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Cyclic Peptides Selected by Phage Display Mimic the Natural Epitope Recognized by a Monoclonal Anti-colicin A Antibody

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Abstract: A 10-mer random peptide library displayed on filamentous bacteriophage was used to determine the molecular basis of the interaction between the monoclonal anti-colicin A antibody 1C11 and its cognate epitope. Previous studies established that the putative epitope recognized by 1C11 antibody is composed of amino acid residues 19–25 (RGSGPEP) of colicin A. Using the phage display technique it was confirmed that the epitope of 1C11 antibody was indeed restricted to residues 19–25 and the consensus motif RXXXPEP was identified. Shorter consensus sequences (RXXPEP, RXXEP, KXXEP) were also selected. It was also demonstrated that the disulfide bond found in one group of the selected peptides was crucial for 1C11 antibody recognition. It was shown that cyclization of the peptides by disulfide bond formation could result in a structure that mimics the natural epitope of colicin A. Copyright © 2004 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: phage display; decapeptide library; epitope mapping; disulfide-linked peptide

INTRODUCTION

Antibody molecules bind their antigens with high affinity and specificity using multiple non-covalent forces. Analysis of tridimensional structures of antibody fragment–protein antigen complexes reveals that the antibody binding site can recognize either a conformational epitope or a continuous epitope. Conformational epitopes usually occupy large areas comprising amino acid residues on several polypeptide segments distant in the primary sequence of the antigen, whereas continuous epitopes, also called sequential or linear epitopes, occupy smaller areas comprising residues adjacent in the primary sequence [1,2]. In general, continuous epitopes can be recognized by antibodies in native as well as in denaturated proteins.

Continuous epitopes can be identified by screening antigen-derived peptide fragments to identify the

Abbreviations: ABTS, [2, 2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid), diammonium salt]; BPB, biopanning buffer; BSA, bovine serum albumin; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography; mAb, monoclonal antibody; PBS, phosphate-buffered saline; scFv, single-chain variable fragment; TFA, trifluoroacetic acid; TU, transducing units.

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minimal reactive sequence segment [3]. This usually involves the use of libraries consisting of either synthetic peptides [4] or cDNA fragments expressed *in vitro* [5] or *in vivo* [6]. In the past decade, however, new biomolecular techniques have been described to generate large libraries of peptides [7–9]. One of these, the phage display technique, has been shown to be a powerful method for the selection of peptide mimotopes that mimic a given epitope [10–13]. This technology is based on the fact that the genome of filamentous bacteriophage can be engineered to encode for a random peptide and display it on its surface. The libraries are a vast mixture of many millions of filamentous bacteriophages and are screened by repeated rounds of selection and amplification.

Phage-peptide libraries have been used successfully to identify specific amino acids involved in protein–protein interactions [14,15] and in protein–nucleic acid interactions [16]. It has also been possible to isolate peptide ligands that bind to various receptors [17–21] and to streptavidin [22,23]. Other isolated peptides have been described such as toxins or enzyme inhibitors [24–26], new protease substrates [27] and an estrogen-like peptide [28].

Colicin A is a bacteriotoxin produced in the cytoplasm of *Escherichia coli* cells and then released into the extracellular medium. This toxin kills sensitive *E. coli* cells by forming voltage-gated ion channels in cytoplamic membranes. Its mode of action involves binding to a specific receptor located in the outer membrane of sensitive bacteria, translocation across the outer membrane and, finally, insertion into the inner membrane to form a voltage-gated ion channel [29]. Fifteen monoclonal antibodies directed against colicin A have been isolated [30]. One of them, the 1C11 mAb, which recognizes the *N*-terminal domain involved in the translocation step, has been extensively used to study the structure–function relationship and the mode of action of colicin A. It was expressed as a single-chain variable fragment (scFv) in bacteria in order to inhibit *in vivo* the lethal activity of colicin A [31]. The scFv exported into the periplasmic space prevents the translocation of colicin A and probably its interaction with the import machinery. Analysis of the structure of the epitope recognized by the 1C11 mAb could allow us to develop inhibitors of this interaction.

Previous results showed that the binding site of 1C11 mAb is localized within the first 70 *N*-terminal residues of colicin A [30] (Figure 1). The putative epitope is restricted to the first 30 *N*-terminal residues of the protein [32] (and data not shown) and is localized between residues 13–26 (TGWSSERGSGPEPG) [33]. Further, the reactivity of 1C11 mAb was assayed by Pepscan analysis against heptapeptides covering residues 8–26 of colicin A, and the results suggested that the antigenic determinant spanned amino acid residues 19–25 (RGSGPEP) [33]. Moreover, when residues 8–21, 17–30 and 13–26 were fused to the PhoE protein to expose these peptides on the bacterial cell surface, only the PhoE protein carrying residues 13–26 reacted with 1C11 mAb in Western blot analysis. Surprisingly, the PhoE protein carrying residues 17–30 did not react with 1C11 mAb. This result indicated that residues 13–16 could be important for proper exposure of the epitope in a protein context. All these results indicate, however, that the epitope is not very clearly defined.

In the present study, a random decapeptide library displayed on phage was used to determine the molecular basis of the interaction between the anti-colicin A 1C11 mAb and its epitope. The screening of a random decapeptide library with the 1C11 antibody indicated that the sequence RXXXPEP was the consensus motif, highlighting a primordial role of these residues in the antigen–antibody interaction. It was also shown that disulfide-linked

1 15

Met Pro Gly Phe Asn Tyr **Gly Gly Lys Gly Asp Gly Thr Gly Trp**

16 30

Ser Ser Glu Arg Gly Ser Gly Pro Glu Pro **Gly Gly Gly** Ser His

31 **35**

Gly Asn Ser Gly Gly

Figure 1 Amino acid sequence of the *N*-terminal region of colicin A. Numbers refer to the position of the residues in the colicin A molecule. The background of the 22colA and 13colA colicin A fragments cloned in fUSE5 are in bold and underlined, respectively. The wild-type decapeptide is boxed.

peptides, although having a consensus sequence shorter than the natural peptide epitope, could bind to the 1C11 antibody with similar affinity.

MATERIALS AND METHODS

Monoclonal Antibody

The 1C11 anti-colicin A mAb (IgG2a, κ) was isolated either as tissue culture supernatants or ascitic fluids as previously described [30] and was purified by protein A affinity chromatography [34] (HiTrap Protein A column, Amersham Pharmacia Biotech, Uppsala, Sweden).The purified mAb was biotinylated according to the manufacturer's instructions (Biotin Protein Labeling Kit, Roche, Basel, Switzerland). The average number of biotin molecules per mAb molecule was eight. The biotinylated 1C11 mAb was used for selection of phage peptides.

Cloning of the Wild-type Colicin A Peptides

The 22colA-phage was constructed by the insertion of paired oligonucleotides (5′ GTAGTGGTGGAGGTGGAAAAGGTGATGGAACCGGCTGGAGCTCAGAACGTGGGAGTGGTCCAGAGCCGGGTGGTGGTGGTGCCCCTTCTG 3′ and 5′ AAGG GGCACCACCACCACCCGGCTCTGGACCACTCCCACGTTCTGAGCTCCAGCCGGTTCCATCACCTTTTCCACCTCCACCACTACCGT 3′) encoding for the polypeptide corresponding to glycine 7 to glycine 28 of the colicin A inserted into the fUSE5 vector between the *SfiI* sites [7]. In the same way, the 10colA-phage was constructed using paired oligonucleotides (5′ GTAGTGGTGGAAGCTCAGAACG TGGGAGTGGTCCAGAGCCGGGTGCCCCTTCTG 3′ and 5′ AAGGGG CACCGGCCTCTGGACCACTCCCACGTTCTGAGCTTCCACC ACTACCGT 3′) encoding for the polypeptide corresponding to serine 16 to proline 25.

Phage-peptide Production

The *E. coli* strain TG1 (*Δ(lac-pro)*, *supE*, *thi*, *hsdD5/F′*, *traD36*, *proAB*, *lacIq*, *lac ZΔM15*) was used as the bacterial host for the bacteriophage. Infective cells were prepared by inoculating 10 ml of 2YT broth with 100 µl of an overnight culture of *E. coli* TG1 and shaking vigorously for 3 h at 37 °C. Cells were transferred to a 37 °C incubator without shaking for 10 min and then stored at room temperature and used within 1 h. For phage-peptide production, TG1 cells were grown in LB broth containing tetracycline at 20 µg/ml overnight at 30 °C. Phage-peptide titration, amplification and purification were performed as previously described [35].

Selection of 1C11 mAb-binding Phage-peptides

The construction of the decapeptide library and the selection of antibody-binding phages were previously described [36]. Briefly, binding phage-peptides were isolated from the decapeptide library by successive cycles of selection and amplification. All incubations were performed in siliconized Eppendorf tubes at room temperature in biopanning buffer [BPB : PBS (1.8 mM KH_2PO_4, 1.2 mM Na_2HPO_4, 137 mM NaCl and 2.7 mM KCl, pH 7.5), 0.1% (w/v) BSA (Sigma-Aldrich, Inc.), 0.1% (v/v) Tween 20 (Sigma-Aldrich, Inc.) and 0.02% (w/v) NaN_3 (Sigma-Aldrich, Inc.)], with gentle shaking. Fifty μl of streptavidin magnetic beads (BioMag Streptavidin, Perseptive Biosystems, Framingham, MA) were washed three times with 500 µl BPB and incubated with 15 µg of biotinylated 1C11 mAb in a final volume of 100 µl. After 1 h, 1 µl of 10 mM D-biotin (Sigma-Aldrich, Inc.) was added for 15 min. After three washes with 500 µl BPB, the beads were incubated with 10 µl of the decapeptide library [4×10^{10} transducing units (TU)] for 4 h. The beads were washed seven times with 500 µl BPB and transferred three times to new siliconized Eppendorf tubes (tubes 1, 3 and 6). Bound phage-peptides were eluted with 100 µl of 0.1 M HCl/glycine pH 2.2, 1 mg/ml BSA, immediately neutralized with 16 µl of 2 M Tris base and used to transfect infective bacteria. After this elution, the beads were washed three times with 500 µl BPB and the remaining phage-peptides were eluted directly by adding infective cells. For the second round of selection, 4×10^9 TU from the first round were used.

ELISA Screening of Clones

Single ampicillin-resistant colonies, resulting from infection of *E. coli* TG1 with eluted phage-peptides were used to inoculate 150 µl of 2YT medium containing tetracycline at 20 µg/ml in 96-well microtitre plates (Nunclon, Nunc, Denmark). After 20 h of growth, the supernatants containing phage-peptides were tested for binding by enzyme-linked immunosorbent assay (ELISA) in Falcon 96-well plates (Becton Dickinson, Oxnard, CA) coated overnight with purified 1C11 mAb at 10 µg/ml in PBS and saturated with 2% (w/v) skim milk in PBS

(PBS-2% milk). Phage-peptide binding was detected with a horseradish peroxidase-labelled anti-M13 antibody conjugate (Amersham Pharmacia Biotech, Uppsala, Sweden).

Phage-peptide Binding Analysis by ELISA

The microplates (96-wells, Maxisorp, Nunc, Denmark) were coated with 1C11 mAb (100 µl per well at 10 µg/ml in PBS) overnight at 4 °C, followed by incubation for 1 h at room temperature with PBS-2% milk to saturate unoccupied sites in the wells. The microplates were washed twice with 0.1% (v/v) Tween PBS (PBS-Tween 0.1%) and then twice with PBS. PEG purified phage-peptides were diluted in PBS-2% milk ranging from 1 : 1 to 1 : 200 and added to the wells and placed on a rotator for 1 h at room temperature. The wells were washed four times with PBS-Tween 0.1% and four times with PBS; next 100 µl of a 1 : 5000 dilution (in PBS-2% milk) of horseradish peroxidase-labelled anti-M13 monoclonal conjugate (Amersham Pharmacia Biotech, Uppsala, Sweden) was added to each well. The microplates were incubated for 1 h at room temperature and extensively washed four times with PBS-Tween 0.1% and four times with PBS. The peroxidase activity was determined using ABTS as substrate followed by measurement of absorbance at 405 nm. Each assay was done in triplicate, and the signal given by the blank well was subtracted.

Competitive Binding Assays using Synthetic Peptides

In competitive binding assays, the peptides used as competitors were diluted in PBS-2% milk with phage-peptides and incubated for 2 h at room temperature, before being added in the wells. The concentration range of each peptide depended on the experiment. The binding curve of the percentage of phage-peptide binding (determined by the adsorbance at 405 nm) versus peptide concentration gave the apparent IC_{50} (concentration of peptide which causes a 50% inhibition of phage-peptide binding to 1C11 mAb). In competitive binding assays using DTT, DTT was added to the phage-peptide and cysteine peptide mixture in PBS-2% milk at concentrations ranging from 1 to 5 mM. The binding curve of phage-peptide binding (%) versus DTT concentration allowed the determination of DTT sensitivity of the phage-peptides and the cysteine peptides. Each assay was done in triplicate, and the signal given by the blank well was subtracted.

Sequence Determination of Displayed Decapeptides

Sequencing was performed using the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (USB, Amersham, UK) with the following oligonucleotides for annealing: 5′ CCCTCATAGTTAGCGTAACG 3′ and 5′ TATTGCCTACGGCAGCCGCT 3′.

Synthetic Peptides

Peptides were synthesized in Fmoc chemistry by the stepwise solid-phase methodology using a multichannel peptide synthesizer [37]. Protected amino acids were coupled by *in situ* activation with (benzotriazol-1-yloxy)tris-(dimethylamino)phosphonium hexafluorophosphate and *N*-Fmoc deprotection was performed as previously described [37]. Side chain deprotection and cleavage of peptides from the solid support was performed by treatment with reagent K (82.5% TFA, 5% phenol, 5% water, 5% thioanisole, 2.5% 1,2-ethanedithiol) for 2 h 30 at 20 °C [38]. Peptides were purified by reversed-phase HPLC using a Perkin-Elmer preparative HPLC system on an Brownlee Prep-10 ODS 15 µm column (100 × 10 mm). They were eluted with a linear gradient of aqueous 0.1% TFA (A) and 0.08% TFA in 80% acetonitrile, 20% water (B) at a flow rate of 6 ml/min with UV detection at 220 nm. The purity of each peptide was controlled by analytical RP-HPLC on a Beckman instrument (Gagny, France) with a Nucleosil C18 5 µm column (150 × 4.6 mm) using a linear gradient of 0.1% TFA in water and acetonitrile containing 0.08% TFA at a flow rate of 1.2 ml/min. Finally, the integrity of each peptide was assessed by Matrix-assisted laser desorption and ionization time-of-flight (MALDI-Tof) spectrometry on a Protein TOF™ mass spectrometer (Bruker, Wissembourg, France).

RESULTS

Selection of the Phage-peptides

The selection of the decapeptide library with 1C11 mAb allowed the selection of 48 phage-peptides displaying decapeptides mimicking the RGSGPEP motif of colicin A (Figure 1), reported to be the minimal antigenic determinant [33]. The sequence alignment of 20 displayed peptides is shown in

Table 1. The peptides were classified into five consensus groups, depending on their amino acid sequences: RXXXPEP, RXXPEP, RXXEP, KXXEP and peptides containing two cysteine residues (cysteine peptides). RXXPEP and the cysteine peptide groups represented, respectively, 27% and 58% of the total sequenced clones. On the basis of the sequence alignment, arginine or lysine, glutamic acid and proline residues were identified as being essential residues of the epitope for the binding of the peptide to 1C11 mAb. Arginine was found in most of the peptides or was sometimes substituted by a lysine in the KXXEP peptides and the cysteine peptide FR. The glutamic acid residue was always preceded by a proline. The PEP motif was localized at three (RXXXPEP), two (RXXPEP) or one residue (for the cysteine peptide LL) downstream from arginine. For some peptides, the first proline was missing, and the EP motif was localized at two residues downstream from an arginine (RXXEP) or a lysine (KXXEP). This EP motif was also found in four cysteine peptides at one residue downstream from an arginine or a lysine. These data suggest that the arginine or lysine residues as well as the PEP or EP motifs are essential for binding to the 1C11 mAb paratope.

In the RXXXPEP consensus sequence, the X residues were different, except that an aromatic residue (tyrosine or phenylalanine) was found just upstream from the PEP motif. For the RXXPEP peptide group, the first X was a hydrophobic residue and the second X was a hydrophilic residue. Concerning the RXXEP motif, a phenylalanine was found downstream from the arginine in three of the four peptides and the second X was a hydrophobic residue (isoleucine or valine).

In the KXXEP peptides, the first X residue was always a tyrosine and the second X was a hydrophobic residue (isoleucine or leucine). Surprisingly, in the cysteine peptide group, only one small hydrophilic or hydrophobic residue separated the arginine or the lysine from the PEP or EP motifs. Moreover, for these peptides, the consensus sequence was very short (RXPEP, RXEP, KXEP) in comparison with the wild-type sequence of colicin A. Nevertheless, the phages displaying peptides efficiently mimicked the colicin A epitope. These results suggest that the X residues might not be directly involved in epitope recognition; however, the nature of these residues is certainly important and adapted to correctly display the consensus motif to the 1C11 mAb binding site.

The apparent affinity (IC_{50}, Table 1) of all the phage-peptides was measured by competitive ELISA using the 22colA synthetic peptide (Table 2). This affinity corresponds to the ability of the 22colA peptide to compete with the phage-peptides for binding to the 1C11 mAb.

Table 1 Selected Phage-peptide Sequences and the IC_{50} Determined by ELISA Using the 22colA Peptide

Phage[a]	Sequence	Apparent IC_{50} (nM)
fd-tet phage:	ADGSGG- - - - - - -GAPSG	NB
ColA wild-type:		
22colA	GGKGDGTGWSSERGSGPEPGGG	24
10colA	SSERGSGPEP	NB
RXXXPEP:		
EP (1)	ERVREYYPEP	20
SP (1)	SSMRKSFPEP	19
RXXPEP:		
GG (6)	GRIAPEPGGG	4
WP (1)	WGNARKSPEP	7
RA (1)	RVNPEPGRWA	11
HR (3)	HITRLSPEPR	3
RH (1)	RMRIDPEPRH	9
VP (1)	VPRVRWSPEP	6
RXXEP:		
PR (4)	PRFIEPRVSR	6
WG (1)	WVSLRQVEPG	14
PT (1)	PRFVEPRKET	7
PS (1)	PRFIEPEGVS	5
KXXEP:		
KR (1)	KYIEPGLRVR	7
KM (1)	KYIEPRFLAM	16
KG (1)	KYLEPWYACG	6
Cysteine peptides:		
LL (6)	LRCPEPGCLL	14
CR (12)	CRTEPGCYVR	16
CC (1)	CSSRVEPRAC	28
AP (1)	ACRSEPRCFP	40
FR (3)	FCKAEPLCAR	23

[a] The number in brackets indicates the number of selected phages. NB: no binding.

Binding Specificity of Selected Phage-peptides

The wild-type epitope containing 22 or 10 residues of colicin A was cloned in the fUSE5 vector in order to obtain the 22colA- and 10colA-phages. These control phage-peptides expressed on their surface the peptides fused to the *N*-terminus of the phage protein 3. Surprisingly, the 22colA-phage but not the 10colA-phage was able to bind to the 1C11 mAb (Table 1). Hence, the wild type 22colA peptide was synthesized (Table 2) and used as a competitor in ELISA tests in order to determine the binding efficiency of the selected phage-peptides. The apparent IC_{50} of all selected phage-peptides

Table 2 Synthetic Peptides

Peptide	Sequence	M_r (theoretical)	M +H $^+$ (found)	HPLC (t_R min)
22colA	ADGSGG**GGKGDGTGWSSERGSGPEPGGG**GAPSG	2802.78	2804.93	13.57
13colA	ADGSGG**TGWSSERGSGPEP**GAPSG	2160.15	2161.65	11.87
10colA	ADGSGG**SSERGSGPEP**GAPSG	1815.78	1817.07	11.48
EP	ADGSGG**ERVREYYPEP**GAPSG	2151.23	2152.68	11.34
GG	ADGSGG**GRIAPEPGGG**GAPSG	1723.78	1724.36	11.22
PR	ADGSGG**PRFIEPRVSR**GAPSG	2070.25	2071.66	10.80
KR	ADGSGG**KYIEPGLRVR**GAPSG	2044.25	2045.87	10.98
LL	ADGSGG**LRCPEPGCLL**GAPSG	1914.14	1914.96	14.27
CC	ADGSGG**CSSRVEPRAC**GAPSG	1921.05	1922.84	11.27

The colicin A and the decameric mimotope amino acids (indicated in bold capital letters) are flanked by the residues recovered in the phage context.

was between 3 and 40 nM (versus 24 nM for 22colA-phage) and was determined by measuring the 22colA peptide concentration required to inhibit 50% of the signal of the phage-peptide binding to the 1C11 mAb (Table 1, Figure 2A and 2B, and data not shown).

The EP- and SP-phages displaying the motif RXXXPEP, that exhibits the best sequence homology with the colicin A epitope RGSGPEP, had approximately the same apparent IC_{50} as the 22colA-phage, although they displayed a shorter amino acid sequence (only 10 residues). Surprisingly, the RXXXPEP motifs displayed by the EP- and SP-phages were efficiently recognized by the 1C11 mAb, whereas the 10colA-phage displaying the SSERGSGPEP sequence was not.

The phage-peptides belonging to the RXXPEP, RXXEP or KXXEP groups were inhibited by a 22colA peptide concentration lower than the concentration required to inhibit the 22colA-phage. This suggests that the binding of these phage-peptides to the 1C11 mAb was weaker than that of the 22colA-phage. The decrease in the length of the motif between arginine or lysine and PEP, or the absence of the first proline residue (upstream from the glutamic acid residue) might explain the decrease in binding activity.

Concerning the apparent IC_{50} values of the cysteine phage-peptides inhibited by the 22colA peptide, the CC- and FR-phages had an apparent IC_{50} value similar to that of the 22colA-phage, whereas the LL- and CR-phages had weaker apparent IC_{50} values than the 22colA-phage. The AP-phage had the highest apparent IC_{50} value, suggesting that the displayed peptide bound more efficiently to the 1C11 mAb than did the 22colA-phage. The presence of the two cysteine residues in the cysteine peptides can probably explain why relatively short peptides (RXPEP or R/KXEP) were selected. Interestingly, the peptides had an apparent IC_{50} value similar to that of the wild-type peptides. The cysteines probably play an important structural role in the RXPEP and R/KXEP motifs.

Inhibition of Phage-peptide Binding by Specific Peptides

In order to further characterize the interactions between the 1C11 mAb and mimotopes, some peptides selected by phage display and wild-type peptides (22colA, 13colA and 10colA) were synthesized (Table 2). They were used in a competitive ELISA format to measure their ability to inhibit the binding of 22colA-phage to the 1C11 mAb (Figure 3). Figure 3A shows that the apparent IC_{50} values of the 22colA and 13colA peptides were 24 nM and 57 nM, respectively. Interestingly, the 10colA peptide did not inhibit the binding of the 22colA-phage (Figure 3A). These data suggest that the length of the peptide is a key factor in its ability to impair the binding of 22colA-phage to the 1C11 mAb.

Figure 3B shows the binding curves of the selected peptides. Interestingly, in contrast to the 10colA peptide, all the selected 10-residue peptides were able to impair 22colA-phage interaction with the 1C11 mAb. Based on the apparent IC_{50} values, the peptides were divided into two groups. In the first group, the LL, CC (peptides containing two cysteines) and the EP peptide (RXXXPEP) had an

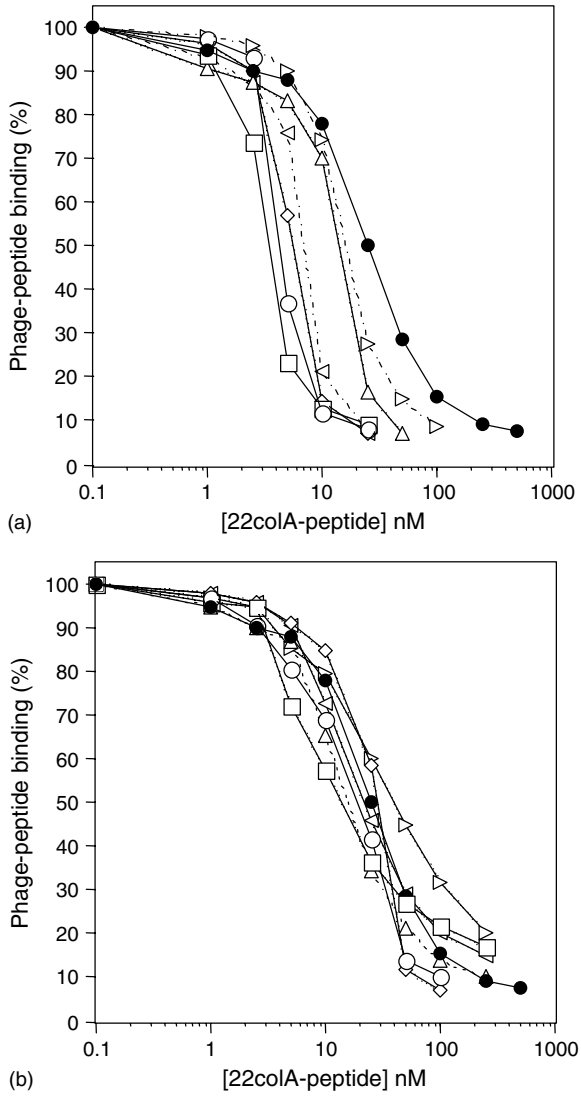

Figure 2 Competition of phage-peptides with the wild type 22colA-peptide. The 1C11 mAb, adsorbed onto microwells, was incubated with the phage-peptides (A) 22colA (●), GG (○), HR (□), WG (△), PR (◊), KM (▷), KR (◁) and (B) 22colA (●), EP (○), LL (□), CR (△), CC (◊), AC (▷), FR (◁) and serial dilutions of the 22colA-peptide. For each measurement, the standard deviation never exceeded 5%.

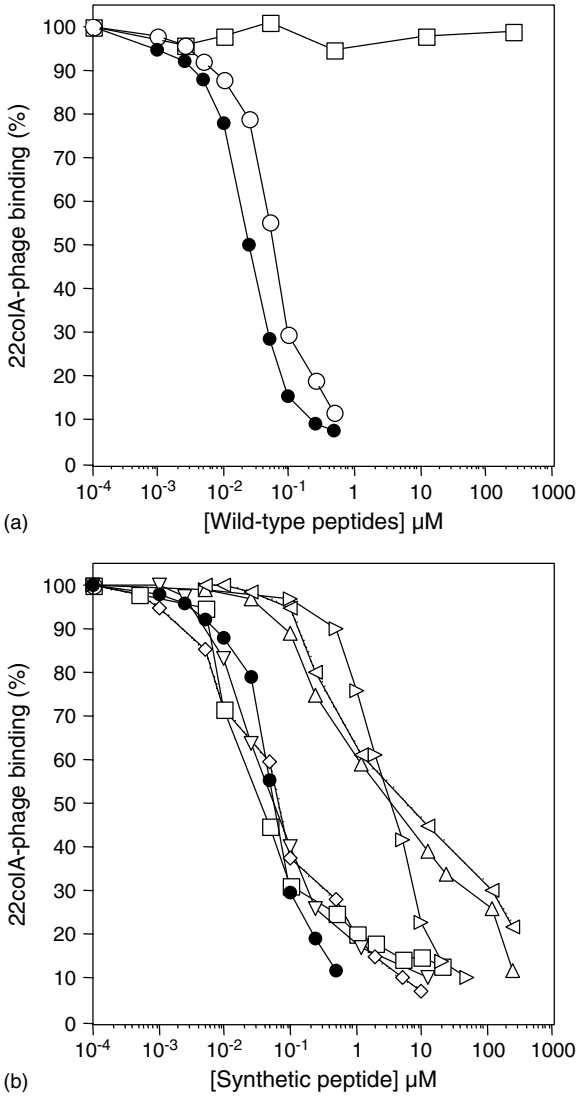

Figure 3 Competition of 22colA-phage with synthesized peptides. The 1C11 mAb, adsorbed onto microwells, was incubated with the 22colA-phage and serial dilutions of (A) the 22colA (●), 13colA (○) and 10colA (□) wild type peptides and (B) the 13colA (●), EP (▽), KR (◁), PR (△), GG (▷), LL (□) and CC (◊) peptides. For each measurement, the standard deviation never exceeded 5%.

apparent IC_{50} value of 40, 50 and 57 nM, respectively. These apparent IC_{50} values were comparable to those of the 22colA and 13colA peptides and indicate that the LL, CC and EP peptides exhibited the strongest binding to the 1C11 mAb. In the second group, the GG (RXXPEP), PR (RXXEP) and KR (KXXEP) peptides had higher apparent IC_{50} values (3500, 3600 and 6200 nM, respectively) (Figure 3B).

Disulfide-linked Peptides

The presence of two cysteines in five of the peptides suggests that these residues might be very important for peptide conformation. It was hypothesized that the cysteine residues form a disulfide bond, required to display the epitope to the 1C11 mAb. ELISA experiments were performed in the presence of DTT with the LL-phage, which contains two cysteines, and with the EP-phage,

which is devoid of cysteine, as a control. The results showed that the binding of LL-phage was sensitive to DTT (Figure 4A), whereas the efficiency of the binding of the EP-phage to the 1C11 mAb was not altered by the DTT concentrations tested. The efficiency of the binding of the EP-phage was always 100%, corresponding to the maximal ELISA signal intensity obtained for this phage-peptide at the concentration used. These data indicate that the presence of DTT did not affect the 1C11 structure, or at least not the 1C11 paratope structure, and consequently did not affect phage-peptides binding to the 1C11 mAb. The same sensitivity to DTT was observed for all the phage-peptides containing cysteine residues (CR, CC, AP and FR) (data not shown). These results clearly indicate that a disulfide bond was formed between the cysteine residues and was necessary for the binding of the mimotopes to 1C11 mAb.

In order to confirm the presence of the disulfide bond in the synthesized LL peptide, a competition ELISA was carried out with the 22colA-phage in the presence of DTT and with or without the LL peptide (at the concentration at which the LL peptide inhibited 50% of the 22colA-phage binding) (Figure 4B). The results showed that the presence of DTT (above 200 nM) prevented the LL peptide from inhibiting the binding of 22colA-phage to the 1C11 mAb. As a control, the same experiment performed in the absence of LL peptide demonstrated that the presence of DTT did not alter the binding ability of 22colA-phage (Figure 4B). The same results were obtained with the CC peptide (data not shown). These results indicate that the disulfide bond is a key structural element and allows the cysteine peptides to acquire the proper conformation for binding to the 1C11 mAb.

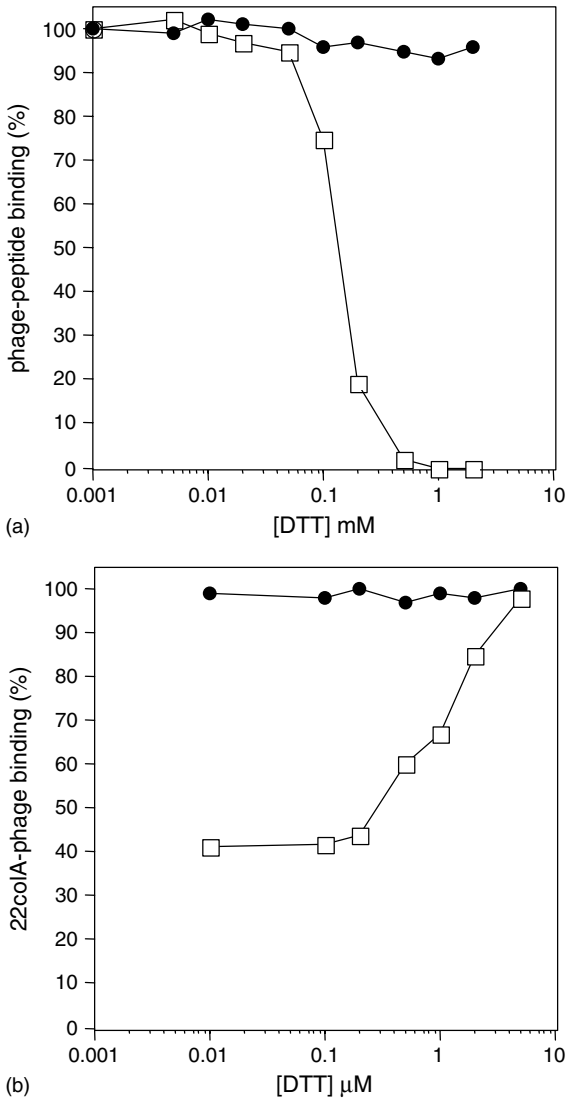

Figure 4 Competition assays with cysteine peptides. The 1C11 mAb, adsorbed onto microwells, was incubated with (A) the EP-phage (●), the LL-phage (□) and serial dilulions of DTT or (B) the 22colA-phage with (□) or without(●) the LL synthetic peptide exposed to different concentrations of DTT. For each measurement, the standard deviation never exceeded 5%.

DISCUSSION

A random decapeptide library displayed on phage was used to further characterize the molecular interactions of the 1C11 mAb with colicin A. The results confirm the fact that the epitope of the 1C11 antibody consists of residues 19–25 of colicin A and establishes the basis of molecular recognition of the 1C11 antibody.

Library Selection

A decapeptide library expressed on the minor coat protein 3 of an fd-derived phage was used. The phage-peptides were screened for their ability to bind to the 1C11 mAb. After two rounds of selection/amplification, positive clones selected by an immunological assay were sequenced, and their relative affinities were compared with that of the natural epitope. Twenty different sequences were obtained and divided into five groups (RXXXPEP, RXXPEP, RXXEP, KXXEP and the cysteine peptide group). The alignment of the consensus sequences

showed that the positively charged residues, arginine (R) or lysine (K), followed by one to three variable residues X and then by an EP or PEP motif were essential for the binding to 1C11 mAb paratope (Table 1). On the basis of sequence alignment, it was observed that the X residues varied considerably. However, in the KXXEP group the nature of the X residues seemed to be conserved. It is concluded that the X residues might not be directly involved in epitope recognition but are certainly important and adapted to correctly display the consensus motif to the 1C11 mAb binding site. The screening of the decapeptide library on one hand confirmed that the epitope of the 1C11 mAb corresponded to the RGSGPEP sequence in colicin A, and on the other hand allowed the identification of key residues (R or K) and key motifs (PEP and EP) crucial for antigen-antibody interaction.

In this study, the 22colA- and 10colA-phages served as controls to compare their affinity with that of the selected phage-peptides. Unexpectedly, the 10colA-phage was unable to bind to the 1C11 mAb, whereas the 10-amino acid phage-peptides selected from the library bound to the 1C11 mAb (Table 1). A similar effect was observed with the peptides. Indeed, the 10colA peptide did not inhibit the binding of the 22colA-phage to the 1C11 mAb, whereas the 13colA and 22colA peptides inhibited this binding (Figure 3A). Remarkably, the 10-amino acid peptides out of the phage context were able to inhibit, with various apparent IC_{50} values, this interaction (Figure 3B). Taken together, these results showed that the synthetic decapeptides selected from the library were able to bind more efficiently to the 1C11 mAb than to the natural colicin A epitope.

Specificity of Non-disulfide-linked Mimotopes

To attempt to determine phage-peptide relative affinities, competitive ELISA tests were performed using the 22colA peptide. These assays, presented in Table 1, Figure 2A and 2B, revealed that among unconstrained peptides (not possessing a disulfide bridge), only the two phage-peptides that displayed the RXXXPEP group (similar to the original sequence RGSGPEP in the antigen) exhibited an apparent IC_{50} not very different from that of the 22colA-phage IC_{50}. The other groups of phage-peptides displaying shorter peptides (RXXPEP, RXXEP and KXXEP) had lower apparent IC_{50} values even though they contained R or K residues and PEP or EP motifs. As expected, without covalent constraint, the EP and SP peptides showed the strongest binding to the 1C11 mAb, suggesting that the optimal conformation for displaying the essential PEP or EP motifs is the RXXXPEP consensus. Nevertheless, the screening of a random decapeptide library showed that constrained cysteine peptides displayed more efficiently the PEP or EP motifs.

Specificity of Disulfide-linked Mimotopes

In phage display experiments, selection of a peptide for binding to a protein requires that the peptide be in the correct conformation. Since linear peptides are usually very flexible and unstructured, their cyclization may be essential for mimotope selection [39]. In this case, the disulfide-bond formation between cysteine residues constrains the conformation of the random sequence. As a result, both affinity and specificity of binding are expected to increase because of the reduced conformational entropy. In this study, using a phage-peptide library expressing unconstrained peptides, peptides were selected containing two cysteine residues. It was demonstrated that these cysteine residues were involved in a disulfide bond absolutely necessary for binding to the 1C11 mAb (Figure 4A and 4B). These observations suggest that this disulfide bond plays a key structural role.

Moreover, the sequence alignment shown in Table 1 reveals that only one residue separates the positively charged amino acid (K or R) from the PEP or EP motifs in the disulfide-linked peptide group. However, despite the shorter length of the cysteine peptides, they were able to inhibit the binding of the 1C11 antibody to 22colA-phage as well as to the 22colA and EP peptides (Figure 3B). In this context, the disulfide bond probably constrained and stabilized sufficiently the structure of the mimotopes to allow a shorter motif like RXEP or KXEP to be efficiently recognized by the 1C11 mAb.

Although it was demonstrated that the disulfide bond plays an important role in peptide recognition, it is not clear how the cyclization induces a constrained conformation that favours antibody binding. Indeed, the residue number separating the cysteines is variable (4, 5 or 8 residues) and does not seem to affect significantly the antibody binding (Table 1). Further experiments are required to answer these questions.

CONCLUSIONS

In the present study, it was shown that the constraints generated by the presence of a disulfide

bond allowed the selection of peptides displaying short and variable epitopes (RXEP and KXEP) with similar or even greater affinity than the RXXXPEP epitope. It was demonstrated that disulfide bond formation was important for peptide recognition, whereas the size of the disulfide loop did not significantly affect 1C11 antibody binding to the cysteine peptide group. The resulting secondary structure provoked by cyclization and the RXXXPEP motif could be the signature of the natural epitope of the 1C11 antibody.

The sequence alignment of selected peptides demonstrated that arginine or lysine and glutamic acid residues as well as the last proline (downstream from the glutamic acid residue) were essential for binding to the 1C11 mAb. These residues might also be key residues for the conformation of the peptide. Since cyclization of the peptides favoured antibody binding, it was thought that the presence of positively (arginine or lysine) and negatively (glutamic acid) charged residues in the selected peptides and in the colicin A might form a salt bridge that could stabilize the structure and display the epitope to the 1C11 mAb. On the basis of this hypothesis, it is proposed that the cyclic cysteine peptides mimic the natural epitope recognized by the monoclonal anti-colicin A antibody. Additional experiments are required to investigate our hypothesis. Our study is another example of an antigen-antibody recognition site analysed by phage display and could extend the possibilities of protein engineering in drug design.

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