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Affinity improvement of the high-affinity immunoglobulin E receptor by phage display

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Abstract

The immunoglobulin E (IgE)-binding site of its high-affinity receptor is localized in the second immunoglobulin-like domain (D2) of the α-subunit (FcεRIα). In this study, the randomized pentapeptides were introduced between Glu^{132} and Ile^{138} of FcεRIα D2 and displayed on a filamentous phage. After eight rounds of panning, a phage clone having a mutation of $Asp^{135}Tyr^{136}Met^{137}$ in FcεRIα D2 was obtained. The binding affinity of the mutant phages to immobilized IgE was approximately 500 times higher than that of the wild type. The mutant phages competitively inhibited the binding of IgE to the soluble receptor at a 50% inhibition (IC_{50}) value of 116 pM. The mutant FcεRIα D2, which had been expressed as a fusion protein with glutathione *S*-transferase in *Escherichia coli*, also showed higher IgE-binding capacity than the wild type. The mutant FcεRIα D2 is expected to manifest its improved IgE-binding affinity together with any fusion partner.

Keywords: High-affinity IgE receptor; Phage display; GST fusion

The binding of immunoglobulin E (IgE) to its high-affinity receptor (FcεRI) on mast cells and basophils is required for the initiation of allergic reactions [1]. Cross-linking of the receptor-bound IgE with multivalent antigen induces cellular degranulation through a series of signal transduction, resulting in the release of chemical mediators, such as histamine, leukotrienes, and prostaglandins. Symptoms of allergic diseases and conditions, such as allergic rhinitis, asthma, and atopic dermatitis, are produced directly with these chemical mediators [1]. Current therapeutic agents, which inhibit release and action of these chemical mediators, are not always effective in allergic diseases and have some side effects [2]. Antisense oligodeoxynucleotides for the α-subunit of FcεRI (FcεRIα) have been a rational therapeutic approach to the IgE-mediated diseases [3]. Prevention of the binding of IgE to FcεRI with antibodies [2,4,5] and synthetic peptides [5–7] has also been investigated as rational therapeutic method. Soluble FcεRIα has been a promising candidate of competitive inhibitor for the IgE–FcεRI interaction [8–11].

FcεRI is a tetrameric complex composed of one α-, one β-, and a homodimer of disulfide-linked γ-subunits [1]. It was demonstrated that the IgE-binding site of FcεRI localized in the extracellular region of the α-subunit [12]. The extracellular region consists of two immunoglobulin (Ig)-like domains. Domain substitution and mutagenesis studies of the subunit demonstrated that the IgE-binding site of FcεRIα was localized in the second Ig-like domain (D2) [13–17]. Together with these mutagenesis studies, the crystal structure analyses of the IgE–FcεRIα complex reveal that FcεRIα binds to IgE at two sites in FcεRIα D2 [18,19]. Seven residues, Lys^{117}, Ile^{119}, Ala^{126}, Tyr^{129}, Trp^{130}, Tyr^{131}, and Glu^{132} interact with IgE in site 1 [19]. Tyr^{131} makes contact with five residues of IgE and is the central receptor residue in site 1 [19,20]. IgE interacts at the residues, Ser^{85}, Asp^{86}, Trp^{87}, Trp^{110}, Trp^{113}, Trp^{156},

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Glu[157], and Leu[158] in site 2 [19]. Trp[87] is the key residue in site 2. Trp[87] and Trp[110] surround Pro[426] of IgE, forming a "proline sandwich" [20]. Inhibitors recognizing sites 1 and 2 might be able to accelerate the dissociation of IgE from the receptor and be useful in the treatment of allergic reactions [19,20]. These inhibitors should interact with Tyr[131] and Trp[87] [20]. However, Trp[87] is completely conserved among the Fc receptors, FcεRI, FcγRI, FcγRII, and FcγRIII [19]. In contrast, Tyr[131] is unique in FcεRI [19]. Tyr[131] seems to be a more important target for the specific inhibitors. Antibodies recognizing these sites, especially Tyr[131] and residues surrounding it, or affinity-improved soluble FcεRIα are promising candidates for the IgE-specific inhibitors.

The functional ectodomain of the recombinant soluble FcεRIα was expressed and purified from transfected Chinese hamster ovary cells (CHO) [8,9], baculovirus expression system [10], and *Escherichia coli* [11]. The regions of FcεRIα ectodomain, which had been displayed on the surface of filamentous phage as fusions with the gene III protein, retained their IgE-binding activities [11,21]. The single-domain (D2) on the phage was able to bind IgE with lower affinity than the two-domain (D1 + D2) fragment on the phage [11,21]. On the other hand, it was observed that some point mutations in FcεRIα D2 increased the affinity for IgE [16,17]. These results indicate that introduction of the larger scale mutation into FcεRIα D2 will increase and improve the binding affinity for IgE. The phage display library has been a powerful way for obtaining such an improved mutant peptide from a large population of randomly mutated sequences [22,23].

In this study, a random mutation was introduced into adjacent residues to the center of binding site 1, the C′ strand including Tyr[131] in FcεRIα D2 [18–20]. Four residues in the C–C′ strands and five residues in the E strand [18] were randomized and displayed on the phage surface. Selection of a mutant FcεRIα D2 showing higher affinity to IgE from the phage display library and the IgE-binding characteristics of the resulting mutant FcεRIα D2 were also examined.

Materials and methods

Reagents. Human IgE (hIgE), murine IgE (mIgE), and the murine anti-hIgE monoclonal antibody conjugated with biotin were produced in our laboratory. Either 6 mg of bovine serum albumin (BSA) (Sigma) or 5 mg mIgE was immobilized on 1 mL CNBr activated-Sepharose 4B (Pharmacia). Oligonucleotides were synthesized on an Applied Biosystems model 380 synthesizer (Applied Biosystems) and used after purification with an OPC cartridge (Applied Biosystems). The 5′-end of oligonucleotides other than primers for PCR was phosphorylated with T4 polynucleotide kinase (Toyobo). Restriction endonucleases and modification enzymes were purchased from Toyobo and New England BioLabs.

Expression and purification of soluble human FcεRIα. Total RNA of the KU812 cells (Riken Cell Bank) was prepared with ISOGEN (Nippon Gene). FcεRIα cDNA [24] was amplified from the total RNA with GeneAmp RNA PCR Kit (Perkin–Elmer Cetus) according to manufacturer's protocol. The following oligonucleotides including either *Sal*I site or *Not*I site were used for PCR primers: 5′-TTAGTCG ACTCCAGCACAGTAAGCACCAGG-3′ and 5′-AAGGCGGCCG CTAAATCCTTGAGCACAGACGTTTC-3′. PCR amplification was performed on Program Temp Control System PC-700 (Astec). Taq polymerase was added at 90 °C before an initial denaturation for 2 min at 95 °C, followed by 30 cycles for 1 min at 95 °C and 1 min at 50 °C, with a final extension for 7 min at 72 °C. The RNA–PCR products were digested with *Sal*I and *Not*I and subsequently purified from agarose gel. The cDNA fragment was cloned between *Sal*I and *Sma*I sites of pUC19 (Toyobo) together with a *Not*I linker (Takara). The *Ban*II/*Not*I fragment of the constructed plasmid was substituted for the oligonucleotides 5′-CCTCAACATTACTGTAATAAAAGCTTAA GC-3′ and 5′-GGCCGCTTAAGCTTTTATTACAGTAATGTTG AGGGGCT-3′ to construct soluble human FcεRIα (shFcεRIα) cDNA (the termination codon is underscored). The shFcεRIα cDNA fragments, which had been obtained by digestion with *Sal*I and *Not*I, were inserted between *Xho*I and *Not*I sites of the vector pBPV (Pharmacia).

The murine C127 cells were co-transfected with a DNA encoding neomycin-resistant gene and pBPV including the shFcεRIα cDNA insert by means of the calcium phosphate precipitation method. The stable transformants were selected by adding 0.5 mg/mL G418 (GIBCO BRL) to the culture medium. The selected clone was cultivated with Dulbecco's modified Eagle's medium (D-MEM) (GIBCO BRL), containing 10% fetal bovine serum, in roller bottles (Becton–Dickinson). When cells reached confluence, the medium was changed with serum-free D-MEM. After cultivation for several days, 300 mL portion of the culture fluid was harvested by centrifugation and passed through 1 mL of a BSA–Sepharose 4B column. The pass fraction was applied to 1 mL of an mIgE–Sepharose 4B column. After washing with phosphate buffered saline (PBS), shFcεRIα was eluted with 0.15 M of glycine–HCl (pH 3.0), followed by immediate neutralization with an equal volume of 1 M Tris–HCl (pH 7.5). The neutralized eluates were combined and concentrated with Centriprep-10 (Amicon).

Construction of phagemid vector. The RNA-PCR products from total RNA of the KU812 cells were used directly as the templates for PCR cloning of the FcεRIα D2 cDNA. The cDNA fragments having *Sfi*I site at the 5′-end and *Not*I site at the 3′-end were amplified by PCR with the primers 5′-TAGCGGCCCAGCAGGCCGACTGGCTGC TCCTTCAGGCCTC-3′ and 5′-ACGTGCGGCCGCAGCTTTTATT ACAGTAATGTTGAGGGG-3′ under the cycle condition mentioned above. The phagemid pCANTAB-DE2 was constructed by inserting the *Sfi*I–*Not*I fragments of the PCR products to the predigested phagemid vector, pCANTAB5 (Pharmacia).

Construction of the phage library. In constructing the Random I phage library, the randomized tetrapeptides were inserted between Tyr[121] and Ala[126] of FcεRIα D2. PCR products were obtained from the *Sal*I–*Not*I fragment of FcεRIα cDNA with the primers 5′-GCTCT TAAGTACTGGTATGAGAACCAC-3′ and 5′-ACGTGCGGCCGC AGCTTTTATTACAGTAATGTTGAGGGG-3′ to introduce *Afl*II site. The *Afl*II mutant of pCANTAB-DE2, pCANTAB-DE2AFL was constructed by inserting *Afl*II/*Not*I digests of the PCR products between the *Bsr*GI and *Not*I sites of pCANTAB-DE2 together with the adaptors 5′-GTACAAGGTGATCTATTATAAGGATGGTGAAGC TC-3′ and 5′-TTAAGAGCTTCACCATCCTTATAATAGATCACC TT-3′. The degenerate oligonucleotide 5′-TTAAG AGC$(MNN)_4$ATA ATAGATCACCTT-3′ was annealed with 5′-GTA CAAGGTGATCT ATTAT-3′ by heating for 5 min at 65 °C and by cooling slowly to room temperature. N stands for equimolar A, C, G, or T and M stands for equimolar A or C. The annealed oligonucleotides were ligated with the gel purified *Bsr*GI/*Afl*II digests of pCANTAB-DE2AFL.

The Random II phage library having the randomized pentapeptides between Glu[132] and Ile[138] of FcεRIα D2 was constructed from the *Afl*II/*Bbs*I digest of pCANTAB-DE2AFL. The degenerate oligonucleotide 5′-TTAAGTACTGGTATGAG$(NNK)_5$ATTACAAATGCC

ACAGTTGAAGACAG-3′ was annealed to 5′-TCCACTGTCTTCA ACTGTGGCATTTGTAAT-3′ and 5′-CTCATACCAGTAC-3′. K stands for equimolar G or T. The annealed oligonucleotides were cloned into the digested pCANTAB-DE2AFL.

The constructed phagemids were transfected to competent *E. coli* (E. P.) JM109 (Nippon Gene) cells by electroporation with a 0.2 cm cuvette and Gene Pulser (Bio-Rad) at a condition of 12.5 kV/cm, 25 μF, and 200 Ω. Transfectants were selected on an SOBAG agar plate (2% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.05% NaCl, 0.01 M $MgCl_2$, 0.11 M glucose, 100 μg/mL ampicillin, and 1.5% Bacto-agar). Grown cells were collected and inoculated to 2× YT medium (1.6% Bacto-tryptone, 1.0% Bacto-yeast extract, and 0.5% NaCl) containing 100 μg/mL ampicillin and 2% glucose. Transfectants were cultivated at 37 °C with vigorous shaking until an absorbance at 600 nm of 0.6–0.8 was achieved. The culture was infected with approximately 3×10^9 phage particles/mL of M13KO7 helper phage and shaken at 150 and 250 rpm for 30 min each. The infected cells were collected by centrifugation and inoculated into 2× YT medium containing 100 μg/mL ampicillin and 50 μg/mL kanamycin. After shaking overnight at 37 °C, bacterial cell debris was removed by filtering through a 0.45 μm filter cartridge. Phage particles were concentrated by polyethylene glycol precipitation and suspended again in 2× YT to obtain the phage libraries.

Panning. Panning of the phage library was performed in a 6 cm petri dish (Falcon) coated with 1 mL of 10 μg/mL hIgE in 10 mM Tris-buffered saline (pH 7.4) containing 1 mM $CaCl_2$ (Ca–TBS) overnight at 4 °C. The dish was blocked with 5 mL of 2% skim milk in Ca–TBS for 1 h at 25 °C. Phages (approximately 10^{12} phage particles/mL) in 10 mM Tris-buffered saline (pH 7.5) containing 0.05% Tween20 (T–TBS) were pretreated with 2% skim milk and added to the IgE-coated petri dish. After incubation for 4 h at 25 °C, unbound or weakly bound phages were washed out by five times of rinsing with T–TBS. The phages bound to the petri dish were eluted by incubation for 5 min with 1 mL of 0.1 M glycine–HCl (pH 2.2). Elution was repeated and the combined eluate was immediately neutralized with 1 mL of 1 M Tris–HCl (pH 7.4). Recovered phages were allowed to infect *E. coli* JM109 for 20 min at room temperature and amplified with the M13KO7 helper phage. The amplified phages were used for the next panning round. After eight rounds of panning cycle, single clones of infected *E. coli* JM109 were isolated from SOBAG agar plate. The mutant FcεRIα D2 cDNA in the selected clone was sequenced with a fluorescent labeled M13 reverse primer and Model 373A DNA Sequencer (Applied Biosystems).

Binding analysis of phages to IgE. Binding assay of the phages was performed by the use of enzyme-linked immunosorbent assay (ELISA). A volume of 100 μL/well was used for all the incubations. Nunc 96-well Maxi-Sorp plates were coated with 10 μg/mL hIgE and blocked with 2% skim milk under the condition described in the methods of panning. Phage diluents were added to each well in duplicate and incubated for 2 h at 37 °C. After five times of washing with T–TBS, horse radish peroxidase conjugated anti-phage antibody (Pharmacia), which had been diluted 1:5000 and preincubated with T–TBS containing 0.1% BSA and 1% 2× YT, was dispensed to each well of the plates. The plates were incubated for 2 h at 25 °C, followed by washing with T–TBS. The absorbance at 492 nm (A_{492}) was measured on a microplate reader after developing with *o*-phenylenediamine and hydrogen peroxide.

For the binding specificity assay, 5 μg/mL hIgE, mIgE, or human IgG (hIgG) was coated to the plates. As a competitor, 10 μg/mL hIgE, mIgE, hIgG, or shFcεRIα was preincubated together with phages before adding to the plates.

Competitive inhibition assay of IgE-binding to FcεRIα was examined on the shFcεRIα coated plates. The plates were coated with 250 ng/mL shFcεRIα in 50 mM sodium carbonate buffer (pH 9.6) overnight at 4 °C. The plates were blocked with 25% Block Ace (Dainippon Pharmaceutical) in PBS for 1 h at 25 °C. After three times of washing with PBS containing 0.05% Tween 20 (T–PBS), 40 ng/mL hIgE, which had been preincubated with equal volume of the phage dilutions in T–PBS, was bound to the coated shFcεRIα in duplicate by an incubation for 2 h at 25 °C. After washing with T–PBS, 1:4000 dilution of the biotin-conjugated anti-hIgE monoclonal antibody was applied to the plates and the plates were incubated for 2 h at 25 °C. Subsequently, the plates were incubated with 1:5000 dilution of peroxidase labeled streptavidin (Miles) for 1 h at 25 °C. A_{492} was measured after developing as described above.

Expression of FcεRIα D2 in E. coli. The phagemids including the wild type or the mutant FcεRIα D2 cDNA were digested with *Sfi*I and blunt-ended with T4 DNA polymerase. Phosphorylated *Bam*HI linker (5′-CGGATCCG-3′, Toyobo) was ligated to the blunt end and subsequently digested with *Bam*HI and *Not*I. Gel purified cDNA fragments were ligated between *Bam*HI and *Not*I sites of the expression vector pGEX-4T-1 (Pharmacia). *E. coli* BL21 competent cells were transformed with the ligated DNAs and the individual colonies were selected. The sequence-qualified clones were cultured in 10 mL of 2× YT containing 2% glucose and 100 μg/mL ampicillin at 30 °C with shaking at 210 rpm. The overnight culture was transferred to 100 mL of fresh 2× YT containing 100 μg/mL ampicillin and shaken in 500 mL Erlenmeyer flask for 3 h at 30 °C. Expression of the glutathione *S*-transferase (GST) fusion protein was induced by the addition of 100 μM isopropyl β-D-thiogalactopyranoside. After shaking for 6 h, cells were harvested from 500 mL culture fluid by centrifugation and suspended in 25 mL of 20 mM Tris–HCl (pH 7.5) containing 1% Triton X-100. The cell suspension was disrupted by sonication for three times, each for 30 s. The cell debris was precipitated by centrifugation and sonicated again. The harvested cell debris by centrifugation was extracted by suspending in 20 mM Tris–HCl (pH 7.5) supplemented with 8 M urea, 5 mM EDTA, and 1 mM dithiothreitol. The cell extracts containing GST-fusion proteins were prepared by centrifugation at 33,000 rpm for 30 min at 4 °C. The cell extracts were dialyzed against 2.2 L of 100 mM Tris–HCl buffer (pH 8.5) containing 1.4 M urea overnight at 4 °C, followed by twice dialyzing against 5 L of PBS. The dialyzed extracts were applied to 1 mL bed volume of glutathione Sepharose 4B (Pharmacia). After washing with PBS, the GST-fusion proteins were eluted out with 10 mM glutathione in 50 mM Tris–HCl (pH 8.0). Concentration of the purified proteins was estimated as that of GST.

Competitive inhibition assay of IgE-binding to FcεRIα with the GST-fusion proteins was performed according to the methods mentioned above. The GST-fusion proteins were preincubated with hIgE instead of the phage particles.

Results and discussion

Expression and purification of recombinant shFcεRIα

The shFcεRIα cDNA encoding 197 amino acids for the signal peptide and the extracellular domain was expressed in the murine C127 cells. The deduced carboxyl-terminal was Ala^{172} of the truncated soluble FcεRIα described by Yagi et al. [10]. From 3.6 L of culture fluid, 1.4 mg of the purified shFcεRIα was obtained using an mIgE-immobilized Sepharose 4B column. The purified shFcεRIα showed a smear band from 41 to 66 kDa on 10% SDS–PAGE (data not shown).

Construction and panning of the phage library

A phage expressing the wild type FcεRIα D2 (FcεRIα D2 phage) was constructed from the PCR products. A point mutation of G_{17} to T was found in the PCR for-

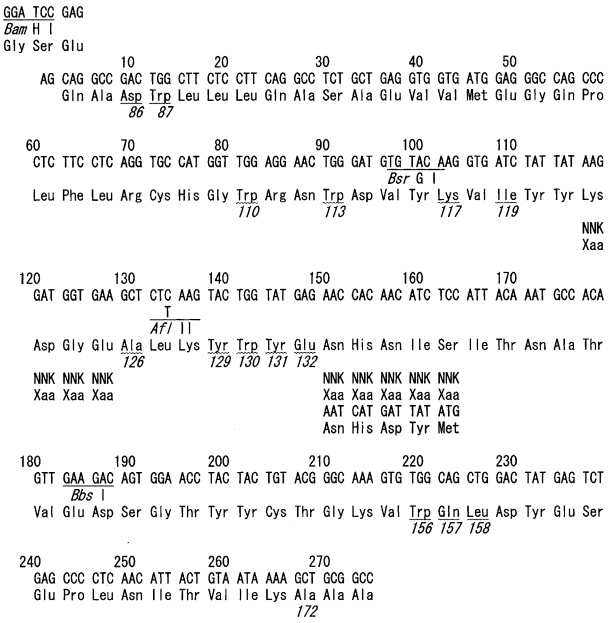

Fig. 1. Nucleotide and the predicted amino acid sequences of FcεRIα D2. Nucleotide and the deduced amino acid sequences of the *Sfi*I/*Not*I insert of the FcεRIα D2 cDNA are shown. See text for meaning of N and K. Xaa stands for any amino acid residue. The amino-terminal Asp^{86} and the carboxyl-terminal Ala^{172} of the FcεRIα D2 are numbered in italics according to the peptide sequence of the soluble FcεRIα described by Yagi et al. [10]. The residues, which interact directly with IgE [19], are numbered in italics and are underscored in waved lines. The *Afl*II point mutation, the randomized residues, the mutant sequences, and the 5′-linking sequence to GST are indicated.

ward primer region of the FcεRIα D2 cDNA (Fig. 1). However, this point mutation occurred at the third letter of the codon for leucine. There was no mutation on the protein level. The phage was able to display FcεRIα D2. FcεRIα D2 phages bound to the immobilized hIgE and the binding was competitively inhibited by the preincubation with hIgE or shFcεRIα (Fig. 2). No inhibition occurred with hIgG (Fig. 2).

In constructing the phage library, *Afl*II site was introduced into the FcεRIα D2 cDNA by PCR (Fig. 1). The resulting single nucleotide substitution placed at the third letter of a codon did not cause any change of amino acid. The *Afl*II mutant phages also bound to hIgE to the same extent as FcεRIα D2 phages (data not shown).

The Random I phage library including randomized tetrapeptides at $Lys^{122}Asp^{123}Gly^{124}Glu^{125}$ was obtained from five times of transformation. The resulting library included 1.0×10^6 independent clones and yielded 2.4×10^{13} phage particles. The size of Random I phage library was able to cover all of the possible combinations of tetrapeptides. After eight rounds of panning cycle against the immobilized hIgE, 45 clones were isolated and examined for its binding affinity to hIgE by the use of ELISA. Almost phage clones showed approximately twice higher A_{492} than FcεRIα D2 phages

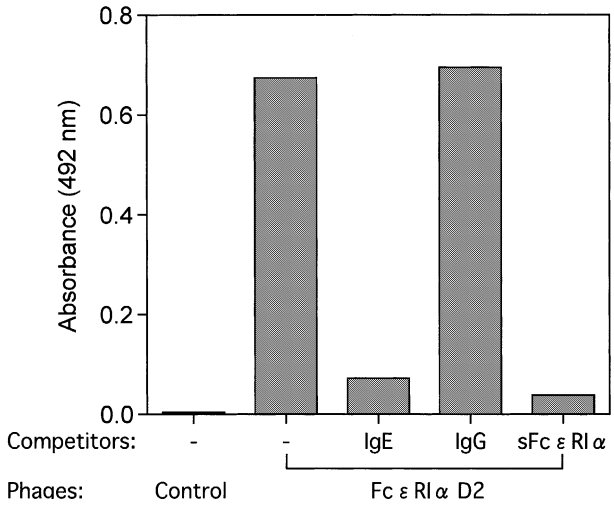

Fig. 2. Competition of FcεRIα D2 phage binding to hIgE obtained by preincubation with hIgE, hIgG, or shFcεRIα. Binding of phage particles (1.0×10^{12} phage particles/mL) to the coated hIgE was measured by the use of ELISA. The control phages were prepared from the pCANTAB5 transfectants. FcεRIα D2 phages were preincubated with the competitors described in Materials and methods.

($A_{492} = 0.336$). The top eight clones were sequenced and they showed identical nucleotide sequence. Almost all of the nucleotides encoding FcεRIα D2 were deleted in the clones. The amino-terminal decapeptide of FcεRIα D2, $Asp^{86}Trp^{87}Leu^{88}Leu^{89}Leu^{90}Gln^{91}Ala^{92}Ser^{93}Ala^{94}Glu^{95}$, connected directly to the gIII protein region of the phage. The resulting deletion mutants expressed hydrophobic residues and seemed to bind on the plastic surface of the panning dish non-specifically. These results indicate that the residues $Lys^{122}Asp^{123}Gly^{124}Glu^{125}$ are important to form the C–C′ strands in FcεRIα D2. Any mutation of these residues will decrease the affinity to IgE by conformational change of the C′ strand which has been reported to form binding site 1 in FcεRIα D2 [18–20].

The Random II phage library was randomized at $Asn^{133}His^{134}Asn^{135}Ile^{136}Ser^{137}$ and constructed from 15 times of transformation. The library consisted of 6.3×10^6 independent clones and 4.8×10^{13} phage particles. The library covered 19% of the possible combination of randomized nucleotides encoding pentapeptide (3×10^7). The library was applied to the eight rounds of panning cycle. Recovery rate of the bound phage was exponentially increasing during four rounds of the panning and slowed down after the fifth round (Fig. 3). On the other hand, the IgE-binding capacity of the recovered phages was constantly increasing throughout the eight rounds of panning (Fig. 3). Phage clones were isolated from the last round and 16 clones out of 40 clones showed an apparently higher IgE-binding activity of $A_{492} > 2.0$ compared with $A_{492} = 0.531$ of FcεRIα D2 phages. The top 11 phage clones were sequenced. They showed the same sequence at the randomized nucleotide,

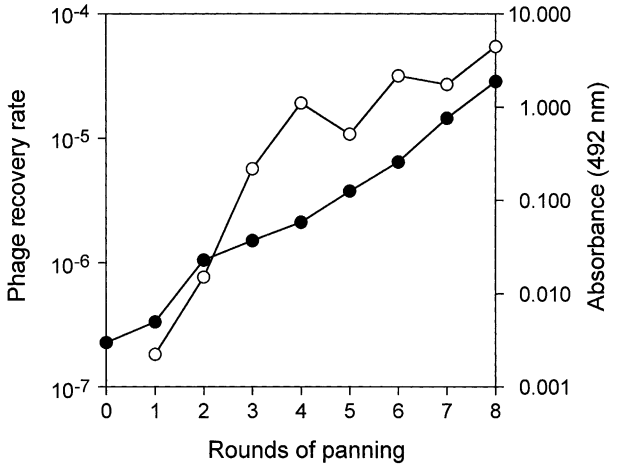

Fig. 3. Panning of the Random II phage library. Phage recovery rate after each round of panning with hIgE (○) was obtained by dividing the recovered phage particles by the applied ones. IgE-binding capacity (●) of the recovered phages (1.0×10^{12} phage particles/mL) was measured using ELISA.

AAT CAT GAT TAT ATG encoding a pentapeptide, $Asn^{133}His^{134}Asp^{135}$ $Tyr^{136}Met^{137}$ (Fig. 1). The mutant FcεRIα D2 and the mutant phage clones obtained in this way were designated as FcεRIα D2DYM and FcεRIα D2DYM phages, respectively. Asn^{133} and His^{134} of FcεRIα D2 were still conserved in the mutant pentapeptide. These residues, which were included in the C′–E loop of FcεRIα D2, were necessary to maintain the C′ strand of FcεRIα D2, the center of IgE-binding site 1 [19,20]. The topology diagram of FcεRIα D2 showed that the ABE strands formed the ABE sheet by hydrogen bonding [18]. The residues Asn^{135}, Ile^{136}, and Ser^{137} in the E strand interacted withthe residues Arg^{106}, Leu^{105}, and Phe^{104} in the B strand, respectively [18]. As a result of the mutation, Asp^{135}, Tyr^{136}, and Met^{137} have interacted with the residues Arg^{106}, Leu^{105}, and Phe^{104} in FcεRIα D2DYM. Three aromatic residues, $Tyr^{129}Trp^{130}Tyr^{131}$, in the C′ strand showed conformational variability among six different crystal forms [20]. The change of interaction between the E strand and the B strand by the mutation will affect conformation of the C′ strand [20] to form a binding site having a higher affinity to IgE. However, the conformational changes observed in the different crystal forms are thought to be independent of the binding of IgE [20].

The binding affinity of FcεRIα D2DYM phages to hIgE was approximately 500 times higher than that of FcεRIα D2 phages (Fig. 4A). FcεRIα D2DYM phages specifically bound to hIgE and mIgE (Fig. 4B). The binding was competitively inhibited by the preincubation with hIgE or mIgE, but not with hIgG. Specificity to IgE was still conserved in FcεRIα D2DYM phages (Fig. 4B) as well as in FcεRIα D2 phages (Fig. 2). The IgE-binding capacity of FcεRIα D2 phages is nearly equal to that of the FcεRIα single-domain (D2) phages,

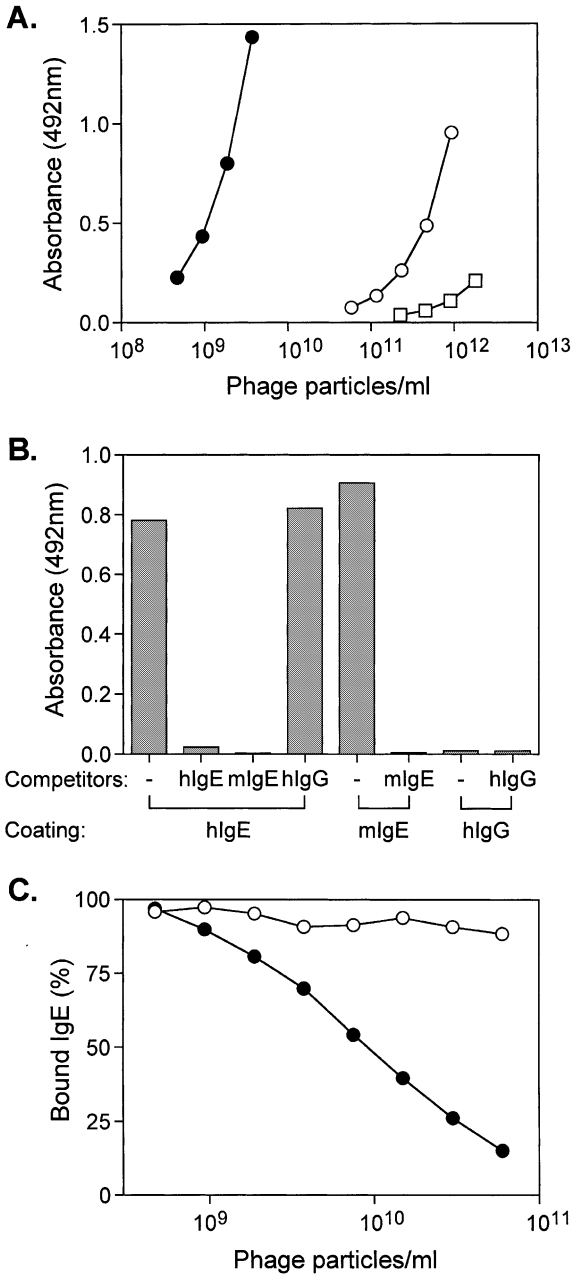

Fig. 4. IgE-binding characteristics of FcεRIα D2DYM phage. All of the assays were performed using ELISA. (A) Serial dilutions of the control (□), FcεRIα D2 (○), or FcεRIα D2DYM (●) phages were applied to the immobilized hIgE. (B) Binding specificities of FcεRIα D2DYM phages (2.4×10^9 phage particles/mL) were examined with the immobilized immunoglobulins. Phage particles were preincubated with the competitors before adding to the ELISA plate as described in "Materials and methods". (C) Inhibition of hIgE-binding to shFcεRIα was examined by the preincubation of hIgE with FcεRIα D2 (○) or FcεRIα D2DYM (●) phages, followed by dispensing hIgE to the ELISA plate coated with shFcεRIα. The amount of bound hIgE without preincubation was taken as 100%.

as described by Scarselli et al. [21]. They also reported that the capacity of IgE-binding was 10 times higher in the two-domain (D1 + D2) than in the single-domain phages (D2). The binding capacity of FcεRIα D2DYM

phages was estimated to be 50 times higher than that of the two-domain (D1 + D2) phages [21]. The FcεRIα D2DYM phages competitively inhibited the binding of IgE to shFcεRIα at an IC_{50} value of 7.0×10^{10} phage particles/mL (Fig. 4C). The IC_{50} value was estimated as 116 pM when one molecule of FcεRIα D2DYM was displayed on a phage particle as a fusion protein with the M13 phage gene III protein. FcεRIα D2 phages did not show any significant inhibition on the hIgE-binding under the condition described in Fig. 4C.

Expression of the GST fused FcεRIα D2 in E. coli

The cDNAs encoding FcεRIα D2 and FcεRIα D2DYM were fused to the 3′-end of GST cDNA together with a linker encoding a peptide GlySerGluAla (Fig. 1). The resulting fusion proteins were designated as GST-IER D2 and GST-IERDYM, respectively. The carboxyl-terminal sequences of the fused proteins after Ala^{172} of FcεRIα D2 were AlaAlaAlaSer. When GST-IERD2 and GST-IERDYM were expressed in *E. coli*, the concentration of the proteins in the cell extracts obtained by sonication and 1% Triton X-100 treatment was relatively low. From 3.3 L of the culture fluid, 949 μg of GST-IERD2 and 1060 μg of GST-IERDYM were purified directly from the cell extracts with 1 mL bed volume of glutathione Sepharose 4B columns. The purified GST-IERD2 and GST-IERDYM inhibited binding of hIgE to shFcεRIα and their IC_{50} values were 1570 and 89 nM, respectively. SDS–PAGE of these purified fusion proteins showed some impurity bands. To improve this, the cell debris obtained by sonication and Triton X-100 treatment of the 500-mL culture was extracted with 8 M urea, as mentioned in "Materials and methods". The urea extracts included a larger amount of denatured fusion proteins than the Triton X-100 extracts. After renaturation by two steps of dialysis, increased amount of purified GST-IERDYM (4656 μg) was recovered from the glutathione Sepharose 4B column. The yield of purified GST-IERD2 did not improved as much as expected, i.e., 288 μg from the 500-mL culture. The bands of impurities were not observed in these renatured fusion proteins other than trace 28 kDa bands derived from the excised GST. The IC_{50} values of the renatured GST-IERD2 and GST-IERDYM were improved to 656 and 31 nM, respectively (Fig. 5). The inhibition of GST-IERDYM on hIgE-binding to shFcεRIα was estimated to be 21 times higher than that of GST-IERD2.

In spite of the difference of fusion pair, the mutant residues $Asp^{135}Tyr^{136}Met^{137}$ increased the capacity of IgE-binding. The first Ig-like domain 1 (D1) of FcεRIα has been reported to play a structural function in maintaining the integrity of the domain interface and the ligand-binding site [25]. As mentioned above, the two-domain (D1 + D2) phages exhibited IgE-binding capacity at a higher level than the single-domain (D2)

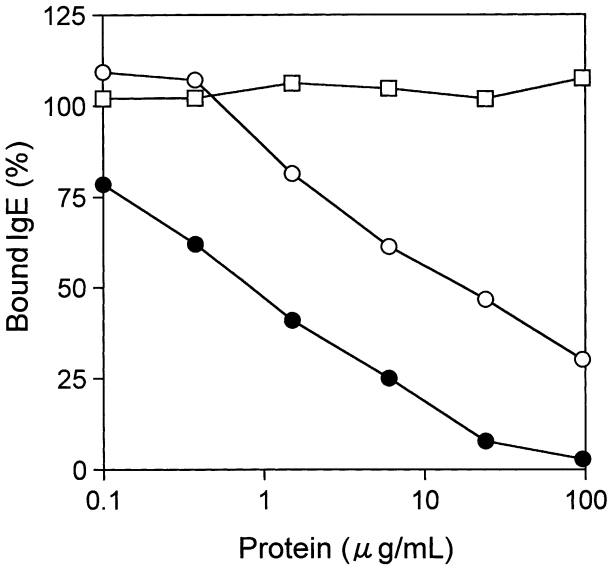

Fig. 5. Competitive inhibition of hIgE-binding to shFcεRIα obtained by preincubation with GST-fusion proteins. Inhibition of hIgE-binding to shFcεRIα was examined by preincubation of hIgE with GST (□), GST-IERD2 (○), or GST-IERDYM (●), followed by detection of bound hIgE using ELISA. The amount of bound hIgE without preincubation was taken as 100%.

phages [11,21]. The soluble FcεRIα, which consisted of two domains, has been produced from the transfected insect cells and CHO [10]. These soluble receptors have shown 50% inhibition of IgE-binding to the immobilized FcεRIα at 0.74 ng/well (insect cells) and 1.17 ng/well (CHO) [10]. The estimated IC_{50} value of the soluble receptors was approximately 200 pM. If FcεRIα D2D YM manifested its high IgE-binding activity together with any fusion partner, it is expected that the FcεRIα D1 fused with FcεRIα D2DYM, i.e., the mutant shFcεRIα will be able to inhibit IgE-binding to FcεRIα at an IC_{50} value lower than pM level.

Synthetic peptides including the C–C′ region have been synthesized and examined as inhibitors for the IgE–FcεRIα interaction [5,6]. The peptide including Tyr^{121} to Asn^{140} did not inhibit IgE-binding to FcεRI [5]. However, the cyclic peptide including Ile^{119} to Tyr^{129} acted as a competitive inhibitor for IgE-binding [6]. The peptide exhibited a dissociation constant of approximately 3 μM for IgE and inhibited IgE-mediated mast cell degranulation at an IC_{50} value of approximately 30 μM [6]. However, the linear peptide consisting of the same residues showed no inhibition on the IgE-binding [6].

In conclusion, by the introduction of randomized oligopeptides to FcεRIα D2, we have demonstrated the importance of $Lys^{122}Asp^{123}Gly^{124}Glu^{125}$ in the C–C′ strands and $Asn^{133}His^{134}Asn^{135}Ile^{136}Ser^{137}$ in the E strand. Substitution of $Asn^{135}Ile^{136}Ser^{137}$ for $Asp^{135}Tyr^{136}Met^{137}$ is expected to produce the mutant shFcεRIα that has a higher affinity to immobilized IgE.

Such a high-affinity mutant shFcεRIα will be a promising candidate as an inhibitor of the IgE-mediated allergic reactions [19].

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