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Journal of Immunological Methods 299 (2005) 21–35

JIM
Journal of
Immunological Methods

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Research paper

Requirement of multiple phage displayed peptide libraries for optimal mapping of a conformational antibody epitope on CCR5

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Received 14 April 2004; received in revised form 30 September 2004; accepted 30 November 2004
Available online 1 February 2005

Abstract

In the absence of information from crystallography, conformational epitopes can often be discerned by antibody screening of phage displayed random peptide libraries. However the context in which the peptide is displayed, and the number of copies displayed in the library, can influence results and interpretations. Here, the monoclonal antibodies 3A9 specific for the transmembrane chemokine receptor CCR5, and CII-C1 specific for type II collagen, were used to screen multiple phage-displayed peptide libraries in which peptides were displayed in either the pIII or pVIII coat proteins. ELISA was used to test for reactivity and cross-inhibitory activity of isolated phage clones. Based on sequences of reactive phage inserts, epitope motifs were initially inferred from a molecular model of CCR5 and subsequently confirmed experimentally using mutagenesis to alanine. For each mAb, phage sequences from pIII biopannings were more diverse than from pVIII biopannings. Notably, sequences from either biopanning were cross-inhibitory despite a lack of linear sequence homology. For CCR5, residues ^{88}H and ^{94}W in the first loop of CCR5 were identified by pIII biopannings, and ^{7}S^{9}IYD^{11} at the N-terminus by pVIII biopannings. Thus conformational epitopes can be identified using phage display, but optimal mapping of complex epitopes can require the use of multiple peptide libraries.

Keywords: Epitope; Phage library; Chemokine receptor; CCR5; HIV infectivity; Type II collagen

Abbreviations: ABTS, 2,2-azino-di-(3-ethyl-benzthiazoline 6 sulfonate); BSA, bovine serum albumin; CCR5, CC chemokine receptor 5; CII, type II collagen; mAb, monoclonal antibody; DMEM, Dulbecco's Minimal Essential Medium; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; HRP, horseradish peroxidase; MIP, macrophage inflammatory protein; *A*, absorbance; PBS, phosphate buffered saline; PEG, polyethylene glycol; pfu, plaque forming units; smpPBS, skimmed milk in PBS.

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[2] J.A.I. is supported by The Cancer Council Victoria as a Cancer Council Victoria Postdoctoral Research Fellow.

0022-1759/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jim.2004.11.025

1. Introduction

The identification of antigenic determinants, or epitopes, is an important procedure for the design of immunogens to be used as vaccines and for the molecular analysis of functionally important domains in complex proteins. In particular, epitopes of antibodies that neutralize certain biological functions such as virus entry into cells are expected to be functionally implicated in those processes. Therefore, epitope mapping for such antibodies should help to delineate functionally important regions of a complex protein, noting that antibody epitopes mostly represent discontinuous sites brought into contiguity by the folded state of a protein molecule (Rowley et al., 2004), and that amino acid sequences (as mimotopes) can mimic an epitope without necessarily exhibiting identity or homology with the original antigenic epitope (Geysen et al., 1986).

Various strategies have been used to identify epitopes, including comparing naturally occurring variants of similar proteins, as applied to bird egg-white lysozyme (Smith-Gill et al., 1982; Benjamin et al., 1984), dissecting the antigen into overlapping polypeptides in the form of recombinantly expressed fusion proteins or truncation mutants (Surh et al., 1990), or testing of short synthetic peptides spanning the sequence of the antigen (Tribbick, 2002). However conformational epitopes are mostly mapped only to sequences of some 100 or more residues, and complete definition of an epitope requires the use of X-ray crystallography. X-ray crystallography of well characterized antibody–protein complexes has indicated that most epitopes contain 15–22 protein residues in contact with the combining site of the antibody, and that a small subset of residues scattered over two or three discontinuous polypeptide segments contributes most of the free binding energy (Novotny et al., 1989; Jin et al., 1992; Braden and Poljak, 1995).

In the absence of information on protein structure by crystallography, a conformational epitope of an antigenic protein can often be discerned by antibody screening of random phage-displayed peptide libraries, and sequencing the DNA inserts of reactive phage (Rowley et al., 2004). Whilst a single phage library should amply express sufficient peptides in the coat proteins for epitope mapping, both the context in which the peptide is displayed and the number of copies displayed can affect the peptides selected (Bonnycastle et al., 1996; Rowley et al., 2004), so prompting the use of multiple libraries for epitope analysis (Bonnycastle et al., 1996; De Ciechi et al., 1996). Here we establish the requirement for use of more than one phage library to identify optimally the conformationally distributed contact residues for a given antibody. The two illustrative monoclonal antibodies (mAb) chosen were mAb 3A9 raised to the transmembrane chemokine receptor–CCR5–that acts as a co-receptor for entry into cells of the human immunodeficiency virus (HIV-1) (Zhou et al., 2000), and blocks the binding of HIV-1 to CCR5 without inhibiting the function of CCR5 as a chemokine receptor (Wu et al., 1997), and mAb CII-C1 raised to the major structural protein of cartilage, type II collagen (Schulte et al., 1998). The screening with these mAbs of different libraries in which peptides are expressed in the pIII or pVIII coat proteins yielded different sets of peptides that, notably, were cross-inhibitory despite a complete lack of linear sequence homology.

2. Materials and methods

2.1. Monoclonal antibodies

The mAb 3A9 was donated by Charles Mackay and Lijun Wu, and CII-C1 by Rikard Holmdahl (see Acknowledgements). The conformational epitope for 3A9 has not been defined, but has been localised by mutagenesis using chimeric receptors to the N-terminus of CCR5 (Wu et al., 1997), and by phage display to the N-terminus and the first extracellular loop (Königs et al., 2000); the amino acids identified by phage display were present in all chimeras (Wu et al., 1997). We used a control mAb 2D7 (BD Pharmingen, San Diego, CA) that has been mapped to the second extracellular loop of CCR5 (Wu et al., 1997) to monitor expression of CCR5 mutants in transfected cells; as mutagenesis was restricted to residues outside this loop, the reactivity of 2D7 should not be affected by the CCR5 mutants used in this study. The mAb CII-C1 reacts with a conformational epitope on triple helical type II collagen (CII) defined by a minimal linear sequence of ARGLT at aa

359–363 of the collagen α-chains, but is non-reactive with heat-denatured CII (Schulte et al., 1998).

2.2. Sources of phage libraries

Three different random phage-displayed peptide libraries, each expressing peptides at the N-terminus of the five copies of the pIII coat protein of the filamentous coliphage M13, were purchased from New England Biolabs (Beverly, MA). These included a heptapeptide library (PhD-7), a dodecapeptide library (PhD-12), and a cysteine-constrained heptapeptide library (PhD C-7-C). The libraries contained 2×10^{13} pfu/ml with a complexity of $>2\times10^{9}$ transformants and were used at a concentration of 8×10^{10} pfu/ml. Phage were propagated in *Escherichia coli* strain ER2537 which was provided with the library kit as a non-competent glycerol stock. The DNA inserts from individual non-selected colonies were sequenced from the library to confirm that they contained random peptide sequences. All phage sequenced contained inserts, although several had the amber stop codon (TAG) that is expressed as glutamine in these libraries.

Linear and cysteine-constrained nonapeptide phagemid libraries expressing peptides on many of the ~2800 copies of the pVIII coat protein (Felici et al., 1991) were kindly provided by Alessandra Luzzago, Instituto di Richerche di Biologia Molecolare (IRBM), Rome, Italy. Phagemids were propagated using the related helper phage M13 KO7 (Pharmacia, Uppsala, Sweden). The pVIII libraries contained $>10^{13}$ ampicillin transducing units/ml, and sequences from individual non-selected colonies showed that about 20% of phages contained inserts that encoded peptides; the remaining phage lacked inserts or contained stop codons. For each of the libraries, pIII or pVIII, all 20 amino acid codons were represented in the random sequences close to their expected frequencies based on the genetic code.

2.3. Antibody screening of phage libraries

The libraries were screened by biopanning with successive rounds of positive selection using the antibody of interest, and negative selection using only the biopanning materials without the target antibody, to remove phage that bound to other components of the system. The combined pIII libraries were screened using 3 μl of each library and 100 μg of mAb 3A9 in phosphate buffered saline (PBS), pH 7.3, supplemented with 1 mg/ml BSA (PBS–BSA). The mixture was left overnight at 4 °C and phage–antibody complexes were isolated using paramagnetic beads coated with anti-mouse IgG (Chemicon, Temecula, CA). The beads were washed 10 times in PBS–BSA, and phage were eluted from the antibodies with 100 μl of 0.1 M HCl adjusted to pH 2.2 with glycine and neutralized immediately with 40 μl of 1 M Tris–HCl pH 9.9. ER2537 bacterial cells in log phase were infected with the eluted phage and the culture was grown for 4.5 h. The bacterial cells were pelleted twice by 15 min centrifugations at $4000\times g$. Phage were precipitated from the supernatant using 25% polyethylene glycol (PEG) 6000 and 2.5 M NaCl on ice for 1 h and pelleted by centrifugation for 15 min at $10{,}000\times g$. The phage pellet was resuspended in 10 mM Tris, 1 mM EDTA, pH 8. Precipitation was repeated and the final phage pellet was resuspended in PBS and used for further rounds of biopanning. Three rounds of positive selection were performed, and negative selection was performed after the first and second rounds of positive selection, each followed by amplification. Phages were used to infect the host bacterial strain and single phage clones were isolated (Ikuno et al., 2001). Biopanning with CII-C1 was performed similarly, using 10 μg of mAb and three rounds of positive selection.

The pVIII constrained library was screened with mAb 3A9 using five rounds of positive selection and three rounds of negative selection, as described previously (Königs et al., 2000), and both linear and constrained libraries were screened with mAb CII-C1 (Cook et al., 1998). In brief, after biopanning, bound phage were eluted with 1 mg/ml BSA in 0.2 M glycine HCl, pH 2.2, neutralized using 1 M Tris–HCl, pH 9.1, propagated in *E. coli* strain K91 with helper phage M13KO7, and grown selectively with 100 μg/ml ampicillin and 70 μg/ml kanamycin; phage were isolated as described, and used for further rounds of biopanning.

2.4. Sequencing of DNA inserts

Phagotopes of interest were selected for sequencing. Single-stranded (ss) DNA was prepared by phenol

extraction (Sambrook et al., 1989) and sequenced using the Sequenase version 2.0 T7 DNA sequencing kit (Amersham, Cleveland OH). The M13 (−28 gIII) or the M13 (−40 gVIII) sequencing primers supplied with the kit were used, and [α-^{35}S]-dATP Redivue (Amersham) was used as label for phagotopes selected from the pIII or the pVIII libraries, respectively. Sequencing was conducted using dGTP, and ambiguous sequences were resequenced using the nucleotide analogue dITP to minimize secondary structure formation. Samples were run on a 4.5% acrylamide denaturing gel. Amino acid sequences of inserts were aligned visually to the sequence of CCR5 or type II collagen.

2.5. Statistical analysis

The frequency of selection of particular amino acids in the phage peptides was compared with the expected frequency of that amino acid occurring randomly based on codon usage by Chi squared. Each peptide sequence isolated was included only once, and no correction was made for multiple comparisons.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Two different ELISAs were used to identify reactive phagotopes, a direct ELISA in which plates were coated with the phagotopes, and a capture ELISA in which phage particles were tethered to the plates employing the antibody used to select the phage, 3A9 or CII-C1. The two assays were used because the direct ELISA identifies highly reactive phagotopes that are likely to contain a strong motif, whereas the capture ELISA is more sensitive where there is only weak reactivity (Rowley et al., 2004). For the direct ELISA, phage preparations were diluted 1/20 in sterile PBS and duplicate samples of 100 μl/well were added to a 96-well microtitre plate (Maxisorp, Nunc, Roskilde, Denmark). The high concentration of phage used to coat the plate was selected since it had been shown to give maximal phage binding for all phage preparations used (data not shown). Plates were left overnight at 4 °C, blocked for 2 h at room temperature with 1% skimmed milk powder and 0.05% Tween 20 in PBS pH 7.4 (smpPBS-Tween), and washed three times in Tris buffered saline, pH 7.4, containing 0.05% Tween 20. 100 μl of mAb diluted to 1 μg/ml in 1% smpPBS-Tween were added and left overnight. The plates were washed as before and exposed to horseradish peroxidase (HRP)-conjugated anti-mouse Ig (Silenus, Hawthorn, Australia) diluted 1/2000 in smpPBS without Tween 20 for 2 h, washed six times and developed with 0.5 mg/ml 2,2-azino-di-(3-ethyl-benzthiazoline 6 sulfonate) (ABTS) (Diagnostic Chemicals, Charlottetown, Canada) in 0.03 M citric acid, 0.04 M Na_2HPO_4, pH 4 and 0.003% H_2O_2. Absorbances were read at 415 nm after 30 and 60 min. Phage with stop codons or no inserts were included in each assay and were used as negative controls.

For the capture ELISA, as previously described (Königs et al., 2000), mAb 3A9 or CII-C1 immobilized on microtitre plates was used as capture antibody, and bound phage were detected using polyclonal sheep antibodies to M13 phage (anti-M13, Pharmacia, Uppsala, Sweden) and HRP-conjugated anti-sheep/goat Ig (Silenus) for colour development. Wells of microtitre plates were coated with 100 μl/well of the selecting mAb diluted to 1 μg/ml in PBS pH7.4, left at 4 °C overnight, blocked and washed. Duplicate samples of 5 μl of phage diluted 1/20 in sterile PBS were added and left overnight at 4 °C. The plates were blocked and washed before adding 100 μl of antibody to M13 phage (Pharmacia) diluted 1/2000 in mpPBS-Tween. Phage bound by the mAb were detected by exposure to HRP-conjugated anti-sheep/goat antibodies diluted 1/2000 in smPBS for 2 h, followed by development with 0.5 mg/ml 2,2-azino-di-(3-ethyl-benzthiazoline 6 sulfonate) (ABTS) (Diagnostic Chemicals, Charlottetown, Canada) in 0.03 M citric acid, 0.04 M Na_2HPO_4, pH 4 and 0.003% H_2O_2.

2.7. Capacity of phage to cross-inhibit phage from different libraries

An inhibition ELISA based on the direct ELISA was performed to show that phage isolated from different libraries competed for antibody binding. Plates were coated with phagotopes and blocked as for the direct ELISA. 5 μl of inhibitor phage were added to 100 μl of mAb 3A9 diluted to 1 μg/ml in 1% mpPBS-Tween. 100 μl replicates (4–6) of antibody/inhibitor combinations were added to the wells and left overnight. The plates were washed and developed as for the direct ELISA. The reactivity of mAb 3A9 with no addition of inhibitor phage was taken to represent 100% reactivity. As

negative controls, we used an irrelevant phagotope derived by screening the 9mer (pVIII) library with the mAb CII-C1 for the 3A9 selected phage, and phage without inserts for the CII-C1 selected phage.

2.8. Homology model of CCR5

In order to identify residues that are likely to be surface exposed and proximate to one another on the extracellular face of the receptor, a homology model of CCR5 was built. Rhodopsin is the only member of the G-coupled protein receptor family for which the structure has been solved. This protein (pdb accession, 1L9H; Okada et al., 2002) was therefore used as a template for the generation of a homology model, as has been performed elsewhere for the purpose of predicting residue positions and orientations for mutagenesis (see, for example Paterlini, 2002). A PSI-BLAST (Altschul et al., 1997) search of the SWISS-PROT database at the NCBI (default parameters; two iterations) was performed and matches with $E<10^{-6}$ retained. Representatives from this list were selected such that no two proteins had greater than 25% sequence identity, and these sequences were combined into a multiple alignment using T-COFFEE (Notredame et al., 2000) (lalign_id_pair and fast_pair options), a program well-suited to aligning remote homologues. This 'seed' alignment was subsequently used to guide a progressive alignment of 126 sequences with <50% identity in CLUSTALW (Chenna et al., 2003). The pairwise alignment between CCR5 and rhodopsin, extracted from the multiple alignment, was used to guide molecular modeling. No attempt was made to model the C-terminal region (^{336}Y to ^{352}L) on the intracellular face.

Homology modeling was undertaken using MODELER6 (Sali and Blundell, 1993). Where CCR5 contained an insertion with respect to rhodopsin, MODELER6 automated loop-building was undertaken, with loop conformation refinement using fast molecular dynamics annealing and conjugate gradients energy minimization. Distance constraints (2.5 Å) were imposed for the ^{20}CYS-^{269}CYS and ^{101}CYS-^{178}CYS disulfides and for the Cα atoms of residues identified previously as being members of conformational epitopes (<20 Å): ^{2}D-^{168}R, ^{2}DYQ^{7}SP^{13}N-^{176}YT^{177} (Olson et al., 1999), and ^{176}D-^{171}KE^{172}(Lee et al., 1999). The multiple alignment between G-protein coupled receptors was used to identify conserved sites in the structure, i.e. amino acid positions with >70% identity or >90% physicochemical similarity across aligned proteins. Distance constraints were imposed for conserved close-packing pairs, defined as residues capable of interacting with interatomic distances <4.0 Å in the rhodopsin structure, and the model in its entirety was subjected to rigorous refinement with slow molecular dynamics annealing and minimization. The stereochemistry of the resulting structure was deemed acceptable using PROCHECK (Laskowski et al., 1993). Due to constraints imposed by disulfide bonds, packing of highly conserved residues, and epitope-derived distance limits, the model is expected to provide a good approximate location for the residues considered in this study.

2.9. Site directed mutagenesis

Based on the sequences of the peptide inserts of phage selected by biopanning, and by reference to the homology model, surface-exposed amino acids were selected for mutagenesis. Amino acids were deliberately mutated in combination from the start, since the mutation of one contact residue among 5–6 contributing to antibody binding might not cause a detectable decrease in the energy of binding. The human CCR5-cDNA of 1019 bp was cloned into the pcDNA3-vector using *Hin*dIII and ApaI restriction sites (Wu et al., 1997). The position of altered amino acids in CCR5 is shown in Fig. 1. Mutagenesis was performed using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The following amino acids of CCR5 were exchanged for alanine: ^{7}S^{9}I; ^{7}S^{9}IYD^{11}; ^{96}FG^{97}; ^{7}S^{9}IYD^{96}FG^{97}; ^{274}RLD^{276}; ^{274}RLD^{88}H^{94}W. The following PCR primers for mutagenesis were obtained from Thermo Electron Corporation (formerly Thermo-Hybaid, Milford, MA); underlined sections represent the codons exchanged for alanine, and the amino acid changes are shown in bold in the name of the primer: CCR5/3A9**SI**d: GGATTATCAAGTGTCAGCTCCAGCCTATGACATC; CCR5/3A9**SI**r: GATGTCATAGGCTGGAGCTGACACTTGATAATCC; CCR5/3A9**YD**d: GTCAGCTCCAGCCGCTGCCATCAATTATTATACATCGGAGCCC; CCR5/3A9**YD**r: GGGCTCCGATGTATAATAATTGATGGCAGCGCTGGAGCTGAC; CCR5/3A9**FG**d:

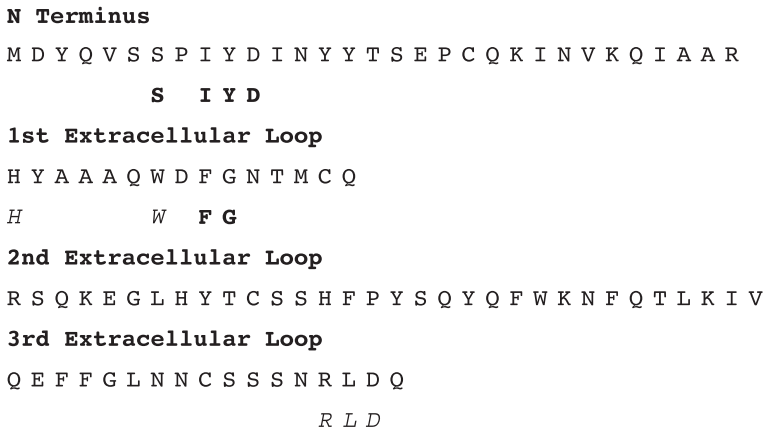

Fig. 1. Alignment of mAb 3A9-specific sequences to CCR5. Motifs observed repeatedly in sequences of 3A9-specific phage were visually aligned to the known sequence of CCR5. Italics indicate motifs identified by biopanning of the pIII libraries and bold indicates motifs identified by biopanning of the pVIII library.

GCCGCCCAGTGGGACGCTGCAAATACA-ATGTGTC; CCR5/3A9**FG**r: GACACATTGTA-TTTGCAGCGTCCCACTGGGCGGC; CCR5/3A9**HW**d: CTTCTGGGCTGCCTATGCTGCCGCC-CAGGCGGACTTTGGA; CCR5/5C7**HW**r: TCCAAAGTCCGCCTGGGCGGCAGCATAGGCA-GCCCAGAAG; CCR5/5C7**RLD**d: GCAGTAGCTC-TAACGCGGCGGCCCAAGCTATGCAGGTG; CCR5/5C7**RLD**r: CACCTGCATAGCTTGGG-CCGCCGCGTTAGAGCTACTGC. Mutation of the second motif in plasmids that already contained one mutated motif generated double mutants stepwise. All mutations were confirmed by sequencing.

2.10. Transfection, cell culture and sorting of cells

HEK293 cells were transfected with 2 μg DNA from the above-mentioned plasmids using Superfect according to the manufacturer's instructions (Qiagen, Hilden, Germany). Transfected cells were cultured in the presence of 700 μg/ml of the antibiotic G418. When cells grew well under selection, 6×10^6 cells were incubated with 5 μg/ml mAb 2D7 in PBS and left at 4 °C on a rotating wheel for 30 min. Cells were pelleted, washed, resuspended in 2 ml PBS containing 2.4×10^7 magnetic beads coated with anti-mouse antibodies (pan-mouse, Dynal, Oslo, Norway) and left rotating at 4 °C for 30 min. Cells were then separated with a magnet and washed five times with 2 ml PBS. Cells were resuspended in pre-warmed media (DMEM, 10% FCS, 1% Penicillin/Streptomycin, 2% L-glutamate and 700 μg/ml G418) and grown at 37 °C in 5% CO_2. This procedure was repeated three times and CCR5 expression was monitored by FACS using mAb 2D7 (see below).

2.11. Fluorescence activated cell sorter (FACS) analysis

HEK 293 cells stably transfected with wild-type CCR5, or CCR5 mutants, were labelled with mAb 3A9 or mAb 2D7 at 5 μg/ml for 30 min on ice. Cells were washed twice with PBS. Bound antibody was detected with 1/500 diluted, FITC-labelled anti-mouse IgG antibody (Dianova, Hamburg, Germany). Labelled cells were then detected, on a FACSCalibur (BD Biosciences, Heidelberg, Germany) using 10,000 cells for each experiment. Gating was not performed. The data were analysed using Cellquest Pro (BD Biosciences). Each mutant was tested at least twice, with similar results. Since mAb 2D7 binds predominantly to the (non-mutated) second extracellular loop of CCR5, it was used as a control to monitor expression of transfected CCR5.

3. Results

3.1. Reactivity of individual phagotopes by ELISA

After biopanning the combined pIII phage libraries with mAb 3A9, 36 phagotopes were isolated and

tested for reactivity by ELISA (Fig. 2); those most reactive were selected for further study. By direct ELISA, two phagotopes were highly reactive with absorbance values >2.0 (3A9/6 and 3A9/16), and a further two had values >1.0. By capture ELISA, all phagotopes derived from the pIII phage libraries were positive when compared with phage with no insert. Of 20 phagotopes that were highly positive by capture ELISA (absorbance>1.4), 17 were sequenced to determine the peptide inserts. The results of biopanning the pVIII library were as previously described (Königs et al., 2000). In brief, there were 48 phagotopes highly positive by capture ELISA, and for 19 the DNA was sequenced to determine the peptide inserts.

3.2. Peptide sequences from phage displayed libraries biopanned with 3A9

Of the 20 phagotopes from the pIII libraries selected by ELISA, DNA sequences were obtained for 17; of these, 16 were derived from the 7mer linear library, one from the 12mer linear library, and none from the 7mer constrained library. The peptides encoded by the 17 DNA sequences differed, but a motif HW was evident in most. In accord with this, the frequency of most amino acids in the peptides was close to their expected frequency in the library, except for significant increases in histidine (H, 20% vs. 3%) and tryptophan (W, 17% vs. 3%) ($p<0.0001$), and marginally significant decreases in cysteine (C), glycine (G) and arginine (R) ($p<0.05$). The several sequences that contained the motifs RLD, LR, or LD were of interest, since the motif HWLRDL occurred in 15 of 19 of the phagotopes obtained previously (Königs et al., 2000) by biopanning with another mAb to CCR5, 5C7, that reacts similarly to 3A9 (Table 1). For the pVIII phage library biopanned using mAb 3A9, there were 19 peptides sequenced after five rounds of positive selection; 18 were identical, C-HASIYDFGS-C, and the other expressed C-VYA-LIMPPL-C; notably, randomly selected phagotopes sequenced after even two of positive selection with mAb 3A9 exclusively contained the sequence C-HASIYDFGS-C (data not shown).

3.3. Inhibition of mAb binding

To confirm that the phagotopes selected by the two sets of biopannings were indeed reacting with the

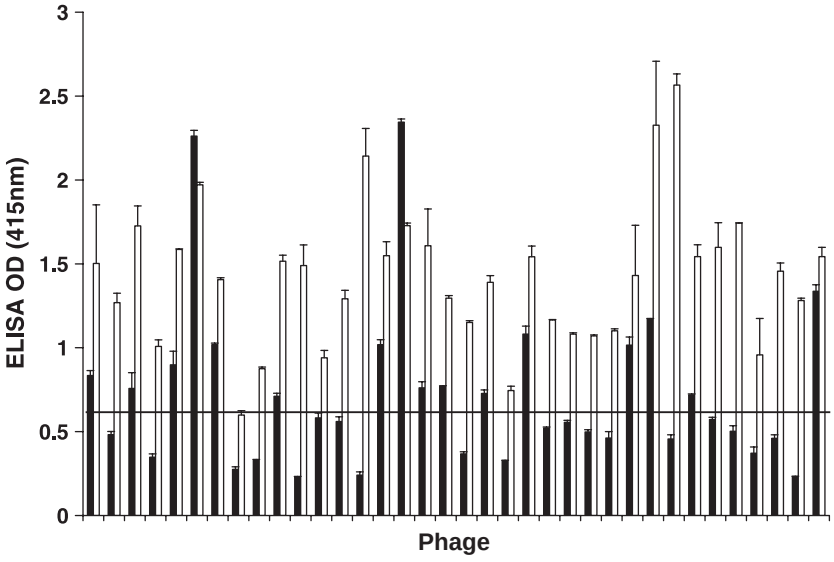

Fig. 2. Reactivity by ELISA (mean and maximum of duplicates) of phagotopes isolated by biopanning with mAb 3A9 of phage library in pIII coat protein. The 36 clones isolated from the pIII biopanning were tested for reactivity by direct and capture ELISA at times shown. The cut-off for positivity by direct ELISA was an absorbance of 0.6 at 415 nm, indicated by the solid line.

Table 1
Sequences of phage clones isolated by biopanning: frequency and reactivity by capture ELISA

Phagotope	Sequence (times selected)	Absorbance[a]
PIII biopanning		
3A9/1	QHWLAQ**R** (1)	1.5[b]
3A9/7	QHWAI*H*N (1)	1.4[b]
3A9/27	QHWA*H*SM (1)	1.4[b]
3A9/6	EHWTWPV (1)	1.9[b]
3A9/16	WHWTSAT (1)	1.7[b]
3A9/10	WH*LTK*PT (1)	1.5[b]
3A9/5	NHWASAEP**LD**VV (1)	1.5[b]
3A9/29	APWAWYP (1)	2.5[b]
3A9/14	LAPWNS**D** (1)	2.1
3A9/17	FH*F*T**RLD** (1)	1.6[b]
3A9/15	HHWVMS**D** (1)	1.5[b]
3A9/22	HHWASSN (1)	1.5[b]
3A9/32	HHWTSSN (1)	1.7
3A9/28	THWAS**LR** (1)	2.3
3A9/36	THWA*H*DS (1)	1.5[b]
3A9/3	THWTG**D**P (1)	1.7[b]
3A9/18	SH/Y**R**TS (1)	1.4[b]
PVIII biopanning		
3A9/1	C-HASIYDFGS-C (18)	2.7[b,c]
3A9/8	C-VYALIMPPL-C (1)	2.6

[a] Values indicate units (415 nm) by capture ELISA measured at 30 min.
[b] Phagotope also positive by direct ELISA.
[c] All 18 phagotopes with this sequence were highly reactive (absorbance>2.5).

paratope of mAb 3A9, phage clones 3A9/16, WHWTSAT and 3A9/22, HHWASSN from the pIII libraries were tested by direct ELISA for their capacity to inhibit the binding of mAb 3A9 to phagotopes carrying C-HASIYDFGS-C derived from the pVIII library. Absorbance values after the addition of mAb 3A9 to the phagotopes without the inhibitor phage were taken as 100%, and the absorbance values in the presence of inhibitor were expressed relative to this, so that the degree of inhibition was 100% minus reactivity in the presence of inhibitor. The positive control was inhibition of the reactivity of the phagotopes by the test phagotope itself, and the negative control was inhibition by an irrelevant phagotope with the sequence RRLPFGSQM (see Materials and methods). The experiment was repeated on four occasions, using 4–6 replicates of each inhibitor, with similar results. Results in Fig. 3 show that levels of inhibition obtained using pIII-selected phagotopes against the pVIII phagotope almost matched those of the positive control; similar results were obtained when the pVIII-selected phagotope was used as inhibitor of the reactivity of pIII-selected phagotopes. Inhibition with the negative control phagotope was negligible.

3.4. Prediction of epitope using phage displayed peptide sequences

The 17 phagotope sequences derived from the biopanning of the pIII libraries showed a much greater diversity in peptide sequence than those (two only) derived from the biopanning of the pVIII library. For the pIII libraries these 17 included the presence of histidine (H) and tryptophan (W) that occurred, respectively, in 15 and 14 of the 17 sequences, and a preponderance of the three related amino acids, alanine (A), serine (S) and threonine (T).

To predict which particular amino acids contributed to the epitope, we constructed a molecular model of CCR5, based on the crystal structure of bovine rhodopsin (Protein Data Bank accession, 1L9H) (Fig. 4). Amino acids in the N-terminal, first

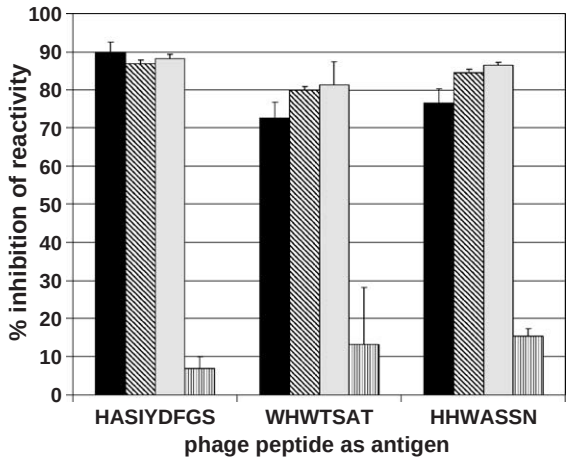

Fig. 3. Representative experiment showing percentage inhibition (mean and S.D. of four replicates) of mAb 3A9 reactivity by phagotopes. The prevalent phagotope isolated from the pVIII biopanning was strongly inhibited by addition of either of two phagotopes isolated from the pIII biopanning and vice versa, whereas an irrelevant control phagotope (see Materials and methods) had minimal inhibitory effect (pVIII phagotope HASIYDFGS, solid black; pIII phagotopes WHWTSAT and HHWASSN, cross-hatch and open; control phagotope RRLPFGSQM, vertical hatch).

and third extracellular loops that corresponded to those predicted from the phage display motifs were identified visually, with particular reference to the following considerations. (i) The observation of the same amino acids in multiple phage sequences would strongly suggest that the actual epitope contains identical amino acids rather than ones that are merely chemically similar; (ii) amino acids that would contribute to an epitope are likely to be surface exposed and within a diameter of 20 Å (Laver et al., 1990), and two residues adjacent in the phage sequence most likely indicate residues separated in the structure by no more than 5 Å; (iii) the predicted position of amino acids in the model would be most accurate near 'anchor points' for the extracellular loops at the termini of the transmembrane helices, the two disulfides, and highly conserved residues.

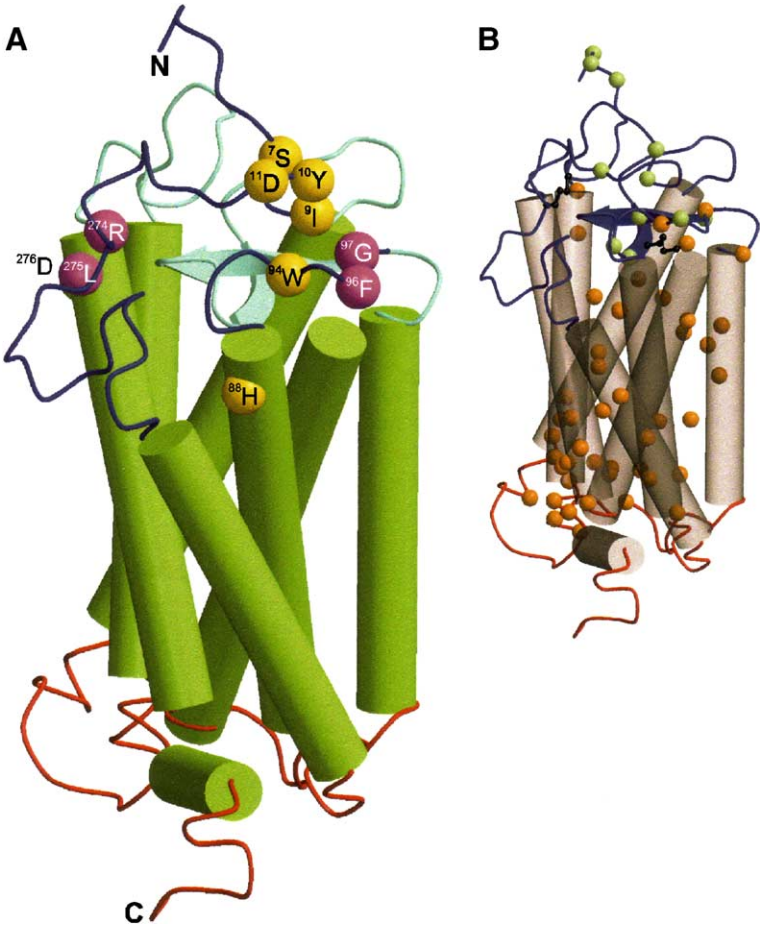

Fig. 4. Cartoon representations of the CCR5 molecular model, prepared using MOLSCRIPT (Kraulis, 1991), oriented with the extracellular domain towards the top of the page. (A) The residues mutated in this study are shown: yellow spheres indicate the predicted Cα positions of residues ascertained to contribute to the mAb 3A9 conformational epitope and magenta spheres indicate candidate residues for which mutagenesis had little effect on binding. Extracellular loops forming part of the epitope are coloured blue, those not identified as contributing to the epitope are cyan, and the intracellular region is shaded red. All residues identified as members of the conformational epitope occur in regions that could be modeled using the rhodopsin template. (B) The location of residues involved in distance restraints during homology model refinement are indicated; light green spheres highlight positions previously identified as contributing to conformational epitopes (Olson et al., 1999; Lee et al., 1999), orange spheres indicate conserved positions that form close-packing pairs, and the two disulfide bridges are shown in ball-and-stick. The extracellular region is coloured blue and the intracellular loops are red.

The motifs derived from the pIII library differed from those derived from the pVIII library. The pIII motifs included HW that was aligned visually to sequences $^{88}H^{94}W$ of the first extracellular loop. Several sequences that contained the C-terminal amino acids R, L, and D, in differing configurations, were of interest, since the motif HWLRDLR occurred in 15 of 19 phage clones obtained in a previous study (Königs et al., 2000) using the 5C7 mAb to CCR5; the LRD motif was provisionally aligned to $^{274}RLD^{276}$ of the third extracellular loop of CCR5 (Fig. 4). The predominant pVIII sequence included SIYDFG that could be aligned to $^{7}S^{9}IYD^{11}$ of the N-terminus and $^{96}FG^{97}$ of the first extracellular loop (Königs et al., 2000).

3.5. Mutagenesis of amino acids within peptide sequences identified by phage display

Based on the location of particular amino acids in the model, six alanine mutants were derived to test the contribution of 11 amino acids to the epitope. The substitutions involved $^{7}S^{9}IYD^{11}$ in the N-terminus, $^{96}FG^{97}$ in the first extracellular loop, $^{7}S^{9}IYD^{96}FG^{97}$ that combined the above two, $^{274}RLD^{276}$ in the third extracellular loop, and $^{88}H^{94}W^{274}RLD^{276}$ that combined mutagenesis of $^{274}RLD^{276}$ and $^{88}H^{94}W$. Mutagenesis of amino acids predicted to be part of the 3A9 epitope was carried out on a cDNA for CCR5. Both wild type CCR5 and CCR5 mutants were stably transfected in HEK293 cells and tested for reactivity with 3A9 by FACS analysis. The results of a representative experiment in which all mutants were tested together are shown in Fig. 5. Mutagenesis of $^{7}S^{9}IYD^{11}$ in the N-terminus, and mutagenesis of motifs $^{88}H^{94}W^{274}RLD^{276}$, reduced reactivity of 3A9 with CCR5 to less than 30% compared with wild-type, whereas mutagenesis of $^{96}FG^{97}$ in the first extracellular loop and $^{274}RLD^{276}$ in the third extracellular loop had no effect. Thus, the proposition that $^{88}H^{94}W$ is part of the 3A9 epitope, whilst well supported by the evidence, is not conclusively proven.

3.6. Biopanning phage libraries in pIII and pVIII with the mAb CII-C1 to type II collagen

To confirm that the results obtained by biopanning different libraries would be representative of results

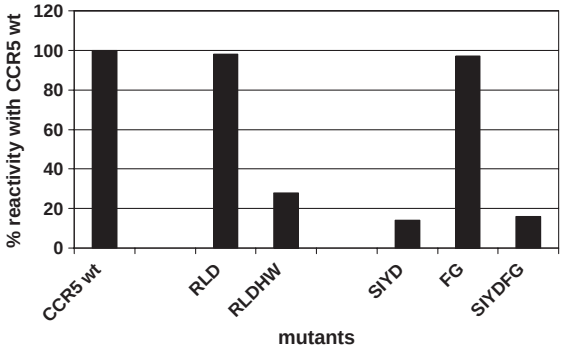

Fig. 5. Results of a representative experiment showing reactivity of mAb 3A9 with CCR5 mutants. Transfected HEK293 cells were labelled with mAb 3A9 and positive control mAb 2D7 (see Materials and methods) and analysed by FACS. Expression of mutant CCR5 was monitored by 2D7 labelling, for which the epitope was not altered by site-directed mutagenesis; for each experiment the values obtained with mAb 2D7 were set at 100% for all cell lines to standardize for CCR5 expression. The mean fluorescence measurements for mAb 2D7 labelling for this experiment were 17.52 for non-mutated CCR5, 20.12 for RLD, 13.44 for RLDHW, 19.43 for SIYD and 20.99 for SIYDFG. The columns show the level of labelling of cells transfected with various mutants of CCR5 expressed as a percentage of that of cells transfected with non-mutated CCR5.

obtained with other mAbs, we compared the selection of phagotopes from libraries in pIII and pVIII with another mAb, CII-C1. We performed two biopannings with libraries in pIII, using either the combined libraries from which most of the derived sequences were contributed by the 7mer linear library, or using only the 7mer library. Table 2 shows the sequences isolated using the 7mer library. Among the phagotopes isolated from either biopanning, none was reactive by direct ELISA, and the reactivity by capture ELISA varied. The peptide sequence STALPGL occurred six times among the most reactive peptides, and TATTWTG occurred twice. No clear peptide motif was identified and, whilst the amino acids ARGLT from the CII-C1 epitope occurred frequently, only threonine (T) was significantly increased above that expected (13% vs. 6%, p=0.047); the frequency of arginine (R) was actually decreased (3% vs. 9%, p=0.033).

Biopanning the combined linear and constrained pVIII libraries with CII-C1, described previously (Cook et al., 1998), yielded a selection of peptides that differed markedly from those obtained from the

Table 2
Sequences of phage clones isolated by biopanning with CII-C1: frequency and reactivity by capture ELISA

pIII library sequence (times selected)	Absorbance[a]	pVIII library sequence (times selected)
TWHDFPL	1.54±0.13	RRLPFGSQM (4)
GKWAPLF	1.33±0.02	RYAFGSQIA (2)
SALFMTP	1.33±0.02	RRLPFGSSL
IPLNTTM	1.15±0.01	RAGRFGYQR
VQGPWPM	1.0±0.07	TRSFGIQAT
WVQTPLT	0.94±0.24	SRLAFGDQL
SAQDWWQ	0.88±0.001	HEHTFGRQW
RPPAIYP	0.84±0.12	TRAFGNEAT
STALPGL (6)	0.82±0.33[b]	RAAPFGNQW
TVEHTPF	0.81±0.11	HRLAFGQNT
NISNEVY	0.78±0.01	HRLAFGQYT
SHMFATP	0.72±0.05	c-IAPKRHNSA-c
TATTWTG (2)	0.75±0.05	c-ESAQRPFGC-c
ELARQNP	0.7±0.06	
VDLLPRE	0.69±0.05	
TMLLPTK	0.58±0.10	
FPWHTQS	0.57±0.04	
NHSKAFW	0.56±0.06	
APERSPQ	0.51±0.004	
KLVTPSS	0.42±0.04	
NGLRLVT	0.39±0.019	

All sequences derived from the pVIII library were reactive by direct ELISA, and were not tested by capture ELISA.
[a] Mean±range for two determinations.
[b] S.D. of means for six isolates.

pIII libraries (Table 2). Of 18 phagotopes sequenced, 15 contained linear peptides, 2 cysteine-constrained peptides, and 1 contained a stop codon. All phagotopes that encoded a peptide were reactive by direct ELISA, and most contained two particular motifs, consisting of two basic amino acids and a hydrophobic residue in the first part of the insert, and the FGxQ or FGQ motif in the second part. From previous studies, including molecular modeling and using a second antibody that maps to precisely the same epitope (Cook et al., 1998; Davies et al., 1999; Xu et al., 2004), only the first motif was considered likely to represent the epitope, whereas the FGxQ motif was bound to the heavy chain CDR3 that would not be in contact with the collagen antigen. Although several amino acids occurred at higher frequency than expected, (A, 12% vs. 6%; F, 4% vs. 3%; Q, 9% vs. 6%; G 11% vs. 6%; R, 15% vs. 9%), none of the differences was statistically significant.

Even though the phagotopes selected from either the pIII or the pVIII libraries differed completely in sequence, they likely contained contact residues from the epitope, since phagotopes from each library clearly inhibited the reactivity of CII-C1 with type II collagen (Fig. 6A). The four phagotopes isolated from the pVIII library strongly inhibited the reactivity of CII-C1 to collagen (58–88%), and the pIII-derived sequences likewise gave significant, albeit weaker inhibition, with a mean of 34% for the phagotope STALPGL isolated six times. Mean inhibitions for phage con-

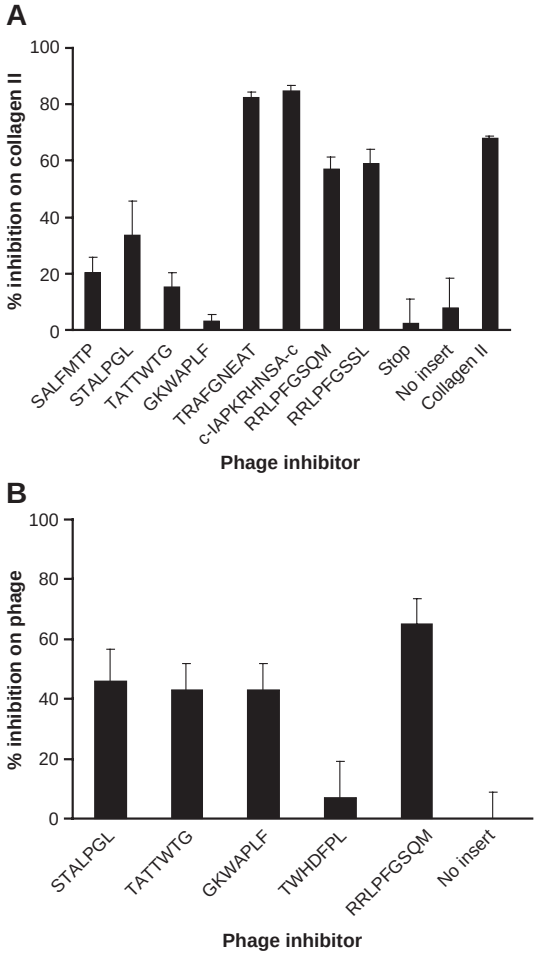

Fig. 6. Percentage inhibition (mean and S.D. of four replicates) of mAb CII-C1 with type II (collagen II) or with a phage clone RRLPFGSQM selected from the pVIII library. (A) Phagotopes selected from either the pIII library (SALFMTP, STALPGL, TATTWTG) or the pVIII libraries (TRAFGNEAT, cIAPKRHNSAc, RRLPFGSQM, RRLPFGSSL) inhibited the reactivity of CII-C1 with type II collagen. (B) Phagotopes selected from the pIII library (STALPGL, TATTWTG, GKWAPLF) inhibited the reactivity by direct ELISA of the phagotope RRLPFGSQM.

taining stop codons, or no inserts, were <1%, whereas intact type II collagen gave 68%. Also, as shown for the phagotopes selected with mAb 3A9, those derived from one library clearly inhibited the reactivity of CII-C1 with those derived from the other library (Fig. 6B).

4. Discussion

Our screening of phage libraries with mAb 3A9 to CCR5 was predicated on the idea that the epitope would be conformational, and that sequences of DNA inserts in phagotopes would reflect mimotopes of the antibody epitope. We drew on knowledge that CCR5 is a member of the rhodopsin family of 7-transmembrane G-protein coupled receptors and, by analogy with the crystal structure of the archetypal member bovine rhodopsin (Okada et al., 2000), would have seven transmembrane helices arranged in a circular fashion, three extracellular loops and an N-terminal 'tail'. In a previous study two mAbs to CCR5, 3A9 and 5C7, were used to screen a single nonameric cysteine-constrained phage peptide library expressed in the pVIII coat protein but, for 3A9, just a single high-affinity phagotope was obtained, even as early as the second round of positive selection. A synthetic peptide corresponding to the selected insert inhibited the binding of mAb 3A9 to CCR5-expressing cells and bound to HIV-1 gp120 (Königs et al., 2000). From the known overlap of the binding sites on CCR5 for mAb 3A9 and HIV-1 (Alkhatib et al., 1997; Wu et al., 1997) we proposed that the synthetic peptide derived from the phage library screening might inhibit infection of an HIV-1 susceptible cell line, but the single peptide initially identified failed to inhibit infection (Sonza, C., Königs, C., and Mackay, I.R., unpublished).

On the basis that the single phagotope isolated originally did not represent the complete epitope, a second screen with 3A9 was performed using libraries that present the phagotope in a different context. The peptide sequences of phagotopes derived by biopanning of the multiple phage libraries with mAb 3A9 were used to predict likely contact residues on the extracellular domains of CCR5, with reference to the primary sequence (Samson et al., 1996) and the homology model. The sequence C-HASIYDFGS-C that had been identified by biopanning from the pVIII library suggested contact residues on the N-terminus (^{7}S^{9}IYD^{11}) and first extracellular loop (^{96}FG^{97}) (Königs et al., 2000), whereas sequences of the peptide inserts derived from the combined pIII phage libraries contained the motif HW, and possibly the amino acids R, L, and D in differing configurations. HX_5W could be aligned to the first extracellular loop, and RLD to the third extracellular loop of CCR5. Alanine mutagenesis indicated that ^{7}S^{9}IYD^{11} from the N-terminus and ^{88}H^{94}W from the first extracellular loop contributed to the epitope.

We previously identified an RLD motif (as well as HX_5W) by screening the pVIII library with an alternative mAb to CCR5, 5C7 (Königs et al., 2000); however, by reason of the minimal effect that mutation of this motif had on the binding of 3A9, and the remoteness in the homology model of ^{274}RLD^{276} from other residues confirmed to contribute to the epitope, this motif was considered unlikely to form part of the 3A9 epitope. Also, mutation of the ^{96}FG^{97} residues did not measurably perturb antibody binding, despite their occurrence in close sequence proximity to residues that did form part of the epitope. Despite the lack of effect, these amino acids may contribute to the epitope, since X-ray crystallography has shown that epitope regions cover some 15–20 amino acids, whereas a much smaller number of residues, perhaps 5–8, may constitute critical contact residues that contribute most of the binding energy (Rowley et al., 2004). The point is that phage display can provide at least an approximate localisation of an epitope on the surface of an antigen based on knowledge of the critical contact residues, and the typical epitope dimension of 700 Å^2; even use of multiple libraries cannot reveal every component of a conformational epitope nor exclude the possibility that residues of the parental antigen not revealed by multiple library biopannings also contribute to the epitope. Such information is obtainable only by knowledge of the shape of the paratope of the antibody revealed by crystallography.

The utility of multiple libraries was verified by the capability of phage clones from the two libraries to cross-inhibit reactivity with the selecting antibody 3A9, indicating that each reacted with the antibody paratope; thus amino acids recognized by the product of each biopanning contributed to the epitope of 3A9. This is consistent with the observation that these regions have been found, by alanine scanning mutagenesis of a different epitope, to occur in close

proximity to one another (Olson et al., 1999). Moreover, these would be predicted from the homology model to be surface exposed and occur within the diameter spanned by an antibody epitope.

The observation that biopanning of multiple phage libraries that have different characteristics can provide additional information was confirmed using another system, the mAb CII-C1. Thus, with phagotopes with quite different motifs selected from peptide libraries in the pIII and pVIII coat proteins, phagotopes from each library were shown to inhibit binding of CII-C1 with the "parental" antigen, type II collagen, and were also cross-inhibitory. The structure of the C1 epitope has been modeled (Xu et al., 2004), and the amino acids A, R, L and T found in the linear sequence can be aligned on the surface of the helix, such that an antibody paratope could contact dimers of these amino acids. The phagotopes from the pIII library contained dimeric configurations of TT, LL and LT, and arginine occurred rarely. By contrast, phagotopes from the pVIII libraries contained the motif RRL, and also a clear motif FGQ for which the binding site is located on the CDR3 region of CII-C1 that is unlikely to contact the collagen helix (Cook et al., 1998). These results suggest that differences in the peptides selected from different libraries are related to the different configuration of the peptides on the phage particle.

The present study demonstrates the utility of phage display, when combined with structural information about an epitope, to predict a small number of possible contact residues for antibody binding; such residues can then be confirmed by mutagenesis. As phage display has been used here to identify amino acids representing critical contact residues for antibody binding, rather than to select optimum mimotope(s), we used some technical shortcuts to reduce the volume of work required. Thus, preliminary studies revealed that the titre of phage particles in preparations obtained at the same time were similar (data not shown), so that phagotopes were tested for reactivity with mAbs by ELISAs, and in inhibition assays, at a single dilution using a standard volume of phage that contained an excess of phage particles, rather than by titration of the number of phage particles. Also, to determine whether phagotopes derived from different libraries reflected the same epitope, we studied cross-inhibition with phagotopes rather than with peptides. Whilst inhibition might occur due simply to steric hindrance from the large phage particle binding to regions other than the paratope, it is our experience (unpublished) that after negative selection with IgG (or IgG-coated beads) during the biopanning, there are selected very few phage particles that do not react with the Fv-region of the antibody. Hence the amino acid motifs identified using the different libraries were considered as potential contact residues for further assessment by homology modeling and mutagenesis.

In conclusion, our results indicate that identification of a conformational epitope using phage display is indeed feasible, but can require the generation of a panel of reactive phagotopes derived from different types of peptide presentation in phage coat proteins. For two exemplary mAb, 3A9 to CCR5, and CII-C1 to type II collagen, the combination of two different sets of phage libraries was needed to ascertain the epitope. This, for CCR5, comprised disparate contact residues in the N-terminus (^{7}S^{9}IYD^{11}) and in the first extracellular loop (^{88}H^{94}W, and possibly at lower affinity binding ^{96}FG^{97}), as shown in Fig. 4. Among the practical implications of our CCR5 finding, the derivation of appropriate mimotope peptide(s) by phage display could facilitate the design of small molecular antagonists that could inhibit entry of HIV-1 into susceptible cells.

Acknowledgements

Prof. Charles Mackay and Dr. Li-Jun Wu provided mAb 3A9 derived at LeukoSite Inc., Cambridge MA, Prof. Rikard Holmdahl, Lund University, Lund, Sweden provided mAb CII-C1, and Dr. Alessandra Luzzago, Instituto di Richerche di Biologia Molecolare (IRBM), Rome, Italy provided the libraries in pVIII. We thank Dr. Mark Myers, Dr. Janet Davies and Prof. Charles Mackay for their collaborative interest, and Ms. Marita Scealy and Ms. Margot Landersz for technical advice and assistance.

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