogenicity of the replaced IgE B-cell epitopes of antigenized GFP can be expediently evaluated as an *in vitro* translated product.

This is achieved via one single tube reaction of coupling the *in vitro* transcription/translation procedure according to the following three engineering steps: (i) PCR1 fragment utilizing a pair of primers that amplify the half molecules spanning the T7 transcription start site, Shine/Dargalno sequence (He and Taussig, 1997) and the beginning of the GFP (delineated by the 5' primer) up to the upstream GFP sequence to site 6 (delineated by the 3' primer). PCR1 consists of 5' regulatory sequence and upstream of wide type GFP from loop 6, which was produced using pIVEX control GFP DNA as a template with forward primer, gfp-f and the reverse primer, gfp#6-r (Fig. 3B and D, lane 5). (ii) PCR2 fragment utilizes 5' primer consisting of GFP sequences (that pair with the 3' primer of the first PCR fragment), the eight amino acids corresponding FcεRIα-binding IgE loop, and 23 oligonucleotides of gfp sequence downstream and thus deleting the native loop 6 sequences. And the 3' primers include His-tag and T7 terminator (Fig. 3B, D, lanes 1 to 4). (iii) Assembly of the two half molecules of GFP, the stuffer PCR1 fragment and PCR2 (Fig. 3C). Fig. 3E showed assembled 1 kb fused products from PCR1 and PCR2 exhibiting antigenized IgE sequences: gfp-6-bc8; gfp-6-de8; gfp-6-fg8; gfp-6-ce2-3L8, respectively (Fig. 3E, lanes 2–5) and GFP control without insert (lane 1).

Next, the PCR-amplified DNA can then be directly employed for transcription/translation-coupled reaction for *in vitro* protein expression. Fig. 4A confirmed the constructs of the assembled PCR products as illustrated in Fig. 3; and base sequences of these newly assembled products were determined by DNA sequencing (Fig. 4B). Further, antigenicity of the *in vitro* expressed transcribed/translated products (in capital letter): GFP-6-BC8, GFP-6-DE8, GFP-6-FG8, GFP-6-C2-3L8 was then evaluated. Fig. 4C showed the detection of translated antigenized GFP constructs ~27 kDa (Fig. 4C,

Construction of *in vitro* Expression Cassette for Displaying Human IgE Epitopes using GFP as a Scaffold Molecule

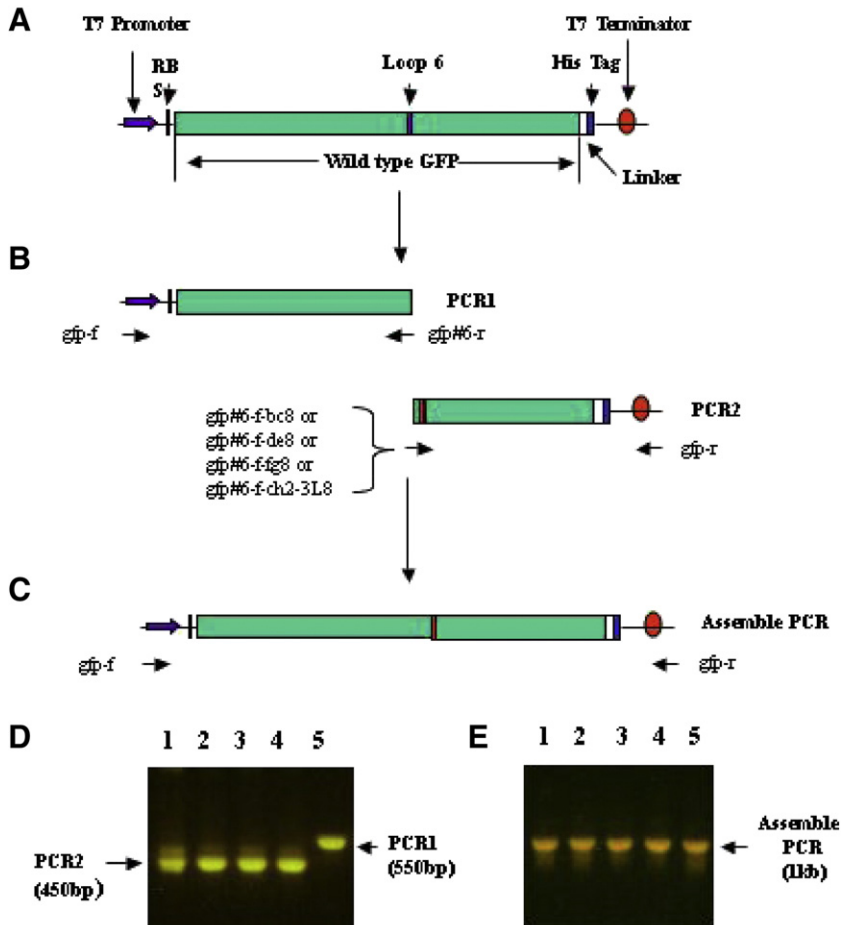


Fig. 3. Construction of *in vitro* expression cassette for IgE B-cell epitopes in GFP scaffold vector. A complete expression cassette of wide type GFP was generated using pVEX control vector GFP as a template (Panel A). The primers and designs were described in Materials and methods. Oligonucleotides encoding different IgE B-cell epitopes prepared with identical 5' and 3' of the flanking sequences were inserted in PCR2 fragment (Panel B). Strategy for reassembled complete antigenized cassette (Panel C), and PCR2, PCR1 fragments and the assembled products were shown (Panels D and E).

lanes 1 to 4), positive control transcribed/translated wild type GFP (lane 5), control purified GFPUV protein (lane 6), and negative control of translated lysate only without added template (lane 7).

To determine the antigenicity of antigenized 8-mer IgE sequence constrained within GFP scaffold, the translated products were evaluated under native gel separation conditions. It is noteworthy that although expression was observed with four PCR assembled products according to the detection of GFP scaffold (Fig. 4C), FG loop, DE loop antigenized GFP were not detected by polyclonal anti-IgE, distinct reactivities were observed with antigenized GFP encompassing C2-3L8 linker loop, and a barely

detectable signal with BC loop antigenized product (Fig. 4D). Furthermore, no reactivities with polyclonal anti-IgE were noted with translated GFP, purified GFP protein and lysates.

2.3. Preparation of purified C2-3L antigenized GFP

GFP exhibits 10 histidine residues that interact favorably with chelated metals such as Ni (II), in particular, with histidines at the top of the β -barrel, His₇₇, His₈₁ and His₂₃₁ as an intrinsic His-tag (Tsien, 1998). Thus, immobilized metal affinity chromatography (IMAC) was employed for purification of antigenized GFP (Li

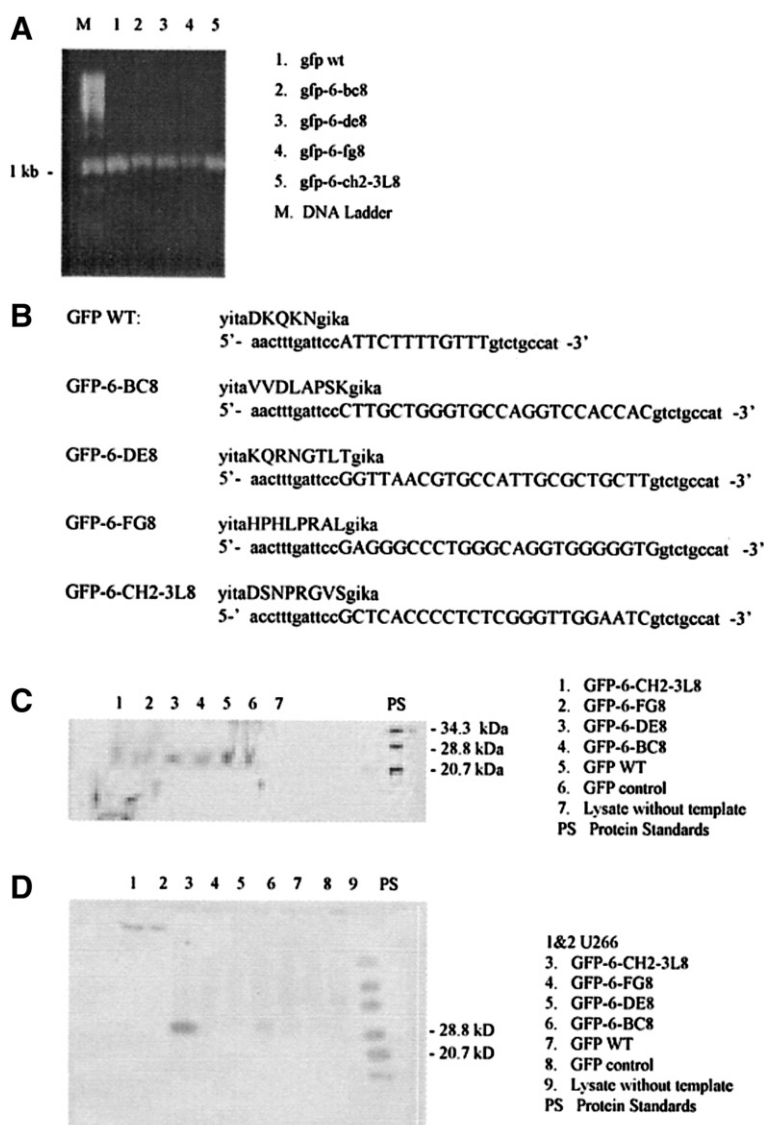


Fig. 4. Evaluation of IgE B-cell epitopes constrained in the GFP scaffold. The amplified DNA fragments:gfp-6-bc8; gfp-6-de8; gfp-6-fg8; gfp-6-ce2-3L8 and gfp wt were verified by length (Panel A), and by sequencing (Panel B). Translated IgE B-cell epitopes-GFP chimeric proteins: GFP-6-BC8, GFP-6-DE8, GFP-6-FG8, GFP-6-CH2-3L8, and wild type GFP (GFP WT), were prepared and evaluated by western blot with anti-GFP (Panel C) or with polyclonal anti-IgE (Panel D) as described in Materials and methods.

et al., 2001). Recombinant protein was solubilized and prepared as described (Material and methods). Antigenized GFP was observed to distribute homogeneously in the cytoplasm and the fluorescence-emitting material was localized in the Triton X-100 clarified lysates and not in the inclusion bodies pellet. C2-3 linker-antigenized GFP can be conveniently enriched via passage of clarified lysates via IMAC column due to intrinsic His-tag. Fig. 5A showed the fractionation profile of C2-3L antigenized GFP by adsorption to Ni-based gel bed, followed by elution in 250 mM imidazole

buffer in fractions 7 to 11 on IMAC column. Fig. 5B showed that this antigenized GFP was relatively enriched in fractions 6 to 8; and was highly enriched in tubes 9 and 10, as a 27–29 kDa band away from the contaminating bands shown by SDS-PAGE. C2-3L antigenized GFP was notable across all the fractions by immuno-blot with a polyclonal anti-GFP. Thus, IMAC affinity adsorption did not yield highly pure antigenized GFP product in our hand.

Next, IMAC-enriched material was further separated by sizing chromatography by Sephacryl S-200 HR due to

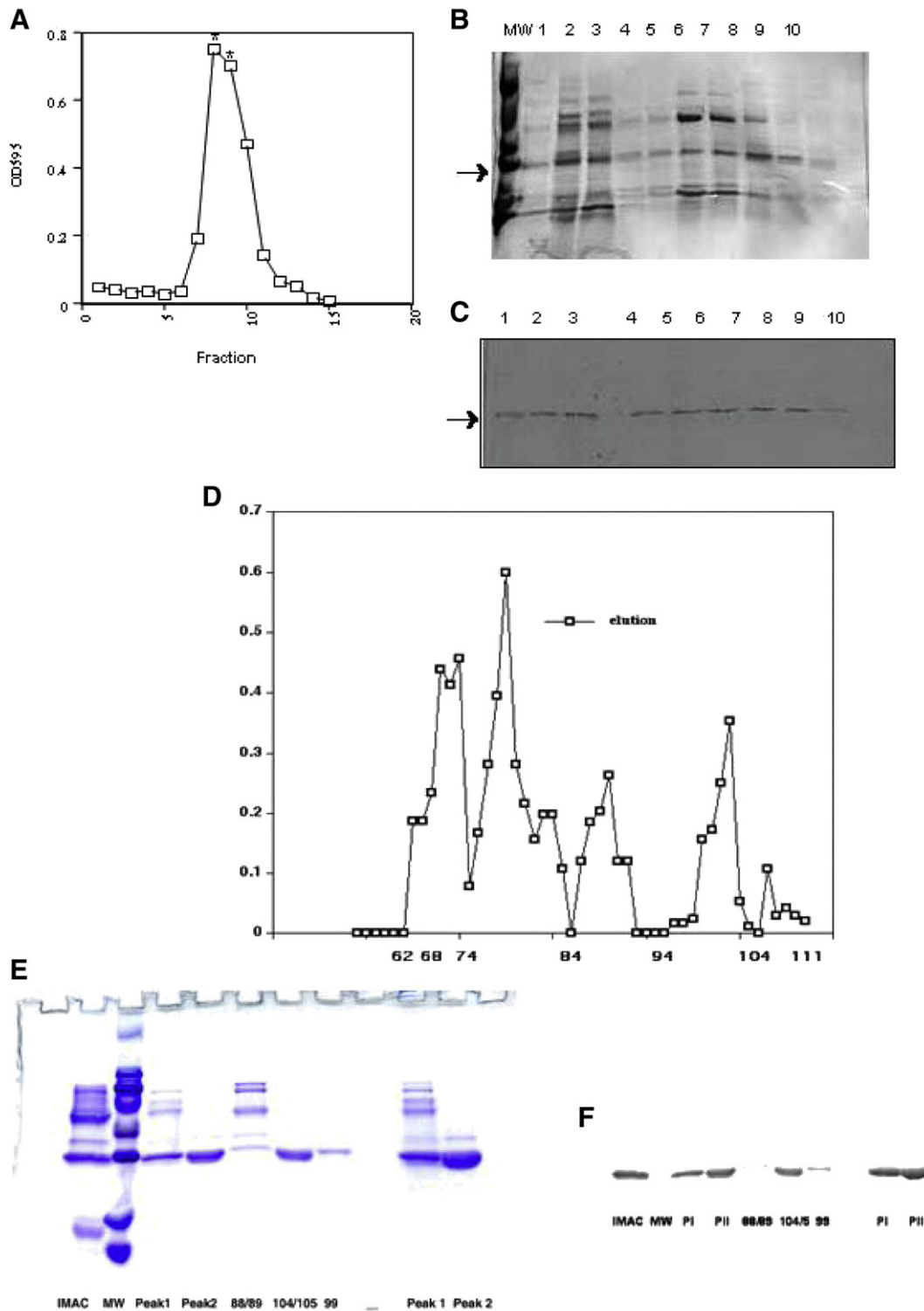


Fig. 5. Purification of C2-3L antigenized GFP. C2-3 linker transformed DH5 α was selected. Procedures of enrichment on IMAC column and gel filtration on Sephacryl S-200 HR were described in Materials and methods.

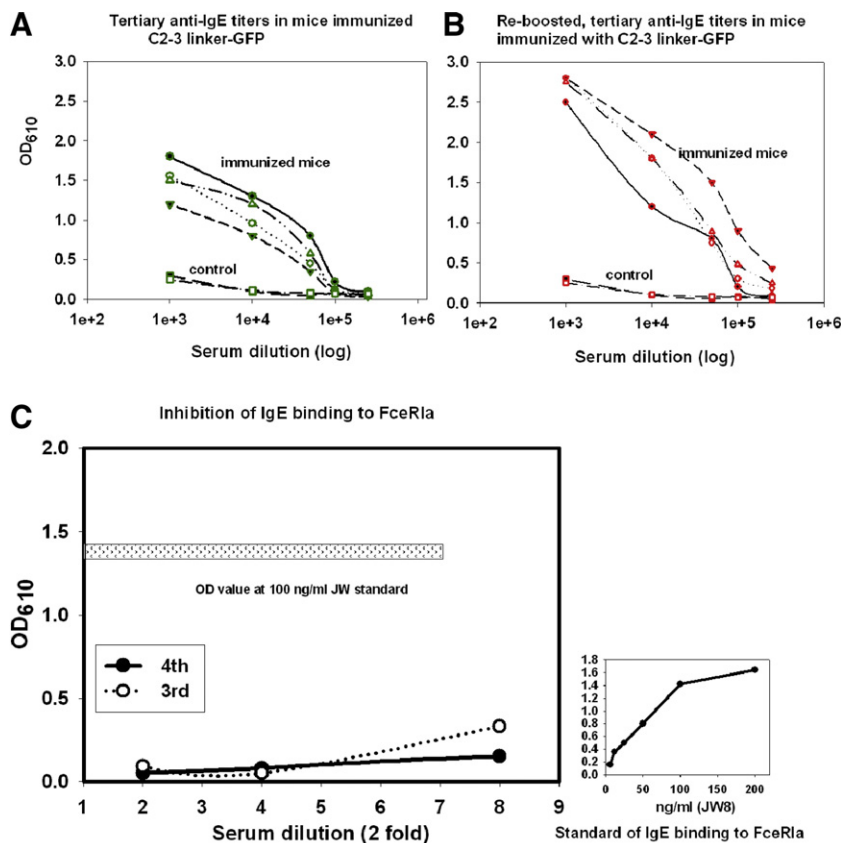


Fig. 6. Immune sera were prepared in BALB/c mice, immunized with 10 μ g of C2-3L antigenized GFP in 2 mg alum and boosted twice at the intervals of 14 days. Immune sera were collected day 10 after the third challenge (Panel A) or fourth challenge (Panel B). ELISA for measuring anti-human IgE was described in Materials and methods. ELISA for measuring IgE bound to recombinant receptor (Panel C) was described in details in Materials and methods, and the standard curve was presented as an inset of Panel C.

its resolution of separating ~ 30 kDa globular proteins from neighboring species. As shown in Fig. 5D, five distinct peaks were resolved under the current operation conditions. As noted before, the IMAC-enriched, pooled starting material exhibited major contaminated molecular species (lane IMAC). The peak (tubes 85–90) with approximately 4.5 mg determined according to OD₂₈₀ exhibited a significant amount of antigenized GFP with other contaminated proteins. Importantly, pure C2-3L antigenized GFP was indeed isolated in peak 2 (tube 100–103) with approximately 3.8 mg determined at OD₂₈₀ and shown as a single ~ 27 to 29 kDa band in SDS-PAGE (Fig. 5E). Thus the total yield of peaks 1 and 2 combined was approximately 8.3 mg from the 25 mg of IMAC-enriched material following sizing column according to the chromatogram (Fig. 5D) monitored by OD₂₈₀. Thus the sizing step contributed to ~ 3 fold enrichment. Thus, the overall two-step purification yielded 50 fold enrichment, as calculated from the starting material of ~ 400 mg clarified protein prior to the IMAC column.

Assuming no loss of materials during steps of handlings and purification, the recombinant antigenized GFP is estimated to be at least 2.1% abundance in the total clarified protein lysate. In summary, the efficacy of the present scheme of purification is approximately 50 fold of enrichment following IMAC and Sephacryl-S-200 HR. This result indicates the adequacies of expression levels and the suitability of the step-wise procedures for enrichment. To corroborate the purity of the material, western blots reacted with antigenized GFP as an ~ 27 to 29 kDa band, present in IMAC eluted material, peak 1 mixture as well as in high intensity in peak 2 pure antigenized GFP (Fig. 5F).

To further substantiate the demarcation of the peak 2 purified antigenized GFP, Fig. 5D to F showed that tubes 88/89 delineating the right boundary of peak 1 contained contaminating materials along with significant presence of antigenized GFP. In contrast, material demarcating the boundaries of peak 2, i.e., material from left boundary tube (#99) as well as from the right

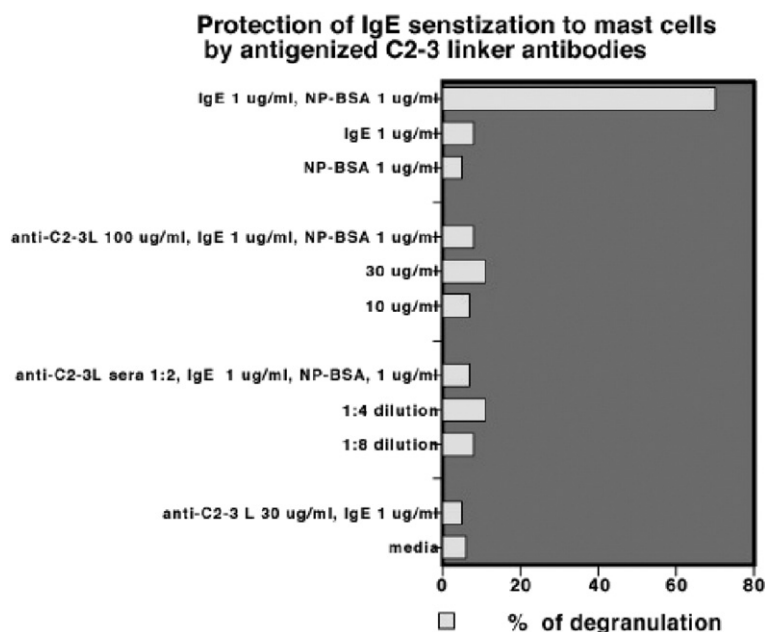


Fig. 7. HuFcεRIα-transfected mast cells were pre-treated for 24 h with dexamethasone. Equal volume of anti-C2-3L antibodies was mixed with JW8 at the final concentrations from 10 to 100 μg/ml anti-IgE to 1 μg/ml JW8 (to mimic human allergic IgE levels) for 30 min at r.t. Mast cells (0.5×10^6) were then sensitized to the above mixture for 30 min at 37 °C in a CO₂ incubator, washed and then challenged with 1 μg/ml NP-BSA and specific hexosaminidase release was determined.

boundary tubes (#104 and #105), exhibited only a single band of purified antigenized C2-3L GFP, albeit at a less concentration than the pooled peak 2 material containing the major purified antigenized GFP, i.e., tubes #100 to #103). Thus Fig. 5D to F demonstrate unambiguously an effective separation of C2-3L antigenized GFP according to a first enrichment by metal affinity column, followed by a high resolution gel filtration on Sephacryl S-200 HR column. It may be noticed that recombinant C2-3L antigenized protein was enriched and purified under native condition throughout preparations from the clarified lysate to final yield.

2.4. Antibodies elicited by the C2-3L antigenized GFP block IgE binding to FcεRIα

Next, we evaluate whether the purified C2-3L antigenized GFP may be employed as immunogens in eliciting neutralizing, protective anti-IgE antibodies in BALB/c mice. Fig. 6 showed that individual mice that received tertiary immunization of C2-3L antigenized GFP exhibited a titer of 1 to 50,000 (Fig. 6A), and upon a re-boost, serum titers were elevated to a range of 1/50,000 to 250,000 (Fig. 6B). Concentrations of anti-IgE in immune sera were measured at 245 ± 37 μg/ml upon tertiary immunization, and around 784 ± 95 μg/ml following a re-boost with ELISA of human IgE-coated plate. Similar

observations with comparable results were repeated in BALB/c and C57BL/6 mice (not shown). The concentrations of *in vivo* anti-IgE in these experiments may be relevant to the clinical setting of employing passive anti-IgE, MAb-E25, i.e., effective blood concentration of MAb-E25 is maintained at a steady state around 30 mg/ml blood in patients (300 kU/l IgE) that received 300 to 400 mg bi-weekly injection up to four times.

Next, It is pertinent to determine the protective effect of antisera in neutralizing binding of human IgE to human FcεRI. Thus the neutralizing activities against IgE binding to recombinant FcεRIα receptors were examined. The outset of Fig. 6C showed the establishment of a solid phase recombinant receptor capture assay in that high sensitivity of binding of JW8 IgE to solid phase receptor was shown with soluble IgE (100 ng/ml) captured to plate-bound recombinant receptor, exhibiting an OD ~ 1.4. Importantly, Fig. 6C showed that binding of JW8 IgE to recombinant receptors was profoundly abrogated by tertiary antisera up to 1/4 dilutions, and re-boosted hyperimmune sera at 1/8 dilution. This observation suggests that *in vivo* protection of serum IgE from binding to mast cells by the undiluted, neat sera may be even more effective than the *in vitro* correlate.

Next, we examine the role of anti-C2-3L on protection JW8 IgE to receptors on mast cells. Mast cell

degranulation via human IgE and human FcεRIα was performed with RBL-2H3 was transfected with human FcεRIα (Robertson, 1993; Nechansky et al., 2001). Thus, the mast cell-based *in vitro* protection assay was set with serum dilutions as well as with affinity pure anti-C2-3L in a range of 10 to 100 mg/ml in neutralizing 1 mg/ml JW8 IgE. The dosages of protective neutralization assay was set according to a clinical setting of employing passive anti-IgE, maintained around 30 mg/ml blood in treated patients. As shown in Fig. 7, ~95% protection was observed in JW8 incubated with affinity purified anti-IgE at the tested concentrations from 10 to 100 mg/ml. In contrast, in the absence of anti-C2-3L antibodies, about 68% of enzyme release was noted in IgE-sensitized, huFcεRIα-transfected mast cells followed by NP-BSA challenge, and approximately, 5% nonspecific B-hexosaminidase release was noted in JW8-sensitized mast cells without allergen challenges. This observation indicates that anti-IgE elicited by C2-3L antigenized GFP prevent IgE sensitization to mast cells; and furthermore, the pre-incubated immune complexes also did not activate mast cells, suggesting that elicited antibodies do not react with non-receptor-binding region of JW8 IgE and may be safely employed for human use.

3. Discussion

3.1. Suitability of GFP as antigenic scaffold

Loop sequences spanning part of the scaffold protein are long known to play a critical role in the biological, i.e., enzymatic, ligand/receptor, antibody, and antigenic specificities. These amino acid residues within the scaffold molecules are characterized by being hydrophilic in nature, surface-exposed, and mobile. Earlier, Zanetti utilized the immunoglobulin fold as a scaffold to present a grafted oligopeptide epitope in an immunologically accessible and conformationally suitable manner (Billetta et al., 1991; Lanza et al., 1993). Later, Brent and Kamb constrained peptide aptamers on the loop region of thioredoxin and GFP respectively that exhibit a pertinent biological conformation to modulate signal transduction to modulate cell growth and differentiation (Abedi et al., 1998; Geyer et al., 1999). Recently, Bradbury showed that grafting lysozyme binding sequence from a camel antibody in GFP loop confer recognition capacity of grafted GFP (Kiss et al., 2006).

GFP is a stable proteolysis-resistant single chain of 238 amino acids exhibiting an 11-stranded β-barrel wrapped around a single central helix, forming a rigid β-barrel. Herein, we study whether receptor-binding loops of IgE may be inserted and protected within the confine

of the GFP scaffold insert and constrain protective IgE sequences into the solvent-exposed loops of GFP as antigens (Abedi et al., 1998; Peelle et al., 2001). The chromophore, consisting of a modified tripeptide, is buried inside the relatively rigid β-barrel structure. Insertion of six oligopeptides into site 6 of GFP significantly quenched and diminished the intrinsic fluorescence. Antigenized GFP with inserts of 16 amino acids in length were expressed and detected with anti-GFP in the immuno-blot (Fig. 2); however, they did not react with polyclonal sources of anti-human IgE employed in this study (not shown). This may reflect lack of the constrained conformation of longer inserted peptides even though GFP is known thermostable and can accommodate selectable aptameric peptide library of shorter length (Peelle et al., 2001). On the other hand, at least one of the four 8-mer cassettes, C2-3 linker can be constrained in site 6 of GFP scaffold, and weak reactivity to BC loop antigenized GFP was detectable. It is possible that the reactivity of BC loop may be improved with shorter core sequence insertion. The analyses of the constrained antigenicity can be conducted by the *in vitro* transcription/translation bypassing steps of laborious cloning and protein production. With the present *in vitro* transcription/translation system, future studies may be expediently conducted with even shorter oligopeptide inserts, and/or aided with further constraining disulfide loops flanking the inserts.

3.2. Anti-IgE active antibodies protect against IgE binding to FcεRIα

To test the feasibility of antigenized GFP as active vaccine, antibodies elicited to the receptor-binding loops of IgE constrained by GFP scaffold may lead to inhibition of IgE binding to FcεRI receptors (Garman et al., 2000; Wan et al., 2002). The CHε2–CHε3 linker region, C2-3L (residue 332 to 337) is critically involved in the docking of solution-phase or circulating IgE to the cognate receptor. Antibodies raised to C2-3L antigenized GFP, appropriately constrained, indeed prevent JW8 chimeric murine/human IgE from binding to recombinant FcεRIα coated on solid phase as well as those expressed on the cell surface of transfected RBL-2H3.

Formation of immune complexes is essential for protection by anti-IgE. It is possible that anti-C2-3L form effective immune complexes with free circulating IgE by neutralizing each respective determinant on the half molecule of the solution-phase IgE. Indeed, MAb-E25 is capable of forming hexameric antigen-antibody complexes via reacting to two identical interaction sites of circulating IgE even though the physical binding

sequences to which MAb-E25 reacts are not known (Corne et al., 1997; Chang, 2000; Garman et al., 2000; Wan et al., 2002). Moreover, since antibodies elicited by C2-3L antigenized GFP recognize conformation on solution-phase, i.e., circulating but not cell-bound IgE, the current construct therefore offers a promise to serve as a safe pan-IgE vaccine for human use. Observations shown in Figs. 6 and 7 substantiate this notion since actively produced antibodies elicited by antigenized C2-C3L did not cross-linked IgE on IgE-sensitized mast cells, while effectively abrogating sensitization of solution-phase JW8 IgE to huFcεRIα-transfected mast cells.

3.3. Relevance of concentrations of active anti-IgE in protecting against IgE-mediated allergy

High antibody titers were frequently observed with optimal immunizations in studies conducted in our lab and by others, including anti-peptide antibodies (Alexander et al., 2000a). Concentrations of anti-IgE present in immune sera were in the range from 250 to 800 μg/ml according to immunization protocol (Fig. 6), constituting 1.5 to 4% of IgG fraction (Chen et al., 1984; Alexander et al., 2000b; Takasuka et al., 2004).

The data presented herein offers a feasible model for the *in vivo* protection of active immunization against IgE-mediated allergy. MAb-E25 recommended for patients higher than 75 kU/l (or 75 IU/ml, i.e., 250 ng/ml). Such patients are administered with MAb-E25 subcut. at 300 to 400 mg per injection resulting in a blood level of anti-IgE of 30 μg/ml, i.e., about 100 M excess of antibodies to circulating IgE. To ensure sufficient protection, patients should receive four bi-weekly or at least on the monthly of MAb-E25 (half life ~2 to 3 weeks). With regard to the active vaccination, the advantage of undiluted neat sera, extrapolating from the rodent study herein is likely to prevail over the passive treatment since 800 to 3,000 fold molar excess of endogenous anti-IgE to circulating IgE may be anticipated (Figs. 6 and 7).

In conclusion, this study provides a feasible *in vitro* and *in vivo* model for studying protection of IgE-mediated allergic diseases via active immunization with appropriately constructed antigenized GFP vaccine. Further this study may promise a general method of molecular engineering for studying conformationally-constrained B-cell epitopes of other protein antigens.

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References

- Abedi, M.R., Caponigro, G., Kamb, A., 1998. Green fluorescent protein as a scaffold for intracellular presentation of peptides. *Nucleic Acids Res.* 26, 623.
- Alexander, J., del Guercio, M.-F., Maewal, A., Qiao, L., Fikes, J., Chesnut, R.W., Paulson, J., Bundle, D.R., DeFrees, S., Sette, A., 2000. Linear PADRE T helper epitope and carbohydrate B cell epitope conjugates induce specific high titer IgG antibody responses. *J. Immunol.* 164, 1625.
- Billetta, R., Hollingdale, M.R., Zanetti, M., 1991. Immunogenicity of an engineered internal image antibody. *Proc. Natl. Acad. Sci. U.S.A.* 88, 4713.
- Caponigro, G., Abedi, M.R., Hurlburt, A.P., Maxfield, A., Judd, W., Kamb, A., 1998. Transdominant genetic analysis of a growth control pathway. *Proc. Natl. Acad. Sci. U.S.A.* 95, 7508.
- Chang, T.W., 2000. The pharmacological basis of anti-IgE therapy. *Nat. Biotechnol.* 18, 157.
- Chen, S.-S., Liu, F.-T., Katz, D.H., 1984. Cellular and molecular mechanisms of murine IgE class-restricted tolerance induced by neonatal administration of soluble or cell-bound IgE. *J. Exp. Med.* 160, 953.
- Christmann, A., Walter, K., Wentzel, A., Kratzner, R., Kolmar, H., 1999. The cystine knot of a squash-type protease inhibitor as a structural scaffold for Escherichia coli cell surface display of conformationally constrained peptides. *Protein Eng.* 12, 797.
- Corne, J., Djukanovic, R., Thomas, L., Warner, J., Botta, L., Grandordy, B., Gygas, D., Heusser, C., Patalano, F., Richardson, W., Kilcherr, E., Staihelin, T., Davis, F., Gordon, W., Sun, L., Liou, R., Wang, G., Chang, T.-W., Holgate, S., 1997. The effect of intravenous administration of a chimeric anti-IgE antibody on serum IgE levels in atopic subjects: efficacy, safety, and pharmacokinetics. *J. Clin. Invest.* 99, 879.
- Garman, S.C., Wurzburg, B.A., Tarchevskaya, S.S., Kinet, J.P., Jardetzky, T.S., 2000. Structure of the Fc fragment of human IgE bound to its high-affinity receptor Fc epsilonRI alpha. *Nature* 406, 259.
- Geyer, C.R., Brent, R., 2000. Selection of genetic agents from random peptide aptamer expression libraries. *Methods Enzymol.* 328, 171.
- Geyer, C.R., Colman-Lerner, A., Brent, R., 1999. "Mutagenesis" by peptide aptamers identifies genetic network members and pathway connections. *Proc. Natl. Acad. Sci. U.S.A.* 96, 8567.
- He, M., Taussig, M.J., 1997. Antibody-ribosome-mRNA (ARM) complexes as efficient selection particles for *in vitro* display and evolution of antibody combining sites. *Nucleic Acids Res.* 25, 5132.
- Kiss, C., Fisher, H., Pesavento, E., Dai, M., Valero, R., Ovecká, M., Nolan, R., Phipps, M.L., Velappan, N., Chasteen, L., Martinez, J.S., Waldo, G.S., Pavlik, P., Bradbury, A.R.M., 2006. Antibody binding loop insertions as diversity elements. *Nucleic Acids Res.* 34, e132.
- Koide, A., Bailey, C.W., Huang, X., Koide, S., 1998. The fibronectin type III domain as a scaffold for novel binding proteins. *J. Mol. Biol.* 284, 1141.
- Lanza, P., Billetta, R., Antonenko, S., Zanetti, M., 1993. Active immunity against the CD4 receptor by using an antibody antigenized with residues 41–55 of the first extracellular domain. *Proc. Natl. Acad. Sci. U.S.A.* 90, 11683.
- Li, Y., Agrawal, A., Sakon, J., Beitle, R.R., 2001. Characterization of metal affinity of green fluorescent protein and its purification through salt promoted, immobilized metal affinity chromatography. *J. Chromatogr.* 909, 183.
- Nechansky, A., Robertson, M.W., Albrecht, B.A., Apgar, J.R., Kricek, F., 2001. Inhibition of antigen-induced mediator release from IgE-sensitized cells by a monoclonal anti-Fc epsilon RI alpha-chain

- receptor antibody: implications for the involvement of the membrane-proximal alpha-chain region in Fc epsilon R-mediated cell activation. *J. Immunol.* 166, 5979.
- Nuttall, S.D., Rousch, M.J., Irving, R.A., Hufton, S.E., Hoogenboom, H.R., Hudson, P.J., 1999. Design and expression of soluble CTLA-4 variable domain as a scaffold for the display of functional polypeptides. *Proteins* 36, 217.
- Ormo, M., Cubitt, A.B., Kallio, K., Gross, L.A., Tsien, R.Y., Remington, S.J., 1996. Crystal structure of the *Aequorea victoria* green fluorescent protein. *Science* 273, 1392.
- Peelle, B., Lorens, J., Li, W., Bogenberger, J., Payan, D.G., Anderson, D.C., 2001. Intracellular protein scaffold-mediated display of random peptide libraries for phenotypic screens in mammalian cells. *Chem. Biol.* 8, 521.
- Robertson, M.W., 1993. Phage and *Escherichia coli* expression of the human high affinity immunoglobulin E receptor alpha-subunit ectodomain. Domain localization of the IgE-binding site. *J. Biol. Chem.* 268, 12736.
- Skerra, A., 2000. Engineered protein scaffolds for molecular recognition. *J. Mol. Recognit.* 13, 167.
- Takasuka, N., Fujii, H., Takahashi, Y., Kasai, M., Morikawa, S., Itamura, S., Ishii, K., Sakaguchi, M., Ohnishi, K., Ohshima, M., Hashimoto, S.-i., Odagiri, T., Tashiro, M., Yoshikura, H., Takemori, T., Tsunetsugu-Yokota, Y., 2004. A subcutaneously injected UV-inactivated SARS coronavirus vaccine elicits systemic humoral immunity in mice. *Int. Immunol.* 16, 1423.
- Tsien, R.Y., 1998. The green fluorescent protein. *Annual Review of Biochemistry* 67, 509.
- Wan, T., Beavil, R.L., Fabiane, S.M., Beavil, A.J., Sohi, M.K., Keown, M., Young, R.J., Henry, A.J., Owens, R.J., Gould, H.J., Sutton, B.J., 2002. The crystal structure of IgE Fc reveals an asymmetrically bent conformation. *Nat. Immunol.* 3, 681.