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Mapping epitopes of neutralizing monoclonal antibodies using phage random peptide libraries

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Identification of protective determinants from microbial proteins is a necessary step in the rational design of subunit vaccines. We have previously used a synthetic peptide scan (Pepscan) assay to map a panel of eight neutralizing monoclonal antibodies (mAb; designated as C1.1 to C1.8) to a common motif sequence from *Chlamydia trachomatis*. In the present study, five of the eight mAbs were used to screen phage random peptide libraries. mAbs C1.1 and C1.3 selected a motif sequence of G-L-X-N-D from a pIII-based phage random peptide library and a motif sequence of G-X-X-N-D from a pVIII-based random peptide library while mAbs C1.6 to C1.8 failed to select recognizable motifs from either of the phage libraries. However, C1.6 to C1.8 bound to the same motif sequence displayed on phage when the appropriate conformational constraints were imposed onto the motif sequence. Thus the specificity of the mAbs identified on Pepscan assays correlates with the mAbs' dependence on local epitope constraints displayed on the phage surface.

Keywords: epitope mapping; phage random peptide libraries; *Chlamydia trachomatis*

Introduction

Analysis of microbial protein antigenicity is essential to rational construction of subunit vaccines for preventing microbial infections. The Pepscan technology [2], allowing multiple peptides to be chemically synthesized simultaneously onto plastic pins and to be assayed repeatedly, has provided a convenient way to analyze protein antigenicity and topology of microbial proteins [8,9]. More recently phage display technology has provided an alternate approach for mapping protein antigen epitopes [5]. This technology is based on the surface display of selectable random peptides on filamentous phage [4]. The biggest advantage of the phage display approach is that no prior knowledge of the target protein is required since a known receptor or antibody can be used to capture the desired ligands from random peptide libraries displayed by phage [6]. Although both technologies have been used successfully to map binding determinants for antibodies and other receptors, it is not known whether the determinant sequences identified by the two approaches are comparable. Since the two approaches are based on different presentation mechanisms, and the density of peptide displayed in the two approaches is different, the detection sensitivity and fine specificity of the determinants identified may also be different, which could affect the design of subunit vaccines when using the identified determinant sequences. In the present study, we used a model epitope sequence recognized by a panel of monoclonal antibodies (mAbs) to compare the fine specificities obtained by using the two approaches. The model epitope sequence is from the *Chlamydia trachomatis* major outer membrane protein [1,11] and it has been considered as a component in subunit vaccines against *C. trachomatis* [11]. Antibodies recognizing the same epitope sequence display different binding characteristics with some mAbs (designated as C1.6 to C1.8) binding to the chlamydial antigen only in immunoprecipitation assays but not in Western blot assays (thus termed as conformation-dependent mAbs) and others (designated as C1.1 to C1.5) binding to the chlamydial antigen in both assays (non-conformation-dependent) [7]. Representatives of these mAbs were utilized to select binding motifs from phage display random peptide libraries. Three different libraries presenting peptides of different lengths and density were used. The specificities of the motif sequences selected from these libraries were compared with those previously mapped with the Pepscan assay [7].

Both the non-conformation- and conformation-dependent mAbs can bind to pin peptide with the non-conformation-dependent mAbs requiring fewer contacting residues and the conformation-dependent mAbs requiring more contacting residues [7]. However only the non-conformation-dependent mAbs were able to select motif sequences from the various phage display random peptide libraries. Therefore the Pepscan approach appeared to be more sensitive in detecting antibody-binding sequences. Although the conformation-dependent mAbs failed to select linear binding motifs from the unconstrained peptide libraries, they can bind to the same motif sequence displayed on phage surface upon imposing the appropriate conformational constraints onto the motif sequence. This observation suggests that the conformation-dependent mAb requires the local epitope conformation instead of the entire antigen conformation for binding. Together with the fine specificity analysis obtained from Pepscan assay, we conclude that the dependence of the mAbs on local epitope conformation correlates with the number of critical residues required.

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Since antibodies raised with a linear peptide usually have very limited cross-reactivities with the native organisms [11], it is therefore necessary to identify the appropriate structural constraints for the motif sequence. Phage displayed peptide libraries may be more appropriate for the selection of conformational constraints by using the conformation-dependent mAbs [10] since the conformation-dependent mAbs bind only to the motif sequence displayed on the phage surface in a conformationally constrained form.

Materials and methods

Random peptide libraries displayed by filamentous phage

Three different libraries presenting peptides of different lengths and density (number of the same peptide displayed on each phage particle) were used. All three libraries were provided by George Smith (University of Missouri, Columbia). The first library displays 6-mer random peptides which are presented on the filamentous phage minor coat protein pIII and it is designated as pIII 6-mer library [5]. The pIII protein is normally expressed at five copies per phage particle. The second library displays 15-mer random peptides also presented by pIII protein and it is designated as pIII 15-mer. The third library displays 15-mer random peptides but is presented on a recombinant pVIII protein (S Choukri and GP Smith, manuscript in preparation) and it is designated as pVIII 15-mer. pVIII is the major coat of protein of filamentous phage and is normally expressed at about 3000 copies per particle. However in the pVIII 15-mer library, the peptide-coding region is fused to an additional synthetic copy of pVIII, and both the wild type pVIII and the recombinant pVIII are co-expressed in the same phage. Therefore each phage in the library will carry two types of pVIII proteins. We have previously found that about 10% of the total pVIII are recombinant pVIII [10]. Therefore about 300 copies of peptide are expected on each phage particle in the pVIII 15-mer library. All phage libraries were stored at 4°C.

Two phage clones previously selected from a phage library with random constraints imposed onto the model motif sequence [10] were used. One clone expresses the 18-mer motif sequence with a disulfide bond constraint (DCLSDVAGLQNDPTTWCW) and the other expresses the 18-mer linear motif sequence (WSESDVAGLQNDPTT IYN) [10]. The two 18-mer peptides differ only in the three amino acids at each end.

Biotinylation of mAbs

The non-conformation-dependent mAbs C1.1 and C1.3 and the conformation-dependent mAbs C1.6 to C1.8 were used for selecting motifs from the three phage libraries. The antibodies were purified from ascites fluid and were biotinylated as we described earlier [10]. Briefly, 20 μl of the purified protein (2 mg ml^{-1}) was adjusted to pH 8–9 in a siliconized 1.5-ml tube by adding 4.4 μl 1 M $NaHCO_3$. Sulfosuccinimidyl-6-(biotinamido) hexanoate (Pierce, Rockford, IL, USA) was dissolved at 0.5 mg ml^{-1} in 2 mM sodium acetate buffer and 20 μl was immediately added to the antibody solution. Coupling was allowed to progress for 2 h at room temperature, and was terminated by adding 20 μl 1 M ethanolamine (pH adjusted to 9.0 with HCl) and incubating for 2 additional h at room temperature. Carrier protein (20 μl 50 mg ml^{-1} dialyzed BSA; A-3912, Sigma Company, St Louis, MO, USA) was added and the reaction mixture was diluted with 1 ml TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) and concentrated and washed three times with TBS on a 30-kDa centricon filter (Amicon, Beverly, MA, USA). The concentration of the biotinylated antibody (bio-mAb) was calculated from the final volume (usually less than 100 μl), assuming no loss during biotinylation.

Affinity selection

The biotinylated mAbs were used to affinity-select target clones from the various phage display random peptide libraries essentially as described [5,10]. Since the peptides from both pIII 6-mer and pIII 15-mer libraries are presented by pIII at similar density, we mixed the two libraries in equal proportions and the mixed library (designated as pIII 6-mer/15-mer) was treated as a single library and subjected to antibody selection. For the first round of selection, about 40 pmol of biotinylated IgG in 400 μl was reacted at 4°C overnight with a 35-mm polystyrene petri dish (Falcon) that had previously been coated with streptavidin [6]. Unbound IgG was washed away, and approximately 10^{12} tetracycline resistance transducing units of library phage were reacted with the dish for 4 h at 4°C. After washing out unbound phage, bound phage was eluted in 400 μl elution buffer (0.1 N HCl adjusted to pH 2.2 with glycine, 1 mg ml^{-1} BSA). Half of the first eluate was amplified and 10^{12} phage units of the amplified first eluate were served as the input phage for the second round of the affinity selection. The second round of selection procedure was identical to the first round of selection. For the third round of selection, 20 fmol of biotinylated IgG was mixed with 10^8 phage units of amplified phage from the second-round eluate in a total volume of 20 μl at 4°C overnight. Each IgG-phage mixture was then reacted for 30 min at room temperature with a streptavidin-coated dish, which was washed and eluted as before. Ten to twenty clones from each third eluate were sequenced as described [3]. Motif-containing clones were amplified in K91 bacterial cells in 20 ml NZY with 20 μg ml^{-1} tetracycline and 1 mM IPTG. Phage were precipitated twice with polyethyleneglycol and the final pellets were resuspended in 200 μl TBS-NaN_3 and stored at 4°C until used.

Direct ELISA and competition ELISA

For direct ELISA, antigens were coated onto an ELISA plate (modified flat bottom, Corning Glass Works, NY, USA) in 50 μl of 0.1 M $NaHCO_3$ (unadjusted pH approximately 8.5) at 4°C overnight. Each well was coated with 5×10^9 physical particles of peptide-phage; then blocked with 100 μl of blocking solution (40 mg ml^{-1} BSA in TBS) at 37°C for 2 h. Appropriately diluted biotinylated mAbs (50 μl well^{-1} in incubation buffer; 29 mg ml^{-1} of BSA in TBS) were added to each well and incubated at 37°C for 1 h. Antibody binding was detected by using avidin conjugated with horseradish peroxidase (Pierce, Rockford, IL, USA). The results were expressed as OD at 405 nm using

2, 2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) as substrate (Sigma).

For competition ELISA, ELISA plates were coated with phage as described above for direct ELISA. After incubation with blocking solution, wells were filled with 25 μl of incubation solution with or without one of the two inhibitors: *C. trachomatis* organisms (10^8 per well) and 1 μg 30-mer peptide H1 (SAETIFDVTTLNPTIAGSDVA GLQNDPTTN; [11]), which contains the model motif sequence from *C. trachomatis*. The biotinylated mAbs (25 μl well^{-1}) were added immediately to each well. After the plates were incubated for 1 h and thoroughly washed, the binding was assayed as described above for direct ELISA.

Results

Affinity selection of mAb binding sequences from the pIII 6-mer/15-mer library

All five mAbs (C1.1, C1.3 and C1.6 to C1.8) were able to bind to the model motif sequence synthesized on pins in Pepscan assay [7]. However it was not known whether they could select the same motif sequence from the random peptide libraries displayed by filamentous phage. Five of the eight mAbs were then used to select their binding sequences from filamentous phage random peptide libraries. We first used the libraries in which the random peptide are presented by the minor coat protein III. To eliminate the effect of peptide length on motif selection, two libraries were used with one displaying 6-mer random peptide and the other 15-mer. The 6-mer and 15-mer peptide libraries were mixed at equal ratio and then subjected to selection. After three rounds of selection with the mAbs, 19 or 20 individual clones were picked up from each sample for sequencing. The amino acid sequences of the random peptide region from the selected clones are summarized in Table 1. The non-conformation-dependent mAbs (C1.1 and C1.3 selected phage clones displaying a motif sequence from both the 6-mer and 15-mer pIII libraries. This motif sequence G-L-X-N-D can be found in the *C. trachomatis* major outer membrane protein and also contains the critical residue region required by the same set of mAbs for binding to pin peptide in Pepscan assay, suggesting that the motif sequence selected from random peptide libraries is specific to the mAbs. It is noticeable that the motif is always located at the N-terminus of the random peptide coding region, which may suggest that the N-terminal residues provide more flexibility for antibody binding. In addition, although the same motif sequence was selected from both the 6-mer and 15-mer libraries, multiple identical clones are only selected from the 15-mer library, which may suggest that the 15-mer library is less diversified than the 6-mer library or the single 15-mer peptide from the entire pIII 15-mer library has the highest affinity to the mAbs.

On the contrary, the conformation-dependent mAbs C1.6 to C1.8 did not select phage clones displaying sequences that can fit into any recognizable motif (Table 1; only clones selected with C1.8 are shown since all three mAbs failed to select any recognizable motifs). This is unexpected since the conformation-dependent mAbs are known to bind to the linear motif sequence synthesized onto pins in the Pepscan assay [7]. This discrepancy may be due to one of the following: the phage particles may preferentially interfere with the binding of the conformation-dependent mAbs; although the phage libraries can provide the linear motif sequence (selected by the non-conformation-dependent mAbs), the linear sequence is displayed at much lower density than that in Pepscan assay. Therefore the low affinity interaction between the linear sequence and the conformation-dependent mAbs detected in Pepscan assay is no longer detectable in phage assays.

Table 1 Sequences of the peptides selected from the pIII 6-mer and 15-mer libraries[a]

MAbs	Peptide sequence	Frequency
C1.1	**GLPND**WSGRQASGTR	17
	GLPNDWPCRQASGTR	1
	GLKNDSAVLVLVGS	1
	P**GLKND**	1
C1.3	**GLKND**SAVLVYLVGS	16
	GLLNDY	1
	GLLNDS	1
	GLQNDF	1
	GLANDV	1
C1.8	RSDVVGLQSLFCCEI	8
	FVRFYNLETLQQRYF	3
	SEELLVESSAIRSRE	1
	SPLVLIGGLDTLWSR	1
	WRRWFYQFPTRAWAS	1
	QVADKE	1
	HSGAEF	1
	RLFLHV	1
	DQHGWD	1
	PLAHNS	1

[a]The first column lists the mAbs used. The second column shows the amino acid sequences of the randomized regions covering either 6 or 15 amino acid residues. The sequences that can fit into a motif are in bold. The third column lists the number of times individual clones appear in the third eluate.

Affinity selection of mAb binding sequences from the pVIII 15-mer library

To increase the chance for the conformation-dependent mAbs to bind to the linear motif sequence on phage, we used the pVIII 15-mer library for mAb selection since hundreds of copies of peptide can be expressed on the surface of a single phage particle. In addition, it is known that pVIII clones can be used to coat plates for direct ELISA measurements while it is very difficult to measure pIII clones by direct coating. After three rounds of selection with mAbs C1.1, C1.3 and C1.6 to C1.8, 12 to 20 individual clones were picked up from each sample for sequencing. The sequences selected from the pVIII 15-mer library by the mAbs C1.1, C1.3 and C1.8 are summarized in Table 2. Since C1.6 to C1.8 all selected clones without recognizable motifs, the data for C1.6 and C1.7 are not shown. Both mAb C1.1 and C1.3 selected phage clones mostly containing the motif sequence (G-X-X-N-D) although not every clone sequenced in the third eluate displayed the same motif sequence. Although the motif sequence represents the same region as the motif sequence from the pIII library, it

Table 2 Amino acid sequences of peptides selected from the pVIII 15-mer library[a]

MAbs	Peptide sequence	Frequency
C1.1	LQLIS**GLLND**TSSSN	4
	SHLVG**GLFND**ASWSF	2
	DFPA**GYLND**STLFPP	3
	DFLI**RYLND**STPFPF	1
	FEPSKSISGVSHYLL	1
	XQYSNSKYLSPVLSP	1
C1.3	TP**GYKND**ALSHFHLT	5
	GP**GMKND**LVPPPSLT	3
	SHPG**GLYND**PPRSTL	3
	DLPG**GMLND**KVRGML	3
	EA**GVLND**GPRFTDAF	1
	QSSDSPPG**GLQND**FP	1
	VYASPNTT**GLLND**IL	1
	FPA**GVAND**PSVAGPF	1
	TSPMFSFTTPSYTLR	1
	GPRPSPVPQSVAVGS	1
C1.8	VMRQECWWWPCNDLY	2
	HPWWTWVSDPNATQP	1
	RFPYWFVLPPIATVD	1
	VPKLIAGSSSLAPLG	1
	VCFARPPRPLQVNALKRRIMLRI-RRLAADATPAPAIAKLYGILAK	15

[a]The data are displayed in the same format as described for Table 1. A 45-mer was selected by C1.8. This may be due to either of the following two reasons: the clone with the triple insertion may have superior growth advantages; the 45-mer peptide may be able to fold into a conformation allowing C1.8 binding. However C1.8 failed to bind to the 45-mer phage clone in direct binding assay (data not shown), which minimized the second possibility.

is much less restricted and more replacements are allowed in the motif from the pVIII library. In addition, the motif was not restricted to the N-terminus and it was distributed across most of the 15-mer. These observations together suggest that the multiple display of peptide in the pVIII library decreased the stringency of the selection, allowing peptide with lower affinity to be selected. However the pVIII still failed to allow the conformation-dependent mAbs C1.6 to C1.8 to select the linear motif (only the clones selected with C1.8 are shown in Table 2). A clone expressing a peptide three times as long as the normal 15-mer peptide was selected at high frequency (15 times) with C1.8 but not with C1.6 and C1.7 (data not shown). This may be due to either of the following: the clone with the triple insertion may have superior growth advantages; the 45-mer peptide may be able to fold into a conformation allowing C1.8 binding. However C1.8 failed to bind to the 45-mer phage clone in a direct binding assay (data not shown), which minimized the second possibility. The failure of the conformation-dependent mAbs C1.6 to C1.8 to select the linear motif sequence from the pVIII 15-mer library does not answer the questions we previously raised: it is still not clear whether the phage particle interference or the low density display of the peptide (even in the pVIII library) failed to allow selection of the linear motif by the conformation-dependent mAb.

MAb binding to phage clones expressing motif sequences

We then measured the binding of the mAbs to the selected phage clones and also to a clone expressing the motif sequence with an optimized disulfide bond constraint. This clone was selected from a conformation-constrained library by the conformation-dependent mAb C1.6 but it can be recognized by both C1.6 and C1.8 [10]. Since it is very difficult to measure binding to phage clones from pIII libraries due to low copy numbers of peptide on each phage, only the clones from the pVIII library were measured.

As shown in Table 3, only the ODs above 0.4 indicate significant binding in the ELISA assays. THe non-conformation-dependent mAb C1.3 was able to bind to all clones expressing the motif sequence (with OD above 0.4) but not the background clones (with OD less than 0.05). Furthermore, such binding was specifically blocked by a motif sequence containing peptide (H1) and the chlamydial organisms to which the mAb was raised (data not shown) with the ODs decreased to 0.07 or less. These observations suggest that the clones expressing the motif sequence were selected by the mAbs as a result of specific binding of the peptides to the mAbs. However the conformation-dependent mAb C1.8 failed to bind to any of the clones expressing the linear motif sequence (with an OD of 0.046 or less), which confirms that the peptide density from pVIII library clones is still too low to allow binding by the conformation-dependent mAb. Since there is nothing to inhibit with, the OD readings for the inhibition of C1.8 mAb binding to the non-constrained phage clones with either inhibitor are below 0.06.

We then compared the binding of the mAbs to two phage clones, one expressing the motif sequence without constraints, and the other with a disulfide bond conformational constraint. As shown at the bottom of Table 3, C1.3 specifically bound to both clones but the conformation-dependent mAb C1.8 only bound to the clone expressing the disulfide bond constrained motif sequence. The binding specificity of C1.8 to the constrained motif sequence expressed on phage was further confirmed by the inhibition of binding by the whole chlamydial organisms but not by the linear peptide H1 since C1.8 has been shown to bind to chlamydial organisms but not to the H1 peptide [7]. The specific binding of the conformation-dependent mAb to the clone expressing the conformationally constrained peptide was further observed under electron microscope (data not shown). Both wild type phage and the phage clone expressing the linear motif sequence failed to bind to the bead coated with C1.8. However the phage clone expressing the motif sequence with a conformation constraint was able to bind to the beads, confirming that the conformation-dependent mAb C1.8 can bind only to the motif sequence displayed on phage when the appropriate conformation constraint is imposed.

Discussion

Both Pepscan and phage display random peptide library approaches have been extensively used to identify binding sites of antibodies. A panel of neutralizing mAbs against

Table 3 mAb binding to phage clones in ELISA (OD_{405})[a]

MAbs inhibitors	ELISA with immobilized phage					
	C1.3			C1.8		
	None	H1	EBs	None	H1	EBs
EA**GVLND**GPRFTDAF	0.537	0.034	0.042	0.037	0.039	0.056
TP**GYKND**ALSHFHLT	0.785	0.032	0.045	0.040	0.034	0.027
GP**GMKND**LVPPPSLT	0.438	0.068	0.073	0.032	0.057	0.049
FPA**GVAND**PSVAGPF	0.754	0.035	0.063	0.034	0.033	0.050
SHPG**GLYND**PPRSTL	0.680	0.070	0.034	0.045	0.047	0.052
DLPG**GMLND**KVRGML	0.707	0.056	0.056	0.043	0.058	0.053
QSSDSPPG**GLQND**FP	0.503	0.054	0.054	0.042	0.049	0.046
VYASPNTT**GLLND**IL	0.498	0.039	0.067	0.046	0.060	0.035
TSPMFSFTTPSYTLR	0.032	0.035	0.069	0.043	0.054	0.056
GPRPSPVPQSVAVGS	0.043	0.034	0.037	0.046	0.056	0.048
DCLSDVA**GLQND**PTTWCW	1.201	0.059	0.065	1.109	1.081	0.067
WSESDVA**GLQND**PTTIYN	1.125	0.075	0.053	0.078	0.067	0.058

[a]The binding of mAbs to phage clones was measured in ELISA and competition ELISA as described in Materials and Methods. The first column lists the amino acid sequences of the random peptide coding regions of each phage clone pre-coated onto the ELISA plates. The sequences that can fit into a motif are in bold. The last two phage clones were selected previously [10]. The second column shows the OD_{405} readings of the direct binding of C1.3 to the plate bound phage. The third and fourth columns list the ODs of C1.3 binding to the plate-bound phages in the presence of inhibitor H1 peptide (see Materials and Methods) and *Chlamydia* organisms (EBs) respectively. The binding of C1.8 is similarly listed in columns 5 to 7. The data shown comes from one of the three separate experiments with each in duplicate wells. The variation between the three experiments is less than 20%.

an epitope from the *C. trachomatis* major outer membrane protein was analyzed previously for its fine specificities in Pepscan assay [7]. The Pepscan assay was very sensitive in detecting antibody-binding sequences, and all mAbs were able to bind to the pin peptide with some requiring two critical residues covering a distance of three residues and the other requiring five critical residues. The mAbs requiring fewer critical residues appeared to be less dependent on antigen conformation and thus they were designated as non-conformation-dependent while the mAbs requiring more critical residues appeared to be more dependent on antigen conformation and thus were designated as conformation-dependent [7]. In the present study, we have used the same panel of mAbs to select binding motif sequences from random peptide libraries displayed by filamentous phage. We found that in contrast to the Pepscan assay, the phage random peptide library approach seems to be less sensitive since only the non-conformation-dependent mAbs were able to select a motif consisting of five critical residues while the conformation-dependent mAbs failed to select conserved motifs and were not able to bind to the phage clones expressing the linear motifs (Table 3). The difference in sensitivity between the two approaches is not due to the interference of the phage particles on the binding by the conformation-dependent mAbs but is likely due to the fact that the Pepscan pins display peptide at much higher density (even higher than hundreds of copies per phage particle). High density display of ligands can allow rare events of correct folding of the ligands to be detected and therefore appears to be more sensitive. This is not only true when comparing the two approaches but also true when comparing different phage libraries. The motif clones selected from the pIII libraries are more restricted than those from the pVIII library. A motif sequence of G-L-X-N-D from a pIII-based phage random peptide library was selected while a motif sequence of G-X-X-N-D was selected from a pVIII-based phage random peptide library. PVIII library is known to display 60 times more peptide on each phage particle than pIII libraries do. Therefore the clones expressing low affinity motif sequences are also selected from the pVIII library because of the high copy number of peptide on each phage. Compared to the pin peptides used in Pepscan assay, phage displays peptide at much lower density. The low density peptide display, although being less sensitive in detecting epitope sequence, may provide better differentiation of binding with different requirements for epitope conformation. The failure of the conformation-dependent mAb to bind to a linear motif sequence displayed by phage may provide an opportunity for using phage display to select conformational constraints.

The fine specificity analysis of the mAb binding by Pepscan assays revealed that the non-conformation-dependent mAbs seemed to require less critical residues while the conformation-dependent mAbs required more critical residues for binding to pin peptide. However at this point, it was not clear whether and how the conformation depedence of the mAbs is related to the number of critical residues required by the mAbs since the conformation dependence was determined at the protein level [7]. In this study, we have demonstrated that the conformation required by the mAbs is the local epitope conformation since imposing the appropriate local constraints onto the same motif sequence can enhance the binding of the conformation-dependent mAbs to the motif sequence. Thus the present observation confirms that the multiple critical residues required for binding of the mAbs as identified in Pepscan assay are needed to maintain a local conformation to better fit the mAb binding. More native epitope residues may be required to maintain a more complicated local confor-

mation. Therefore antibodies more dependent on local epitope conformation appear to require more native residues for binding and are less tolerant to epitope residue substitution. This is consistent with the observation that mAbs only binding to the constrained motif sequence but not to the linear motif sequence on phage require more critical residues for binding in the Pepscan assay. However the frequency of the 'correct folding' (the proper folding required by a given receptor) imposed by native epitope residues in a free peptide may be rare because of the random nature of the short peptides. Therefore such rare frequency can only be detected in a high peptide density display assay such as Pepscan. In the case of low density display of peptide ligand such as phage display, imposing the appropriate exogenous conformation constraints to maintain high frequency of the correct folding is necessary to allow the correct folding to be measured.

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