



Fine epitope mapping of humanized anti-IgE monoclonal antibody omalizumab

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ABSTRACT

Omalizumab is a humanized anti-IgE antibody that inhibits IgE binding to its receptors on mast cells and basophils, thus blocking the IgE-mediated release of pharmacologic mediators from these cells. Previous studies have indicated that omalizumab binds to the Cε3 domain of IgE, which is the binding site of IgE receptors, but the precise epitope recognized by omalizumab is unknown. In this study, we employed the phage display peptide library technology to select peptides binding to omalizumab. A striking peptide sequence motif was recovered, which is homologous to the sequence ⁴²⁴HLP⁴²⁶ within the Cε3 domain of IgE-Fc. Our results further indicated that omalizumab specifically bound to the synthesized peptide “⁴²¹THPHLPALMRS⁴³²” containing the ⁴²⁴HLP⁴²⁶ motif in IgE-Fc. We therefore conclude that the ⁴²⁴HLP⁴²⁶ motif is the omalizumab epitope. This epitope overlaps with the high-affinity IgE receptor-binding site, thus providing insights into the structural basis for the mechanism of action of omalizumab.

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Allergic diseases, such as asthma, allergic rhinitis and atopic dermatitis, have become increasingly prevalent in the past few decades. Most allergic diseases are caused by IgE-mediated hypersensitivity reactions [1,2]. IgE is one of the five classes of antibodies in the human immune system, which accounts for a minute proportion of immunoglobulin in the blood. Allergic patients sensitized to an allergen produce increased amounts of IgE specific to that allergen. These IgE molecules circulate in the blood and bind to IgE receptors on the surface of basophils in the circulation and mast cells in various tissues [3,4]. In an allergic reaction, the allergens enter the body and bind to the antigen-binding sites of IgE on mast cells or basophils, crosslink the IgE molecules, aggregate the underlying receptors, and thereby trigger the cells to release histamine and the other pharmacological mediators of the symptomatic allergic response [2,4].

Mapping of the sites of the IgE-Fc that interact with IgE receptors is an important step toward the understanding and possible manipulation of the allergic reaction. The crystal structure of the human IgE-Fc bound to its high-affinity receptor (FcεRI) α-chain reveals that one receptor binds one dimeric IgE-Fc molecule asymmetrically through interactions at two sites, each involving one

Cε3 domain of the IgE-Fc [5]. The binding site for the low-affinity IgE-Fc receptor (FcεRII) in IgE-Fc is not precisely mapped, but also lies in the Cε3 domains [6]. Omalizumab (Xolair) is a humanized anti-IgE monoclonal antibody (mAb) approved for treatment of moderate to severe asthma. Omalizumab tightly binds to free circulating IgE molecules and prevents binding of these molecules to FcεRI on mast cells and basophils and FcεRII on various cell types. It does not bind to IgE already bound by either FcεRI or FcεRII. These binding characteristics allow omalizumab to inhibit allergen-induced responses without causing receptor crosslinking, which could lead to anaphylaxis [7–9]. Previous studies have indicated that omalizumab also binds specifically to the Cε3 domain of IgE, which is the binding site of FcεRI and FcεRII, thus eliminating the ability of free IgE molecules to bind to both types of receptors [8,10]. However, the precise epitope within the Cε3 domain of IgE recognized by omalizumab has not yet been reported.

The phage display peptide library (PDPL) technology is a valuable tool for the exploration of epitopes recognized by antibodies. Phage displaying a peptide that mimics the epitope of an antibody can be selectively enriched by panning on antibody-coated plates [11,12]. In this study, through panning a phage display peptide library with omalizumab, the specific epitope recognized by omalizumab was identified to be ⁴²⁴HLP⁴²⁶ within the Cε3 domain of IgE-Fc. This epitope was observed to overlap with the FcεRI-binding site, thereby revealing the structural basis for the mechanism of action of omalizumab.

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Phage clones	Reactivity with mAb, A450nm (mean)			Deduced amino acid sequences
	Omalizumab	Trastuzumab		
1G3, 1B11, 1G11, 2C2, 2C3, 2F2, 2G2, 2H1, 2H3, 2H8	0.578	0.033		<u>K</u> H <u>L</u> P <u>L</u> Y <u>R</u>
1C5, 1A4, 1A5, 1B3, 1D10, 1E5, 1F8, 1H2	0.536	0.052		<u>K</u> H <u>N</u> W <u>P</u> P <u>P</u>
1A10, 1B5, 1C5, 1D5, 1D8, 1E11, 1H11	0.520	0.066		<u>F</u> H <u>E</u> P <u>K</u> Y <u>R</u>
1D4, 1D6, 1D7, 1F6, 1H6, 1H9	0.484	0.090		<u>G</u> H <u>I</u> A <u>K</u> Y <u>R</u>
1E7, 1B8	0.580	0.068		<u>G</u> H <u>L</u> W <u>K</u> Y <u>V</u>
Consensus motif				(424) <u>H</u> L <u>P</u> (426)
Corresponding segment of IgE				(421) <u>T</u> H <u>P</u> H <u>L</u> <u>P</u> R <u>A</u> L <u>M</u> R <u>S</u> (432)

Fig. 1. Reactivity and deduced amino acid sequences of inserts of selected phage clones. Phage clones were isolated by panning the Ph.D.-7 PDPL with omalizumab. After three rounds of panning, reactivity of positive phage clones with omalizumab was measured by ELISA as described in Materials and methods. Phage clones highly reactive in ELISA were subjected to DNA sequence analysis. Deduced amino acid sequences of inserts from selected phage clones were aligned for the consensus motif, which is indicated by underlined letters.

Materials and methods

Materials. The Ph.D.-7 Phage Display Peptide Library (PDPL) was purchased from New England BioLabs (Beverly, MA). Omalizumab and the anti-HER2 humanized antibody trastuzumab were respectively purchased from Genentech and Roche Ltd. Omalizumab and trastuzumab were biotinylated using a biotin labeling kit (Roche, Indianapolis, IN). Horseradish peroxidase (HRP)-conjugated avidin was purchased from Jackson ImmunoResearch Laboratories (Avondale, PA). The human IgE-secreting cell line SKO-007 was obtained from the American Type Culture Collection (ATCC). The IgE was purified from the SKO-007 cell culture supernatant by affinity chromatography on a sepharose 4B column conjugated with omalizumab. Peptides used in this study were synthesized by Yeli Bio-Scientific Inc. (Shanghai, China). The quality of peptides was determined by analytical reverse-phase chromatography and mass spectral analysis and the purity was greater than 95%.

Phage display library screening. The Ph.D.-7 PDPL is a filamentous phage display system containing a repertoire of 1.2×10^9 sequences of random 7-amino acid peptides fused to the N-terminal sequence of the M13 synthetic minor-coat protein PIII via the flexible linker GGGs. Biopanning of PDPLs with omalizumab was performed as described previously [12]. Briefly, the mAb omalizumab was immobilized on 96-well plates. Then the wells were blocked with 3% bovine serum albumin and incubated with phages (1×10^{11} pfus) from the Ph.D.-7 PDPL at 4 °C for 1 h. The wells were washed for five times and the phages that bound with omalizumab were amplified. Three rounds of selection were performed. In the second and third round of selection, the library was pre-cleared on trastuzumab.

ELISA screening of phage clones. 96-well plates were coated with omalizumab (10 µg/well). After two washings and blockade of free protein-binding sites, 100 µl of supernatant containing amplified particles from each phage clone were added to wells and incubated for 2 h at 37 °C. Following five washings, HRP-conjugated anti-phage M13 monoclonal antibody (Amersham Biosciences) was added and incubated for 1 h at 37 °C. After washing, 3,3',5,5'-tetramethylbenzidine (TMB) was added as a substrate and the absorbance was read at 450 nm using an immunoreader. Finally, phage clones highly reactive in ELISA were subjected to DNA sequence analysis.

Western blot. For Western blot analysis, purified phage particles (1×10^{10} pfus/lane) were electrophoresed on a 10% SDS-PAGE

under nonreducing conditions and then electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences). After blockade of its free protein-binding sites, the PVDF membranes were treated with an appropriate dilution of omalizumab, followed by incubation with HRP-conjugated goat anti-human IgG (Zymed, San Francisco, CA). Finally, the bands were visualized by 3,3'-diaminobenzidine (DAB) (Sigma, St. Louis, MO) as a peroxidase substrate.

Immunochemical assay. The reactivity of omalizumab with synthetic peptides was determined by ELISA assay. Briefly, 96-well plates were coated with KLH-peptide. Following washing and blockade of free protein-binding sites, different concentrations of biotinylated omalizumab was added to each well and incubated for 4 h, followed by the addition of an appropriate dilution of HRP-conjugated avidin. After washing, TMB was added as a substrate and the absorbance was read at 450 nm using an immunoreader.

The ability of synthetic peptides to block the interaction between the omalizumab and human IgE was also investigated. Briefly, biotinylated omalizumab was incubated with different concentrations of synthetic peptides for an hour. Then the mixture

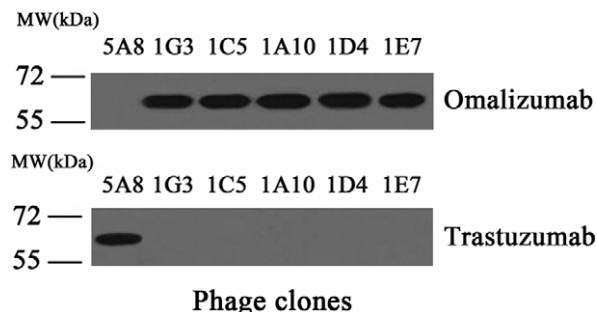


Fig. 2. Western blot analysis of the specific reactivity of omalizumab with phage minor-coat PIII protein-fused peptides from the selected phage clones. Purified phage particles (1×10^{10} pfus/lane) isolated by biopanning omalizumab with the Ph.D.-7 PDPL were electrophoresed on a 10% SDS-PAGE under nonreducing conditions and then electrophoretically transferred onto PVDF membranes. After blockade of its free protein-binding sites, the PVDF membranes were treated with an appropriate dilution of omalizumab or trastuzumab, followed by incubation with HRP-conjugated goat anti-human IgG. Finally, the bands were visualized by DAB as a peroxidase substrate. A trastuzumab-specific phage clone (5A8) was used as control. MW represents molecular weight.

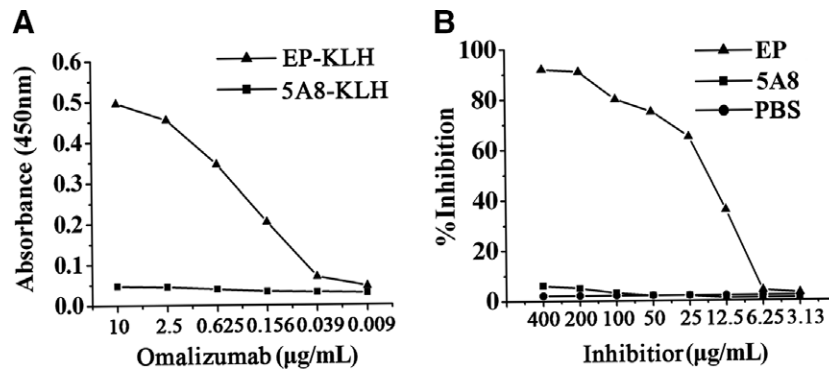


Fig. 3. Reactivity of synthetic epitope peptides with omalizumab. (A) Ninety-six-well plates were coated with EP-KLH or 5A8-KLH. Following washing and blockade of free protein-binding sites, different concentrations of biotinylated omalizumab was added to each well. After 4 h incubation, the antibody binding to peptide was detected by sequential addition of an appropriate dilution of HRP-conjugated avidin. 5A8 is a trastuzumab-specific epitope peptide. 5A8 was coupled to KLH to generate 5A8-KLH. (B) Competitive inhibition assay. Biotinylated omalizumab was incubated with different concentrations of synthetic peptides (EP or 5A8) for 1 h. Then the mixture was added to 96-well plates pre-coated with human IgE. After 2 h incubation, the wells were washed and the antibody binding to IgE was detected with an appropriate dilution of HRP-conjugated avidin. 5A8 is a trastuzumab-specific epitope peptide. Results are expressed as percentage inhibition of binding compared with binding in the absence of inhibitor.

was added to 96-well plates pre-coated with human IgE and incubated for 2 h, followed by the addition of an appropriate dilution of HRP-conjugated avidin. After washing, TMB was added as a substrate and the absorbance was read at 450 nm using an immunoreader. The percentage of inhibition was calculated using the following formula: % inhibition = $(A_{450\text{in the absence of inhibitor}} - A_{450\text{in the presence of inhibitor}}) / A_{450\text{in the absence of inhibitor}} \times 100$.

Results and discussion

Selection of phage-displayed peptides reactive with omalizumab

To identify the peptides recognized by omalizumab, phage clones were isolated by panning the Ph.D.-7 PDPL with omalizumab. After the third round of panning, the binding of the cloned phages to omalizumab was tested in an ELISA assay. The results showed that 33 out of the 46 isolated phages specifically bound to omalizumab (Fig. 1). The results of Western blot analysis also indicated that omalizumab could bind to these 33 phage clones (representative results are shown in Fig. 2). The reactivity was specific since omalizumab did not react with the trastuzumab-specific phage clone 5A8, nor did omalizumab-specific phage clones react with trastuzumab (Fig. 2). All omalizumab-positive phage clones were selected for sequencing.

Mapping of omalizumab-specific epitope on human IgE

Sequence analysis of omalizumab-positive phage clones identified five distinct sequences (Fig. 1). Alignment of the amino acid sequences of these clones resulted in the motif HLP and this could be aligned with the sequence $^{424}\text{HLP}^{426}$ located on the Cε3 domain of human IgE (Fig. 1), suggesting that $^{424}\text{HLP}^{426}$ was involved in the binding of IgE-Fc to omalizumab. Moreover, His was present in all phage-displayed peptides, suggesting that it might be crucial for omalizumab-specific epitope expression.

Reactivity of synthetic peptides with omalizumab

The peptide “ $^{421}\text{THPHLPALMRS}^{432}$ ” containing the $^{424}\text{HLP}^{426}$ motif in human IgE-Fc was synthesized and denoted as EP. The ELISA assay results showed that omalizumab bound to KLH-conjugated EP peptide (EP-KLH) in a dose-dependent manner and the binding was specific since omalizumab did not bind to control peptide-KLH (Fig. 3A) nor did EP-KLH react with trastuzumab (data not shown). The capability of the EP peptide to block the binding of omalizumab to human IgE was also determined. The results revealed that peptide EP effectively inhibited the binding of omalizumab to human IgE. In contrast, irrelevant control peptide did not produce noteworthy inhibition on their interaction (Fig. 3B).

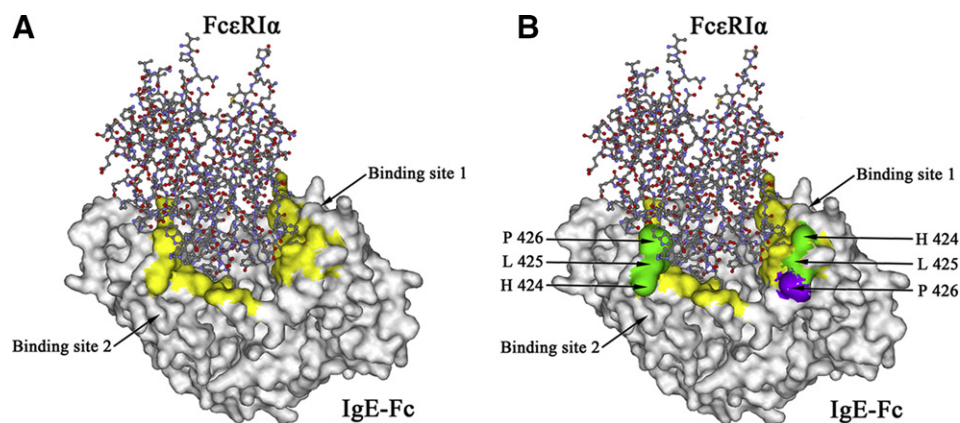


Fig. 4. Model of FcεRIα-binding to IgE-Fc. The model was established using the software Discovery Studio 2.0 (Accelrys, San Diego, CA) based on the previously reported crystal structure of FcεRIα in complex with IgE-Fc [5]. IgE-Fc is shown in a surface representation and FcεRIα is depicted in ball and stick form. (A) The IgE-Fc residues within 5 Å of FcεRIα are defined as FcεRIα-binding site residues and colored yellow. (B) The FcεRIα-binding site residues that do not overlap with the omalizumab epitope residues are colored yellow. The overlap of the omalizumab epitope and the FcεRIα-binding site are shown in green. The omalizumab epitope residues that do not overlap with the FcεRIα-binding site residues are colored purple. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

These results further demonstrated that the ⁴⁵¹HLP⁴⁵⁴ motif was the omalizumab epitope.

The epitope of omalizumab reveals the structural basis for its mechanism of action

Human IgE-Fc has been crystalized in complex with its high-affinity receptor FcεRIα. The crystal structure reveals that one receptor molecule binds one dimeric IgE-Fc molecule asymmetrically through interactions at two sites (binding site 1 and binding site 2), each involving one Cε3 domain of the IgE-Fc [5]. Therefore, it is possible to compare the binding site of FcεRI α-chain to IgE-Fc with the localization of the omalizumab epitope identified in this study. Fig. 4 illustrates the structure of FcεRIα in complex with IgE-Fc. The IgE-Fc residues within 5 Å of FcεRIα are defined as FcεRIα-binding site residues and colored yellow (Fig. 4A). The omalizumab epitope identified here comprises the three amino acids “⁴²⁴HLP⁴²⁶” (Fig. 4B, green and purple), which include two of FcεRIα-binding site residues ⁴²⁴HL⁴²⁵ in binding site 1 (Fig. 4B, green) and three of FcεRIα-binding site residues ⁴²⁴HLP⁴²⁶ in binding site 2 (Fig. 4B, green). Therefore, it can be concluded that the overlap of the omalizumab-specific epitope and the FcεRIα-binding site within the Cε3 domain of IgE-Fc is the structural basis for the inhibition of IgE binding to FcεRI by omalizumab. As a consequence of the inhibition, the expression of FcεRI on basophils, mast cells and dendritic cells is also markedly downregulated [13–17]. Thus, IgE effector functions are inhibited since the binding of IgE to FcεRI on IgE effector cells and the following activation of mast cells and basophils are blocked. Moreover, omalizumab does not bind to IgE already bound by FcεRI, so it does not crosslink FcεRI-bound IgE and sensitize mast cells and basophils [10]. The overlap of the omalizumab epitope and the FcεRIα-binding site can also explain why omalizumab is unable to bind to receptor-bound IgE.

In summary, the ⁴⁵¹HLP⁴⁵⁴ motif within the Cε3 domain of IgE-Fc has been identified as the omalizumab epitope in the present study. This epitope overlaps with the FcεRI-binding site, thus providing insights into the structural basis for the mechanism of action of omalizumab.

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