

The use of phage-peptide libraries to define the epitope specificity of a mouse monoclonal anti-Der p 1 antibody representative of a major component of the human immunoglobulin E anti-Der p 1 response

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Summary

Background More than 80% of individuals who are sensitive to the dust mite *Dermatophagoides pteronyssinus* produce immunoglobulin (Ig) E antibodies to Der p 1, the most significant domestic allergen. There is therefore considerable interest in elucidating the interaction between human IgE and Der p 1, as a basis for developing strategies for therapeutic intervention.

Objectives We have therefore sought to determine the Der p 1 epitope recognized by a mouse monoclonal anti-Der p 1 antibody (mAb 2C7) representative of a major component of the human IgE anti-Der p 1 response.

Methods M13 15mer and T7 9mer bacteriophage-peptide display libraries were screened with mAb 2C7. Mimotope sequences were defined and compared with the native Der p 1 sequence and with those of three homologous molecules, namely chymopapain, papain and actinidin. The sequence of a candidate epitope was then located in the three-dimensional model of Der p 1 and the corresponding sequences in the homologous molecules were studied for accessibility in the three-dimensional structure.

Results We have demonstrated that it is possible to isolate phage clones with peptide inserts specific for mAb 2C7. Examination of the sequences obtained and the location of the corresponding epitope within the three-dimensional model of Der p 1 has shown that mAb 2C7 recognizes a conformational epitope comprising the sequence Leu147-Gln160. The relevance of the identified epitope was established by showing that native Der p 1 can block the binding of specific phage clones to mAb 2C7. Similar sequences were identified within the three-dimensional structures of chymopapain, papain and actinidin, thereby providing a structure-based explanation for immunological cross-reactivity.

Conclusion The identification of the Der p 1 sequence Leu147-Gln160 as a potential epitope recognized by a major component of the human IgE anti-Der p 1 response may provide therapeutic opportunities for disrupting the interaction between IgE and this important allergen.

Keywords: Der p 1, dust mite, immunoglobulin E, phage

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Introduction

Mite allergens are a major cause of allergic diseases such as bronchial asthma, allergic rhinitis and atopic dermatitis [1]. More than 80% of individuals who are sensitive to the dust

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mite *Dermatophagoides pteronyssinus* produce immunoglobulin (Ig) E antibodies to Der p 1 [2,3], the most significant domestic allergen. Given the potent IgE-eliciting properties of Der p 1, there is considerable interest in studying the molecular interaction between IgE and this important allergen.

The structure of Der p 1 has been modelled on the crystal structure of papain, with which it shows considerable sequence similarities [4]. The model shows that Der p 1 consists of two domains. One domain consists of mainly α -helices formed by residues Thr21-Tyr116 and the crossing over of the C-terminal portion Val219-Leu222. The N-terminal portion Thr1-Arg20 crosses over into the other domain, which consists mainly of β -pleated sheets formed by residues Cys117-Tyr218. The enzyme active site of this cysteine protease is found at the interface of the two domains.

Recombinant [5–7] and overlapping synthetic [8,9] peptides representative of Der p 1 were previously used to study the repertoire of epitopes recognized by human anti-Der p 1 antibodies. As expected, such Der p 1 epitopes were found to be in the accessible regions of higher hydrophilicity and motility [10–13]. However, available data suggest that human IgE recognizes epitopes on Der p 1 that are discontinuous in nature [7,14]. These conclusions would therefore cast some doubts as to the value of using linear sequences of Der p 1 for epitope mapping.

Recent advances in technology have allowed the use of antibodies to select their specific ligands from vast bacteriophage libraries displaying random peptides [15,16]. In this study, we have used non-constrained (M13; 15mer) [17] and constrained (T7; 9mer) [18] phage-displayed peptides to search for sequences recognized by a mouse monoclonal antibody (mAb 2C7) representative of a major component of the human IgE anti-Der p 1 response [19]. Results show that mAb 2C7 recognizes a sequence corresponding with Leu147-Gln160 on Der p 1. Definition of this epitope should facilitate the development of future therapeutic strategies aimed at disrupting the interaction between IgE and Der p 1.

Materials and methods

Der p 1 preparation

Der p 1 was purified from lyophilized house dust mite culture supernatant by our recently described multistep procedure [20].

Mouse monoclonal anti-Der p 1 (mAb 2C7)

Mouse mAb 2C7 (IgG2b κ) was produced by conventional hybridoma technology as described recently [19].

Phage-displayed random peptide libraries

Two bacteriophage display libraries were used in this study, namely a filamentous library expressing pentadecamer

peptides on gene VIII (f88–4-15; kindly provided by Dr J. Scott, University of Missouri, Columbia, MO, USA) [17] and our newly designed library expressing nonamer peptides on gene X (major capsid protein) of the lytic bacteriophage T7 (T7 select 415b, Novagen Limited, Madison, WI, USA). The inserts generated in this library are flanked by two cysteine residues, thereby promoting disulphide bond formation and constraining the peptide conformation. The M13 15mer library was originally at 2×10^9 pfu and was amplified to 4.9×10^{11} /mL before use. The T7 9mer library was originally at 1.39×10^8 pfu and was amplified to 4.8×10^{10} /mL before use.

Affinity selection, enumeration and propagation of the selected phage

The peptide display libraries were screened using mAb 2C7. The M13 15mer library was propagated essentially as previously described [21], involving five rounds of biopanning using a polystyrene bead (Integra Biosciences, Cramlington, UK) coated with mAb 2C7 (200 μ g/mL) and blocked with BSA (5%) in TRIS-buffered saline (TBS). Selected f88–4 phage were propagated in *Escherichia coli* strain K91kan, and infected cells were selected for tetracycline resistance (access further details via the web at: www.biosci.missouri.edu/smithgp/index.html). The T7 9mer library was propagated according to the manufacturers instructions, involving three rounds of biopanning using Nunc immunotubes (Nunc Maxisorb, Life Technologies Limited, Paisley, UK) coated with mAb 2C7 (20 μ g/mL) and blocked with 5% Marvel milk powder (MMP; obtained from a local retailer) in TBS. T7 phage were propagated in *E. coli* strain BL21.

Detection of specific M13 phage-peptides

For ELISA, individual *E. coli* clones containing f88–4 phage were cultured in 0.5 mL Excel terrific broth (Sigma, Poole, UK) containing 30 μ g/mL tetracycline in a 12 $\times$ 8 array (Micronic Systems, Stockport, UK). After overnight growth, culture supernatant was prepared by centrifugation (800 g for 30 min at 4°C). The phage clones were then assessed for their ability to bind to mAb 2C7 (10 μ g/mL) and isotype control (10 μ g/mL of mouse IgG2b κ , Sigma) coated onto Nunc microtitre plates by overnight incubation at 4°C. The plates were washed with 0.05% Tween in PBS, then blocked with 200 μ L of 1% BSA in PBS and washed again. Fifty microlitres of cell culture supernatant containing phage clones was added to the wells and incubated for 2 h at room temperature. After washing the plates, bound phage was detected using 50 μ L of 1:1000 dilution of rabbit-anti-M13 biotinylated antibody (Sigma), followed by 50 μ L of 1:20 000 dilution of extravidin-alkaline

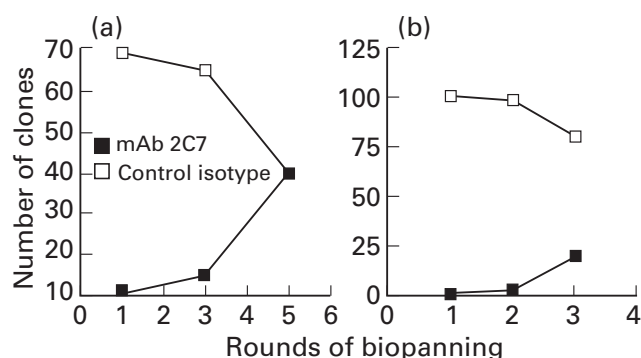


Fig. 1. The increase in the number of specific phage clones obtained from biopanning the M13 (a) and the T7 (b) libraries with mAb 2C7. M13 and T7 phage were detected by ELISA and immunoblots, respectively

phosphatase conjugate (Sigma). The colour reaction was developed using standard methods.

Eighteen clones giving optical density values of 0.75 and above were selected for further analysis. These clones were first propagated in Excel Terrific broth, containing 30 µg/mL tetracycline, PEG (polyethylene glycol 8000) precipitated and 10-fold serial dilutions were assessed as before by ELISA against mAb 2C7, the isotype control and BSA alone. Specific phage clones were then further tested by ELISA-inhibition using different concentrations of Der p 1 in PBS.

Detection of specific T7 phage-peptides

The lack of a suitable monoclonal antibody to T7 phage meant that the phage could not be detected by ELISA. Therefore, selected T7 phage were screened on nitrocellulose blots. Amplified phage from all three rounds of

biopanning were plated out on LB plates, to compare the number of specific clones derived from each round, and about 100 plaques per round were selected for testing. A nitrocellulose membrane (Millipore Corporation, Bedford, UK) was placed on each plate and incubated for 30 min at room temperature. The membrane was removed and blocked with 5% MMP. Prior to use, mAb 2C7 and the antimouse-alkaline phosphatase conjugate were pre-absorbed with the lysate of wild type T7 phage. The membrane was placed in a Petri dish and 10 µL of mAb 2C7 (diluted in 10 mL of 5% MMP/TBS) was added followed by 1 h incubation at room temperature. After extensive washing with 0.1% TBS/Tween and finally with TBS, the membrane was treated in the same way with the antimouse-alkaline phosphatase conjugate. The colour reaction was developed using standard methods.

PCR and DNA sequencing

T7 phage DNA for sequencing was prepared from PEG/NaCl purified virions using standard protocols. DNA sequencing of f88–4 clones was performed on a PCR product obtained by amplification of the insert region of gene VIII with the primers (A) 5'-TCCCCCTGTTGACAATTAATC and (B) 5'-ATTAGGCGGGCTGGGTAT. PCR reactions were 50 µL containing 1 µmol/L of each primer, 0.2 mmol/L dNTPs, 1 unit RH taq polymerase (Life Technologies, Paisley, UK), 10 mmol/L TRIS-HCl pH 8.0 and 50 mmol/L KCl. One microlitre of culture supernatant was added and amplification was achieved with 40 cycles of 30 s at 95 °C, 60 s at 58 °C and 30 s at 72 °C followed by a 5 min soak at 72 °C. Five microlitres of PCR product was purified for DNA sequencing by enzymatic treatment with exonuclease I (1 unit) and shrimp alkaline phosphatase (1 unit) (Amersham International Plc, Amersham, UK) for 20 min at 37 °C and

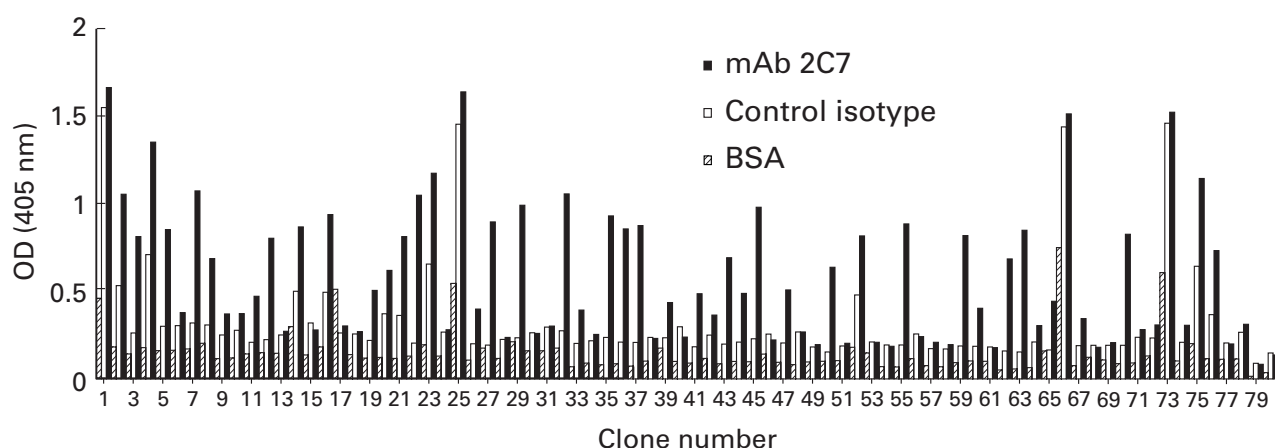


Fig. 2. The specificity of M13 phage clones obtained after five rounds of biopanning with mAb 2C7. The phage clones were detected in the culture supernatant by ELISA

Table 1. Mimotopes selected from the M13 and T7 phage libraries by mAb 2C7. The common motif DXXXR/KXXGR is aligned with the native sequences of Der p 1 (Leu147-Gln160), chymopapain and papain (Gly138-Gly151) and actinidin (Gly141-Gly154)

Library	Clone	Sequence	Frequency
M13	35	E Q S Y P C W L S G T P S T P	5
	27	T P L C D Y A A A R V G A C G	6
	5	K E Q L P T S Y P P E R A G W	1
	3	N C L S S D E P L H I R W C Q	1
	4	E A L S E E E W P R Y T S H P	1
T 7	2	S C D A T Q R A Q G R C S	3
	6	S C D S S Q K K Q G R C S	4
	19	S C D E S R R R Q G R C S	1
	14	S C D A R S E R Y * A C S	1
T7		D A -- R - - G R	3
		D --- K - - G R	4
		D --- R - - G R	1
		D A R -- R	1
M13		- L -- D --- H - R -- Q	1
		-- L - D - A - R - G -- -	6
Der p 1		L D A F R H Y D G R T I I Q (aa147–160)	
Chymopapain		G K P F Q L Y K S G V F D G (aa138–151)	
Papain		G K D F Q L Y R G G I F V G (aa138–151)	
Actinidin		G D A F K Q Y A S G I F T G (aa141–154)	

*stop codon.

for 20 min at 65 °C. The fluorescent DNA sequence was obtained using ABI Big-Dye terminator cycle sequencing reaction mixtures as stated by the manufacturer using the half volume protocol (Perkin-Elmer, Warrington, UK). F88–4 PCR products were sequenced using primer A above, and those of T7 were sequenced with primer 5'-AGCAGAAGCCTGAAGAGAGAGTC. Automated sequence detection was performed with the ABI Prism 310 Genetic Analyser using a 36-cm capillary, POP6 polymer and 20 minute run times.

Sequence translation and alignments

Phage insert sequence alignments and translation to peptides were performed using the Freeware program SeqApp (Don Gilbert, University of Washington, USA). Homologous sequences were searched and aligned using fasta3 program from the SWALL database (Expasy website: www.expasy.ch/sitemap.html).

Der p 1 modelling

A three-dimensional model of Der p 1 has previously been published [4], based on its strong sequence homology with the solved crystal structures of papain (PDB code 9PAP)

[22], actinidin (PDB code 2ACT) [23] and papaya protease omega (PDB code 1PPO) [24]. Although the full molecular co-ordinates of the published Der p 1 model have not been released into the public domain, sufficient information has been disclosed [4] to enable the re-construction of the same model. The molecular co-ordinates for the three structures 9PAP, 2ACT and 1PPO were downloaded from the Brookhaven Protein Databank [25] and the sequence of Der p 1 was then aligned with the sequences of these three structures exactly as published [4]. We then carried out molecular modelling of the Der p 1 sequence using the programs QUANTA97 (version 97.0711) and MODELER [26] distributed by Molecular Simulations Incorporated (Burlington, MA, USA). These programs were run on a Silicon Graphics Iris Indigo workstation running the IRIX 5.3 operating system. The three-dimensional structures of chymopapain [27], papain [22] and actinidin [23] were available from the PDB database and were viewed using the Swiss Pdb Viewer [28–30].

Reactivity of mAb 2C7 with Der p 1 and homologous cysteine proteases

Der p 1 (1 µg/mL) was bound directly onto microtitre plates and the ability of various concentrations of soluble Der p 1,

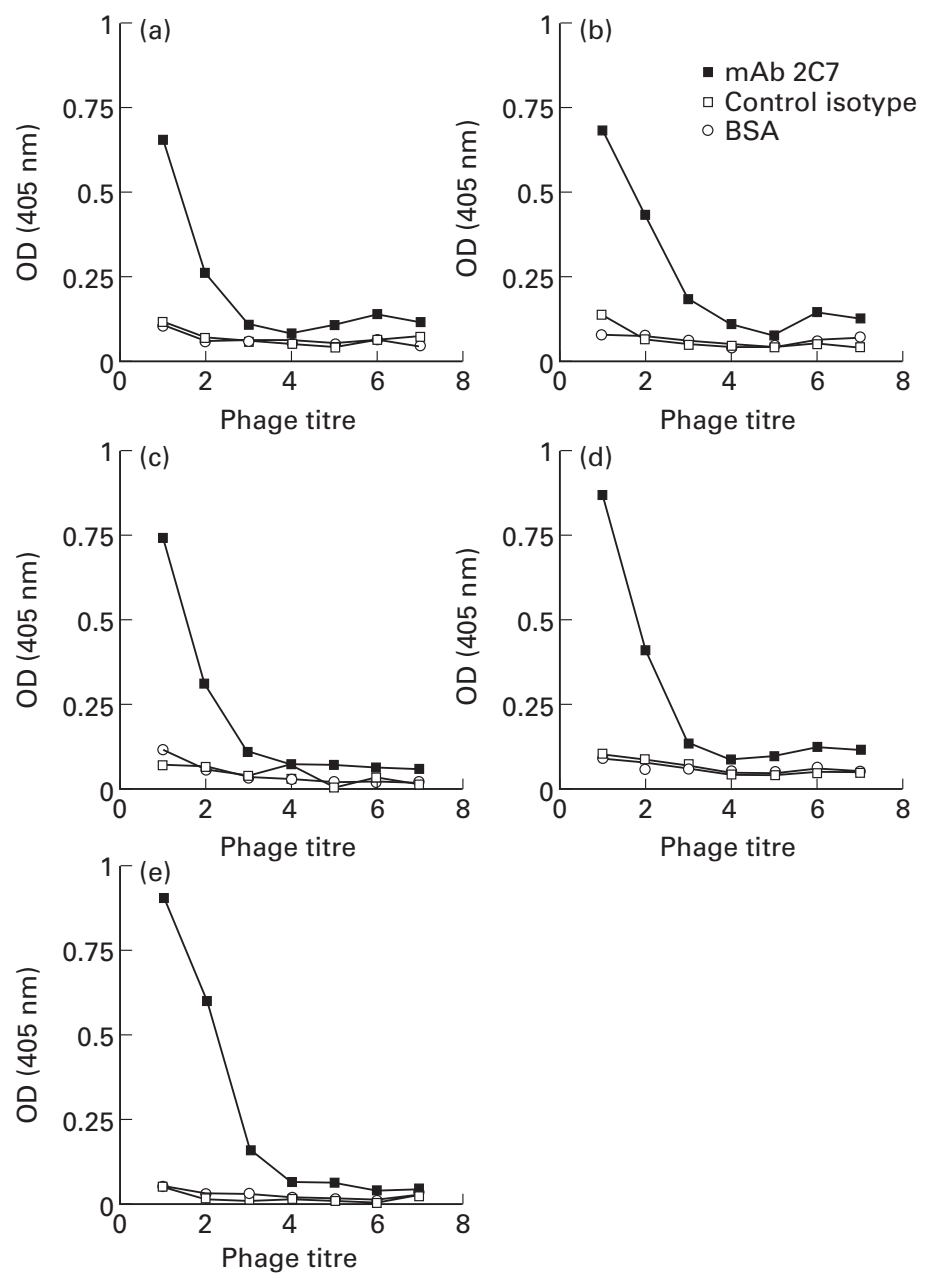


Fig. 3. Titration curves of PEG precipitated M13 phage clones 3 (a), 4 (b), 5 (c), 27 (d), and 35 (e) (see Table 1). Points 1–7 on the x-axis represent the 10-fold serial dilutions starting from the equipotent titre of 2.28×10^{12} . The phage clones were detected by ELISA

chymopapain, papain and actinidin to inhibit the binding of 1:1000 dilution of mAb 2C7 was assessed using 1:1000 dilution of alkaline phosphatase conjugated monoclonal antimouse IgG (Sigma).

Results

Evaluation of the biopanning efficiency

The M13 and the T7 libraries were subjected to five and three rounds of selection and amplification against

immobilized mAb 2C7, respectively. Such biopanning procedure resulted in 30-fold (M13 library) and 20-fold (T7 library) increases in the number of phage clones binding specifically to mAb 2C7 (Fig. 1). In terms of performance, the T7 library has the advantages of superior infectivity and a faster growth rate of clones.

M13 phage sequences selected by mAb 2C7

Of the large number of M13 phage selected by mAb 2C7, 80 clones were randomly chosen from round 5 of biopanning

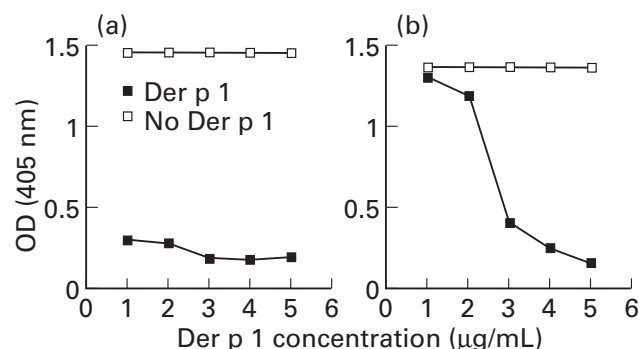


Fig. 4. The biological relevance of M13 phage clones 3 (a) and 27 (b) (Table 1) tested by ELISA inhibition against native Der p 1. The clones were tested at the equipotent titre of 1.64×10^{11} in the presence and absence of Der p 1

for testing in ELISA to ascertain reactivity with mAb 2C7. The vast majority of clones showed binding to mAb 2C7, with 0.820 being the maximum OD₄₀₅ value obtained (Fig. 2). Eighteen clones, giving OD₄₀₅ values of 0.750 or above, were DNA sequenced. Examination of these sequences revealed five motifs (Table 1). The specificity of clones representative of these sequences was ascertained by titration of the PEG precipitate against mAb 2C7 (Fig. 3). The biological relevance of clones 3 and 27, which are potentially constrained, was ascertained by ELISA inhibition against native Der p 1 (Fig. 4); when tested at the same titre non-constrained clones showed no inhibition.

T7 phage sequences selected by mAb 2C7

Of the T7 phage selected by mAb 2C7, 100 colonies were tested by immunoblotting, of which 20 bound specifically to mAb 2C7. These 20 clones were then DNA sequenced. Examination of these sequences revealed four motifs (Table 1).

Alignments of mimotope sequences

Alignments of the sequences obtained from the M13 and the T7 libraries revealed a common motif in which eight of the T7 clones were DXXXR/KXXGR, with the other eight clones showing some similarity with this motif particularly at the N-terminus (D) (Table 1). Collectively, sequence comparison shows that the identified motif corresponds with Leu147-Gln160 in the native Der p 1 sequence (Table 1).

Locating the epitope recognized by mAb 2C7 on the Der p 1 model

The sequence Leu147-Gln160 was located on the three-dimensional model of Der p 1 by automated comparative

modelling via the internet server Swiss Model. Examination of solvent accessibility showed that α -helices and β -sheets comprise the core of the Der p 1 molecule, and that the outer surface comprises mainly the interconnecting loops, of which Ser92-Ser114 and Val140-Tyr169 are the longest. The Der p 1 epitope, Leu147-Gln160, identified by mAb 2C7 is therefore part of one of these loops (Val140-Tyr169) which is located on the surface of the domain comprising mainly β -sheets (Fig. 5). The corresponding sequences in chymopapain, papain and actinidin (Table 1) were also located in the three-dimensional structure of these molecules and their examination showed that they exhibit a variable degree of accessibility (Fig. 5).

Reactivity of mAb 2C7 with homologous cysteine proteases

The ability of mAb 2C7 to recognize chymopapain, papain and actinidin was assessed by ELISA inhibition using Der p 1-coated microtitre plates, and results were compared with that obtained using a concentration of Der p 1 giving 50% inhibition (0.6 µg/mL). To achieve this degree of inhibition, 100-fold molar excess of chymopapain and 183-fold molar excess of papain were needed. On the other hand, actinidin showed no inhibition even at 600-fold molar excess. The extent to which mAb 2C7 recognized Der p 1, chymopapain, papain and actinidin was directly related to solvent accessibility of its epitope (Fig. 5).

Discussion

We have previously established, by cross-inhibition studies and V region gene sequencing, that mAb 2C7 is representative of a major component of the human IgE anti-Der p 1 response [19]. In ELISA, mAb 2C7 is capable of inhibiting, by up to 70%, the human IgE response to Der p 1. Its heavy chain has greater than 70% homology with three members of the human V_H3 family, DP-35, DP-53 and DP-54, and its light chain has greater than 70% homology with 11 human V_K sequences, including the V_KII sequences DPK18, DPK19 and DPK28. These observations have therefore prompted us to map the epitope specificity of mAb 2C7, as a prototype human IgE anti-Der p 1.

The epitope specificity of mAb 2C7 was determined by searching for phage-displayed non-constrained (M13; 15mer) [17] and constrained (T7; 9mer) [18] peptide sequences. The agreement between the peptide sequences selected from these two different phage libraries has served to further confirm the validity of the mimotope sequence obtained (DXXXR/KXXGR). The identified mimotope corresponds with Leu147-Gln160 in the native Der p 1 sequence, which is contained in a loop (Val140-Tyr169) located on the surface of the domain comprising mainly β -sheets. The three-dimensional model of Der p 1 shows that the scaffold

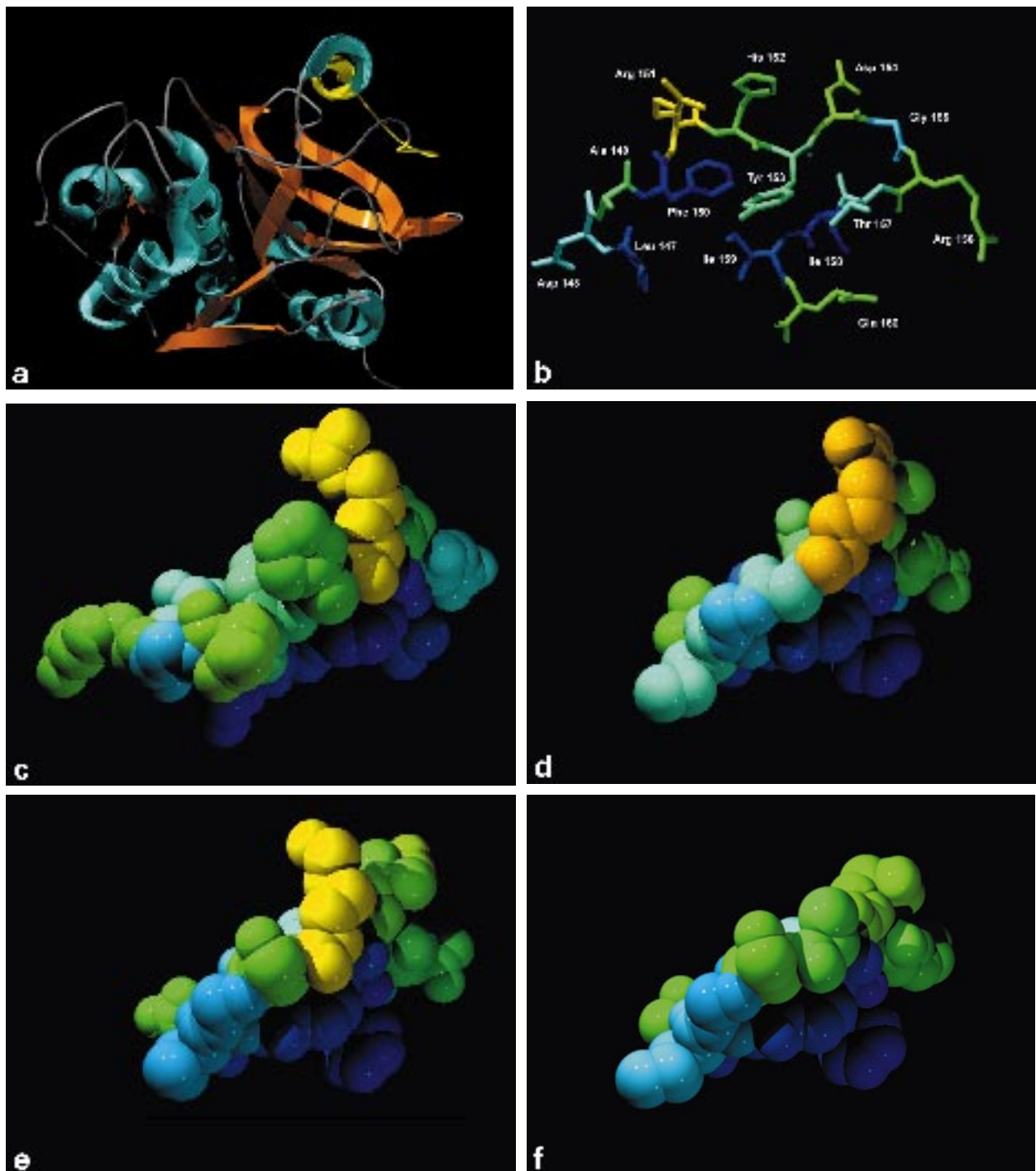


Fig. 5. Three-dimensional model of Der p 1 showing the epitope (Leu147-Gln160) recognized by mAb 2C7 coloured in yellow (a) and its amino acid composition (b). Solvent accessibility (decreasing in the order yellow, green, light blue and dark blue) of the mAb 2C7 epitope on Der p 1 (c) and the corresponding sequences in chymopapain (d), papain (e) and actinidin (f)

of the epitope recognized by mAb 2C7 is formed by nonaccessible, non-polar aromatic (Phe150) and aliphatic (Leu147, Ile158 and Ile159) residues, while the remaining amino acids form the exposed surface of the epitope, with Arg151 and

Arg156 being the most accessible amino acids. The charge distribution of the identified epitope is such that large accessible residues, namely Arg151, His152 and Arg156, form the central part of the epitope. The epitope also

contains two small negatively charged residues, namely Asp148 and Asp154.

Homologous loops corresponding with the site recognized by mAb 2C7 were located on the crystallographically determined three-dimensional structures of chymopapain, papain and actinidin, all of which are cysteine proteases showing sequence similarities with Der p 1. Gly138-Gly151 in chymopapain and papain and Gly141-Gly154 in actinidin correspond with Leu147-Gln160 in Der p 1, which is the epitope targeted by mAb 2C7. The Der p 1 loop containing the mAb 2C7 recognition site shows some differences compared with the corresponding loops in chymopapain, papain and actinidin. For instance, it contains more highly accessible large residues with long side chains (e.g. Arg156) and more charged residues (see above).

The availability in chymopapain, papain and actinidin of loops corresponding with that containing the Der p 1 epitope recognized by mAb 2C7 suggests that this antibody may cross-react with these homologous molecules. This has indeed been confirmed by our ELISA inhibition tests, which showed that the degree of cross-reactivity is directly related to the shape of the loop. For instance, the loop in chymopapain, which is highly reactive with mAb 2C7, is very similar to that of Der p 1; whilst in actinidin it is quite flat and this cysteine protease was least reactive with mAb 2C7. The data presented in this paper are very much in line with other studies showing that allergenic epitopes recognized by IgE, on for instance birch pollen profilin [31] and ragweed allergens [32], contain α -helices and are predominantly conformational, but containing elements of sequential nature [33].

Our data represent the first definition of what is likely to be the epitope recognized by a major component of the human IgE anti-Der p 1 response. It should now be possible to further define, by site-directed mutagenesis, the key residues involved in the interaction of Der p 1 with the Fv region of mAb 2C7, for which we have a three-dimensional model [19]. Such studies should also incorporate attempts to predict algorithmically [34] all possible surface residues involved in antibody interaction which will allow the docking of the mAb 2C7 model [19] to that of Der p 1 [4]. This should provide the structural basis for the rational design of a peptide which may act as a monovalent Der p 1 allergen, capable of disrupting the interaction of human IgE with this ubiquitous inhaled allergen.

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