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Rational Dissection of Binding Surfaces for Mimicking of Discontinuous Antigenic Sites

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Summary

Peptide-based approaches to mimicking protein interactive regions have relied mainly on linear peptides; however, most binding sites are discontinuous and thus not easily reproducible by a linear sequence. Any attempt to replicate those sites by chemical means must not only integrate all residues involved in the recognition but also provide structural organization to native-like levels. Here we describe a surface mimic approach to the reconstruction of such complex molecular architectures, using as a model a discontinuous antigenic site of foot-and-mouth disease virus that is defined by residues belonging to three different capsid proteins. Our surface mimics are synthetic cyclic peptides, designed in silico, capable of binding antibodies directed to this site, and with demonstrated functional capabilities as vaccines in guinea pigs. Further, by saturation transfer difference NMR, we have determined several antibody binding residues on these peptides.

Introduction

Protein-protein interactions play a fundamental role in events such as cell communication and division, signal transduction, protein degradation, and all types of regulatory mechanisms [1]. These interactions are essential in the formation of multiprotein complexes that integrate into cellular machineries and ultimately constitute organelle structures. They are equally crucial in the cellular responses to foreign agents such as those that rely on antigen-antibody recognition.

In characterizing these molecular events, it is not enough to define the interacting partners for a given protein but also how the interaction takes place, that is, the identity and structural orientation of the residues actually involved, in order to design replicas of the binding sites or drugs disturbing the interaction [2]. A minimalist approach to these molecular recognition processes has focused on the interacting protein surfaces. The specific properties of those interfaces, such as predominant interaction types or amino acid composition, are particularly relevant. While the interface core is often characterized by a high content of hydrophobic and aromatic residues, the surrounding rim remains accessible to solvent after complex formation and must also be considered part of the interaction patch [3]. With these assumptions, constructions appropriately mimicking these contact surfaces can be used as protein-protein interaction models as well as simplified but functional replicas of proteins. Peptides are particularly advantageous in this respect, given their synthetic availability, relative flexibility, and high solvent accessibility of most of their structure.

One widely studied example of molecular recognition processes is antigen-antibody interaction. Peptides designed as replicas of antigenic sites of several pathogens have been widely exploited in the development of chemically defined synthetic vaccines [4]. Despite their occasionally lower immunogenicity, peptide-based vaccine approaches, or other strategies involving a simplified rebuilding of the target antigen such as plasmid DNA, recombinant protein subunits, or empty capsids, offer demonstrable advantages over conventional vaccines, especially with regard to safety, storage, and standardization.

The reproduction of antigenic sites by chemical means involves different degrees of difficulty depending on the level of discontinuity of the interacting residues in the protein backbone. Protein folding and viral subunit assembly bring sequence-distant residues into close spatial proximity, which in practice means that few antigenic sites can be easily simulated by linear peptides reproducing consecutive residues of a single antigenic protein. Reconstruction of those discontinuous protein binding sites is a major goal in molecular engineering. Much of its challenge lies in building up chemical groups so that a native-like orientation of the critical interacting residues can be achieved. The complexity of this task is the main reason for the low success rate in such attempts and therefore the few examples existing in the literature [5, 6].

Previous peptide approaches to this problem have been based on grafting together two or more peptide fragments containing residues defining the epitope [7]. Our group used this approach to mimic a discontinuous antigenic site on the capsid of foot-and-mouth disease virus (FMDV) [8]. Five key amino acids located in three

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independent protein loops had been identified by MAR (monoclonal antibody resistant) mutant analysis [9] as defining the antigenic site D. These three fragments were spliced in a single peptide construction, designed on structural criteria, that was able to induce moderate levels of neutralizing antibodies [10], an important requirement for a vaccine candidate [11].

In the present work we have explored a completely different strategy, switching from the concept of loop integration to that of reproducing the composition of the critical interface amino acids, by developing a surface mimic approach to the discontinuous antigenic site of FMDV. We believe that similar design criteria could be applied to the chemical reconstruction of other discontinuous binding sites.

Results

Surface Mimic Peptide Design by Rational Assembly of Exposed Native Fragments

A previous prototype of antigenic site D mimic was designed [10] by assembling into a single molecular entity the three loops (one each from VP1, VP2, and VP3) containing site D residues. Connections among the loops involved residues at the inner capsid region in order to minimally perturb the environment of the interacting residues (see Figures 1D–1F for more details).

In designing our new surface mimic peptide of antigenic site D, we have taken a complementary but conceptually alternative approach to the one described above. First, the new design includes some additional structural elements, such as the most exposed part of the VP2 (130–141) loop. Three lines of evidence led us to favor including this loop as a likely (yet so far unreported) constituent of discontinuous site D: (1) excellent accessibility and central location in the midst of the three loops previously considered; (2) preliminary data proving a positive recognition between peptides including this VP2 (130–141) region and site D antibodies; and (3) the fact that the same loop is a constitutive element of an antigenic site in another FMDV serotype.

Second, in order to preserve as much as possible the physical properties of the recognition interface, its amino acid composition has been maintained, that is, surface-exposed residues, not defined as antigenically critical by mutational studies but comprised in the same region, have also been considered in the design. Third, peptide sections located inside the capsid have been trimmed off, and thus only selected segments containing surface-exposed, interfacial residues have been incorporated. Overall, while in the design of our earlier D8 construct [10] we adopted a "cross-sectional view" (Figure 1D) of the capsid, the present surface mimic approach views the capsid "from the front" (Figure 1A) and deals only with highly exposed elements.

In silico dissection of an approximately 25×25 Å^2 surface area, delimited by the five residues originally defining site D (T193 from VP1, S72, N74, and H79 from VP2 and E58 from VP3), provides as candidate components of a surface mimic four protein loops (VP1 [191–195], VP2 [72–79], VP3 [58–61], and VP2 [132–137]) plus four individual amino acids (P186 [VP2] and Y63, K84, and N88 [VP3]). As shown in Figure 1B, all these elements in the virus can be viewed as adopting a quasicircular layout. A first prototype, the cD1 cyclic peptide, was thus designed with a view to integrating most of the above elements while accomplishing a pseudocyclic, native-like outline. On these premises, the final viral elements included in cD1 were, from the N to C terminus: VP1 (193–195), VP3 (84), VP3 (58–60), VP3 (63), VP2 (72–80), and VP2 (133–137), with intentional sequence reorganization of the latter (SEKDR instead of SDREK) due to better structural fit (Figure 1C). The gap between the two most remote residues in the capsid (T193 [VP1] and K135 [VP2]) was bridged by two disulfide-bonded Cys residues, imposing a certain degree of conformational restriction on the construct, and by a Gly residue, providing some compensatory flexibility. This design resulted in the sequence CTGDKENVYPSQNFGHMHK SEKDRGC, shorter and synthetically simpler, thus better suited than the D8 heterodimer as a vaccine candidate.

cD1 Peptide Synthesis

The linear precursor of cD1, cD1L, with Cys residues at both ends, was prepared by solid phase Fmoc-based methods. After cleavage, deprotection, and purification, oxidation at high dilution and basic pH led to cyclic cD1. Three different oxidation conditions ([1] GSSG-GSH redox pair under N_2, room temperature, [2] same at 5°C, and [3] air oxidation, room temperature) were monitored on a small scale by HPLC, Ellman's test, and MALDI-TOF MS. After 4 hr, cyclizations under inert atmosphere were not completed and a byproduct corresponding to Met oxidation was detected. MS analysis also showed traces of a glutathione-cD1L heterodimeric disulfide. For all of these reasons, method 3 was chosen for large-scale cyclization. Further details are provided in the Supplemental Data available with this article online.

cD1 Peptide-Site D Antibody Interaction Studies by ELISA

The cD1 surface mimic peptide and its linear precursor cD1L were both assayed by ELISA for binding to several monoclonal and polyclonal antibodies directed to the antigenic site D region (Figure 2). A positive control with virus particles is also shown. Given the high specificity of these antibodies, originally generated against the native discontinuous epitope [12], this is quite a demanding test of the structural mimicry achieved by the synthetic constructs vis-à-vis the highly ordered virus epitope. In fact, no previous design of site D mimics has been successful by this stringent criterion [10], despite their ability to induce neutralizing antibodies. In the present case, however, both cD1 and cD1L peptides were recognized by the antibodies (Figure 2). Therefore, compared with our previous D8 prototype, cD1 appears to be a better design in terms of native site D mimicry.

Whereas the polyclonal antibodies showed superior affinity for cD1 and cD1L, probably due to their higher diversity, monoclonal antibodies were also able to bind both peptides, in the order 5C4 > 2E5 > 3E9 > 2A12. On the basis of the mutational studies used to define site D [9], our results indicate that the novel design reproduces especially well the VP2 (72–79) region of the virus, and to a lesser extent the VP1 component (T193 region). Interestingly, neither of the two peptides was recognized by either SD6, a monoclonal antibody directed to antigenic site A of the FMDV, nor by polyclonal

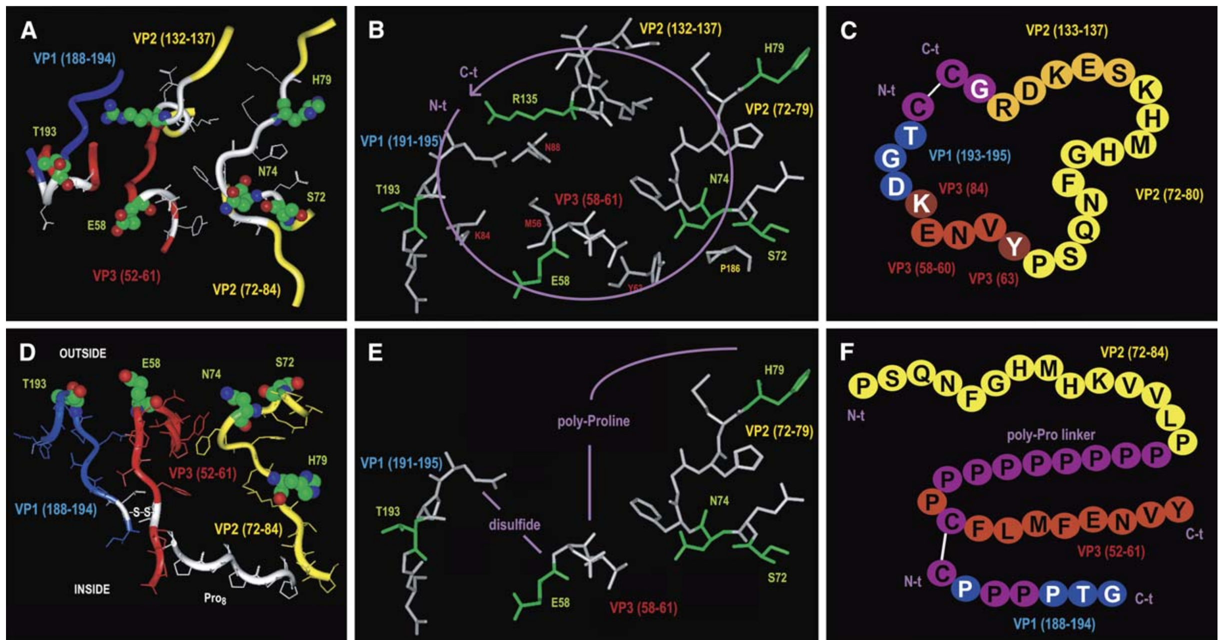

Figure 1. Dissection of a Surface Binding Patch

Design process of a surface mimic peptide (A–C) representing discontinuous antigenic site D of FMDV and comparison to a previous construction, D8 (D–F).

(A) Front view of the antigenic site D of FMDV. The five amino acids defining the site are displayed in CPK models and constitute the limits for the elements to be included in the design. Surface most-exposed fragments comprised in those boundaries are colored in white. The novel loop included in this design (VP2 [132–137]) contains a highly exposed Arg, a candidate to interact with antibodies (see text) and also represented in CPK.

(B) The elements defined in (A), including four peptide fragments and several isolated amino acids from all three structural proteins, can be viewed as defining a cycle, represented by a purple arrow.

(C) Incorporation of surface viral elements into a 26 residue peptide (amino acids in single letter code), which from N to C terminus reproduces VP1 (193–195, TGD), VP3 (K84), VP3 (58–60, ENV), VP3 (Y63), VP2 (72–80, PSQNFGHMHK), and VP2 (133–137, SEKDR, slightly reorganized from native SDREK for structural reasons). The quasicircular structure is closed by a disulfide bond between two nonnative Cys residues inserted at the ends of the arrow and a Gly at the C terminus.

(D) Cross-sectional view of the antigenic site D of FMDV, representing elements included in the previous design, D8. Antigenically relevant fragments were connected via a polyproline module and a disulfide bond (shown in white), all involving residues at the inner side of the capsid.

(E) Front view of the fragments included in D8 and their assembly, as compared to cD1 (B).

(F) In the D8 heterodimer, VP2 and VP3 fragments, located antiparallel to each other, were joined into a single 32 residue peptide chain by means of a polyproline (n = 8) linker, assuming that the relatively rigid poly-Pro II helix accurately spanned the gap between both ends. An additional 7 residue fragment, representing VP1, was connected to VP3 by a disulfide bond. Nonnative elements are displayed in purple.

sera elicited against a linear peptide reproducing site A (A24), thus confirming the specificity for site D antibodies. Somewhat unexpectedly, better recognition was observed for the linear cD1L peptide, suggesting that the cyclic cD1 version imposes too tight a conformational constraint that prevents optimal fitting. To address this latter point we optimized the cD1 design, in order to achieve more flexible constructions.

Exploration of Flexible Versions of cD1 Peptide

The major structural restriction introduced in cD1 design is the nonnative disulfide bridge. In addition to the reduction in mobility inherent to any cyclic structure, disulfide dihedral (Cβ-S-S-Cβ) angles are usually confined to values of 90° or −90° and contribute an extra conformational constraint. Previous studies with cyclic disulfides [13] mimicking the GH loop of VP1, which defines the continuous antigenic site A of FMDV, had suggested that the introduction of ω-amino acids next to either of the cysteines of the disulfide bond increased to some degree the flexibility of the cycle and therefore provided more possibilities for conformational exploration, eventually converging onto the native epitope structure. In line with this, we extended this approach to our search for cyclic surface mimics of the discontinuous D epitope, and thus designed three novel constructions

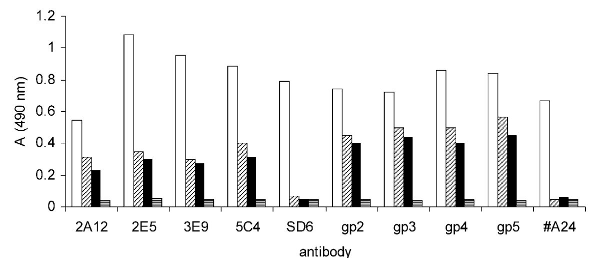

Figure 2. cD1 Peptide Recognition by Monoclonal and Polyclonal Site D Antibodies

Peptide-antibody binding evaluated by direct ELISA between cyclic peptide cD1 (filled bars), its linear precursor cD1L (diagonally lined bars), native virus (open bars), or heterodimeric design D8 (horizontally lined bars) and site D monoclonal (2A12, 2E5, 3E9, 5C4) and polyclonal (gp2, gp3, gp4, gp5) antibodies. Controls with antibodies to site A (monoclonal SD6 and polyclonal A24) are also shown.

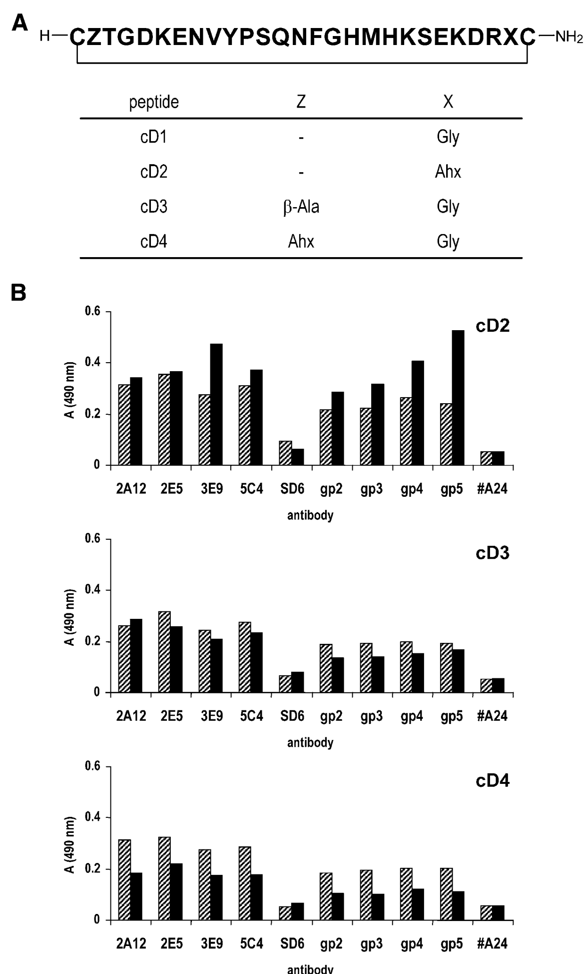

Figure 3. Flexible Cyclic Analog Sequences and Antibody Recognition

(A) Sequences of peptides cD2, cD3, and cD4, proposed as flexible analogs of the surface mimic cD1. For each peptide, residues introduced at positions Z and/or X are indicated.

(B) Recognition by site D antibodies of cyclic peptides (filled bars) cD2, cD3, and cD4 and their linear precursors (diagonally lined bars) cD2L, cD3L, and cD4L. Monoclonal and polyclonal antibodies are shown, including controls with site A monoclonal SD6 and antisera to peptide A24, representative of continuous site A.

with ω-amino acids at either side of the disulfide bridge (Figure 3A), synthesized using the same chemistry as that for cD1. These three peptides, cD2, cD3, and cD4, as well as their linear precursors, cD2L, cD3L, and cD4L, were assayed for interactions with monoclonal and polyclonal antibodies spanning discontinuous site D, as previously done for cD1L and cD1. As expected, all linear peptides showed similar antibody reactivities (Figure 3B). Cyclization had a negative effect on the recognition of peptides cD3 and cD4. Peptide cD2, however, was superior to its linear precursor, evidencing appropriate structural organization for antibody binding. In this particular case, the required flexibility was achieved by a 6-aminohexanoic acid (Ahx) residue inserted next to the C-terminal Cys, while the intramolecular disulfide maintained the optimal conformation for recognition.

In Vivo Evaluation of Peptides as Vaccine Candidates

All four cyclic peptides were taken one step further and assayed as vaccine candidates, by testing in vivo for their ability to elicit neutralizing antibodies and eventually generate in an animal model a protective immune response against viral infection.

For each of the cyclic peptides and controls, guinea pigs 1–16 were inoculated and boosted 21 and 42 days after immunization. Controls included the site D mimic D8 (17) from previous designs [10], a conventional FMDV vaccine based on inactivated virus (21, 22), and antigen-free animals (19, 20). Sera were collected at days 0 and 63. All four cyclic disulfides were highly immunogenic when formulated as free peptides without conjugation to a carrier protein, giving rise to high antibody titers (around 5 by direct ELISA against the corresponding peptide antigen) in the same order of magnitude as for the D8 control (titer defined as the logarithm of the dilution giving a 3-fold absorbance value over the blank). Titers for vaccinated animals with inactivated virus were only slightly higher (around 6). These data are presented in the Supplemental Data. The improved recognition may relate not only to the length (26 residues) and intrinsic immunogenicity of the sequences but also to the resistance to degradation provided by the cyclic structures [14]. This primary level of immunogenicity, however, must be validated by the more demanding requirement of specificity toward the native antigen, if the synthetic peptide is to be of any use as a vaccine candidate.

Viral neutralization assays where reduction of FMDV infectivity was measured were performed in cell culture by combining serial serum dilutions with infectious virus and then incubating baby hamster kidney (BHK) cells with the mixtures. As shown in Figure 4, for peptides cD1 and cD2 (the latter with a flexible Ahx acid residue close to the C terminus), a substantial reduction in viral infective capacity was obtained at high serum (i.e., antibody) concentration. In contrast, for peptides cD3 and cD4, where the flexible Ahx unit is at the N-terminal side, the reduction in infectivity was much lower. Control sera from animals vaccinated with inactivated virus showed 100% neutralization at the dilutions assayed. Negative results with PBS controls are also shown. Finally, comparisons of cD1 and cD2 antibodies with D8 antisera, in terms of viral infectivity reduction, also favor the new surface mimic peptides over the previous design.

A final requirement, prior to evaluating the present surface mimic peptides as vaccines in FMDV natural hosts, was testing them for in vivo protection of immunized guinea pigs directly exposed to FMDV virus. Three weeks after the second boost, animals were challenged with 10^6 plate-forming units (PFU) of a guinea pig-adapted FMDV [15] and monitored for 6 days for appearance of lesions on the inoculation site (primary lesions) and the other paws and tongue (secondary lesions). The time course of emergence of primary and secondary lesions upon guinea pig challenge with a guinea pig-adapted strain of FMDV usually shows animal-to-animal variation [16], and minor, transient primary lesions at the inoculation point can be observed in conventionally vaccinated animals. Thus, the criteria for full protection are based on the absence of lesions at day 6 postchallenge. As shown in Table 1, vaccination with peptides cD3 and

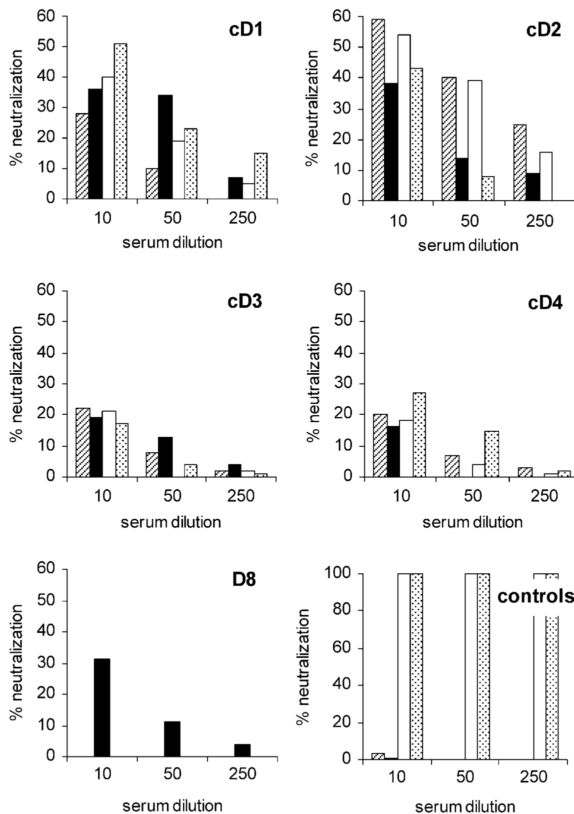

Figure 4. Antipeptide Antibodies Reduce Viral Infectivity in Cells

Viral infectivity reduction in cell culture by sera from guinea pigs immunized with peptides cD1, cD2, cD3, or cD4, extracted 3 weeks after the third inoculation. Controls with preimmune sera revealed neutralization levels below 3%.

Table 1. Protection against Foot-and-Mouth Disease in Guinea Pig Immunized with cDn Peptides

Immunogen	Animal	Protection Index[a] (Days Postchallenge) 3	6
cD1	1	3 3 2 2 0	3 3 2 2 0
	2	2 2 1 0 0	1 1 3 3 0
	3	2 1 0 0 0	2 1 0 0 0
	4	0 0 0 0 0	0 0 0 1 0
cD2	5	0 0 0 0 0	2 2 0 0 0
	7	1 0 0 0 0	0 0 0 0 0
	8	4 4 0 2 0	4 4 2 1 0
cD3	9	2 2 0 0 0	3 3 0 1 0
	10	2 2 0 1 0	2 2 0 0 0
	12	2 3 0 3 0	3 3 1 3 0
cD4	13	0 1 0 2 0	0 2 0 3 0
	14	2 2 0 0 0	3 3 0 1 0
	15	0 3 0 0 0	0 3 0 1 0
	16	2 3 1 1 0	2 2 0 2 0
D8	17	2 2 1 0 0	3 1 2 2 0
None	19	0 1 0 0 0	1 1 1 0 0
	20	0 0 0 1 0	1 2 2 2 0
Vaccine	21	1 0 0 0 0	0 0 0 0 0
	22	0 0 0 0 0	0 0 0 0 0

[a] The clinical score used to evaluate severity of lesions ranged from 0 to 4 (0, no lesion; 4, serious lesion) in the four paws, and from 0 to 2 in the tongue. For each animal the five digits refer, respectively, to rear left paw (inoculation site), rear right paw, front left paw, front right paw, and tongue.

cD4 was quite ineffective, as none of the animals were protected and severe primary and secondary lesions were detected. One out of three animals immunized with cD2 was fully protected, as judged by the absence of lesions in locations other than the inoculation site, and a second one showed minor lesions only. In the cD1 group, one animal developed a single minute lesion.

Poor statistics for D8 in these experiments are compensated by previously published neutralization results with D8 peptide [8] and D8 analogs D2, D4, D7, and D10 [10]. All in all, evidence from the neutralization experiments suggests that the surface mimic is also superior in vivo to the D8 construct.

Peptide-Antibody Interaction and Epitope Mapping by STD-NMR

ELISA is the preferred method to study antigen-antibody interactions, mainly due to its throughput capabilities and sensitivity. However, structural conclusions are difficult to draw on the basis of these experiments alone, and the expected structural bias induced by antigen immobilization on the ELISA plate advises against it. Therefore, we decided to perform a series of NMR experiments in order to identify the structural features that allow cDn peptides to bind site D antibodies. This seems an appropriate technique in that it not only can afford structural information with atomic resolution but also because it does so in a solution environment.

Peptide-antibody systems are difficult to study by NMR with the most common interaction experiments [17, 18], primarily due to antibody production limitations either amount-wise or in terms of isotopic enrichment, but also as a consequence of the large size of the peptide-antibody complex. Nevertheless, recent developments in the NMR field provide a set of experiments that require amenable amounts of antibody and no isotopic enrichment [19]. These techniques have no molecular weight limitation [20] because they rely on the observation of the ligand (in our case the peptide), and are capable of extracting structural information on its bound state. Among several studies using NMR to characterize peptide-antibody recognition [21], saturation transfer difference (STD) experiments are the most widely employed [22]. They consist of the observation of saturation transferred from the protein to the ligand (antibody and peptide, respectively) in the complex. The transfer implies complex formation but also provides information on the mode of ligand binding, thus allowing binding-epitope delineation. However, due to intrinsic limitations of these experiments, binding must occur in the fast exchange regime, which means binders with dissociation constants in the μM range. Our ELISA results suggest that the binding affinity between anti-site D antibodies (monoclonal and polyclonal) and cDn peptides might be in the appropriate range for STD experiments.

With a view to obtaining a full exploration of the antigenic site, we chose to use the wide antibody repertoire provided by the polyclonal sera fraction directed to the discontinuous native epitope. Our approach is somehow the reverse of more usual STD experiments, in the sense that in the present case the combinatorial element is supplied by the protein partner (a mixture of antibodies) instead of the ligand (single peptide),

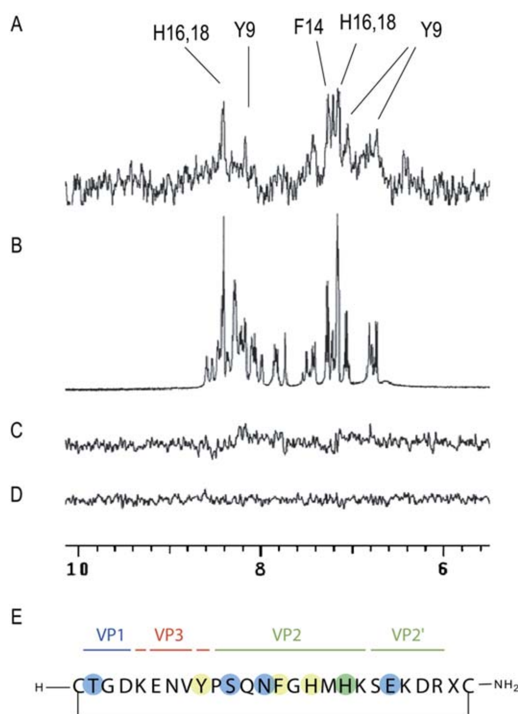

Figure 5. STD-NMR Experiments to Evaluate Binding of cD2 Peptide and Heterogeneous Mixture of Antibodies from Polyclonal Serum gp5

Amide region for experiments:

(A) STD experiment for cD2 peptide in the presence of serum.

(B) ^{1}H-NMR spectrum.

(C) Control STD experiment with a sample containing cD2 peptide in the absence of serum.

(D) Subtraction control using the same on- and off-resonance frequencies.

(E) cD2 sequence, where X stands for 6-aminohexanoic acid. Those epitope residues involved in the interaction with antibodies according to STD-NMR are highlighted in yellow; antigenically critical residues identified by mutational experiments [9] are in blue; residues selected by both methods are shown in green.

thereby affording in a single experiment a full description of the peptide residues interacting with any site D antibody. The peptide of choice was cD2, the one providing the best antibody response.

A number of transients and irradiation frequencies were explored, with 8000 scans selected as a good compromise between signal-to-noise ratio and experiment length, and δ −1 ppm as the most efficient frequency. Control experiments were also performed, showing that internal subtraction during the experiments was devoid of artifacts, and that nonantibody-mediated peptide saturation was also inexistent at the chosen irradiation frequency (Figure 5A).

The large number of scans required can be explained by either weak binding or by the peptide interacting with only a small fraction of the polyclonal antibody population, a fact easily rationalized in the context of the heterogeneous and diverse population of antibodies present in the sample. In our case, the observation of signals in the aliphatic, aromatic, and amide regions of the peptide spectrum clearly implied that recognition between cD2 and site D antibodies was taking place, as seen by ELISA.

One outstanding feature of STD experiments is that they allow locating the epitopes responsible for interaction on the ligand site [21, 23]. Standard 2D TOCSY and NOESY experiments [24] allowed full assignment of peptide resonances (Table S3), which in turn were used to identify the STD signals corresponding to amino acids in close contact with the antibody in the complex. Despite some overlap in the amide region of the STD experiment (Figure 5A), Tyr9 HN and side chain resonances, together with other signals from Phe14, His16, and His18 side chains, could be assigned, altogether defining a hydrophobic binding core that might be easily affected by mutations at adjacent positions.

Besides, NOESY experiments also showed that addition of site D antibodies did not produce significant changes in cD2, neither in terms of nuclear Overhauser effect (NOE) signal (negative, as corresponds to a peptide of the size of cD2) nor in the number of NOESY crosspeaks. The lack of intense transferred NOEs mediated by complex formation agrees with the observation of a weak cross-saturation effect in the STD experiment, and again this is probably due to antibody heterogeneity and the limited fraction of the peptide associating with antibodies.

Discussion

The structural complexity inherent to discontinuous binding sites makes their replication an extremely difficult and challenging task [7]. The particular example studied here deals with antigen-antibody recognition and how peptide replicas can be used as synthetic vaccines.

In those cases where recognition can be achieved by a partial representation of the epitope, extensive screening of peptide libraries for antibody binding serves as a first step to find low-affinity linear segments that can be combined into a higher affinity ligand [6]. However, in most cases, such as the FMDV site D presented here, this approach is not applicable because none of the integrating components of the antigenic site is, by itself, able to recognize antibodies elicited against the virus, nor capable of inducing the production of virus binding antibodies [10].

A possible solution to this problem is to assemble into a single molecule the main components involved in the recognition event, as done in our earlier, partially successful construct [10]. We now show that integrating all surface-exposed residues comprised in the binding area, including parts of the earlier construct [10], achieves a much better functional mimicry of the epitope. Further consideration of the structural features of the fragments to be included in the new construct suggested a cyclic peptide in a crown-like shape as the choice. Insertion of flexible ω-amino acids at appropriate positions provided a precise regulation of the subtle balance between rigidity and flexibility essential for optimal antibody binding.

The exceptional finding of a peptide construction able to bind antibodies generated against a discontinuous epitope encouraged us to examine the binding in solution, in order to describe for the first time how the interaction takes place, that is, which residues are directly exposed to the antibody contact. Because cyclic peptide cD2 interacted best with site D antibodies, we

explored this binding interaction to obtain a more accurate definition of the antigenic site. Epitope mapping by STD-NMR suggests key involvement of aromatic residues Tyr9, Phe14, His16, and His18 in the cD2 interaction with site D antibodies. This network of aromatic amino acids encompasses a series of residues (Ser11, Asn13, and His18 in our designed cD2 peptide, corresponding to Ser72, Asn74, and His79 of the VP2 capsid protein) that have been described as crucial for antigen recognition by site D antibodies, because they tend to undergo mutations under immune pressure [9].

The residues observed in the STD experiment would seem to provide a set of hydrophobic interactions, and consequently important surface burial, to allow the flanking residues (Ser11, Asn13, and His18) the formation of a hydrogen bond network. Prevalence of aromatic resonances in the STD experiments can be understood by the nature of hydrophobic interactions, which generally places ligand atoms closer to the receptor where saturation transfer is more efficient. Biased interactions to the VP2 (72–79) fragment were also observed by ELISA, where monoclonal antibodies 5C4 and 2E5 were the best peptide binders. However, appropriate structural disposition of those residues for productive binding is uniquely achieved by the surface mimic design, as neither individual peptide fragments comprising VP1 (189–194), VP2 (71–84), and VP3 (52–62) nor the earlier D8 construct were able to interact with site D antibodies [10].

Effective peptide-antibody recognition was complemented by an even more relevant functional feature: when assayed on guinea pigs, synthetic cD2 showed vaccine capabilities at three levels: (1) it elicited antibodies that recognized and (2) neutralized FMDV, and (3) it afforded partial but significant protection of the animals against FMDV challenge.

In sum, superiority of cD2 over the previous D8 construct arises not only from synthetic and structural simplicity (a simple 26 residue peptide ring versus a heterodimer of 32 + 7 amino acids) but more importantly from the fact that our new surface mimic constructions are uniquely able to recognize site D antibodies. In addition, neutralization levels of anti-cD2 antibodies are higher than those reported for anti-D8 sera [8].

It has been previously proposed that an ideal FMDV synthetic vaccine must be based on a multiepitope peptide cocktail representing major antigenic sites [25, 26]. Adding site D constructions such as D8 to conventional site A peptides has been shown to induce a more promiscuous and better neutralizing antibody repertory, including T cell response to site D [25]. Such a double epitope combination can be effective in overcoming the challenge posed by highly mutable RNA viruses such as FMDV. Peptide cD2, the best site D peptide replica to date, is thus an obvious candidate for a multicomponent synthetic vaccine. Further, the surface mimic approach used here for FMDV site D can be appropriately extended to other discontinuous binding sites that play key roles in the vast majority of interaction events.

Significance

Although continuous antigenic sites have been classically reproduced by linear peptide sequences, most antigenic sites and other binding regions of proteins are composed of sequence-wise remote elements and thus cataloged as discontinuous. Their integration into a molecule available by chemical means is a tall order in molecular engineering and has to consider structural as well as chemical features.

In this work, a surface mimic approach has been proven superior to other chemically built discontinuous protein binding sites, such as an earlier construction where several loop fragments were assembled. For the first time, the discontinuous antigenic site D from FMDV, constituted by fragments from three different proteins, was replicated by a single peptide able to bind viral monoclonal antibodies. The interaction was characterized by STD-NMR, and key peptide residues directly involved in antibody recognition were identified. These amino acids, mainly aromatic in nature, are at most two positions away from those residues defining site D by mutational analysis, suggesting that such mutations would alter the conformation of the hydrophobic contact pattern on the viral surface and thus allow the virus to escape antibody recognition.

The designed cyclic peptides, with variable levels of rigidity, were not only evaluated for antibody binding but also functionally assayed as vaccines on a guinea pig model. Modulation of flexibility dramatically affected the antibody recognition and general activity, with the candidate having an ω-amino acid inserted next to the C-terminal end showing better protection against viral infectivity both in vivo and in vitro. This peptide will be used in future synthetic FMDV vaccine candidates. The surface mimic approach hereby chosen can be viewed as a general strategy for reconstructing discontinuous binding sites on proteins and multiprotein entities.

Experimental Procedures

Materials

Protected Fmoc amino acids were purchased from Novabiochem (Strasbourg, France) and Bachem (Bubendorf, Switzerland). Rink amide p-methylbenzhydrylamine resin (0.73 mmol/g) was from Novabiochem. Anisole, thioanisole, ethanedithiol (EDT), piperidine, and N,N′-diisopropylcarbodiimide were from Sigma-Aldrich-Fluka (Madrid, Spain). Peptide synthesis-grade dichloromethane (DCM), dimethylformamide (DMF), and HPLC-grade acetonitrile were from SDS (Barcelona, Spain).

Preparative HPLC separations were done on a Phenomenex Luna C_8 reverse-phase column (21.2 × 250 mm, 10 μm particle size) on a Shimadzu SCL-10A system. Analytical HPLC runs were performed on a Phenomenex Luna C_8 reverse-phase column (4.6 × 50 mm, 3 μm particle size) on a Shimadzu LC2010A system. Amino acid analyses of peptide hydrolysates (6 N HCl, 1% phenol, 155°C, 1 hr) were run on a Beckman 6300 autoanalyzer. MALDI-TOF mass spectra were recorded on a Voyager DE-STR instrument (Applied Biosystems, Foster City, CA) using α-cyano-4-hydroxy-cinnamic acid as matrix. Foot-and-mouth disease virus (FMDV) type C-S8c1 was a plaque-purified derivative of isolate C-S8 (C1-Santapau-Sp/70) [27]. Monoclonal antibodies have been previously described [12].

Peptide Synthesis

Linear cDnL (n = 1, 2, 3, 4) sequences were prepared as C-terminal amides following standard Fmoc/tBu protocols on an Applied Biosystems 433A peptide synthesizer, starting from 0.1 mmol of Fmoc-AM-MBHA resin (0.73 mmol/g) and using 10 eq Fmoc-amino acid, 10 eq 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt), and

20 eq N,N-diisopropylethylamine for couplings. Peptide resins with free N terminus were washed with DCM and MeOH and vacuum-dried prior to acidolysis with reagent R (90% TFA, 2% anisole, 5% thioanisole, 3% EDT). The crude peptides were purified by preparative HPLC using linear 10%–35% gradients of acetonitrile in water (both solvents containing 0.1% TFA) at 25 ml/min. Fractions affording 98% homogeneity were selected by analytical HPLC using linear 10%–40% acetonitrile (+0.036% TFA) gradients in water (+0.045% TFA) at 1 ml/min. Further characterization was by MALDI-TOF MS and amino acid analysis. Global yields were 51% for cD1L, 30% for cD2L, 15% for cD3L, and 12% for cD4L.

Cyclic peptides were prepared by air oxidation at room temperature of 30–50 mg lots of the linear precursors under high-dilution conditions (25 μM peptide) in 0.1 M Tris·HCl, 6 M Gn·HCl, 1 mM EDTA (pH 8.0) with continuous agitation, and monitored by analytical HPLC, Ellman's test, and MALDI-TOF MS. Once completed, reactions were quenched by addition of pure TFA to a final concentration of 0.5% (pH < 2). Cyclic peptides were purified by preparative HPLC, with an initial isocratic elution with water (+0.1% TFA) at 15 ml/min for salt removal, followed by a linear 5%–20% gradient of acetonitrile (+0.1% TFA) in water (+0.1% TFA) at 25 ml/min. During elution of the main peptide peak, an isocratic regimen was maintained for better separation. The final products were further analyzed by HPLC and MALDI-TOF MS and quantified by amino acid analysis. Global yields (cyclization and purification) for cD1-cD4 were, respectively, 61%, 35%, 38%, and 22%.

ELISA

Monoclonal and polyclonal antibodies were tested by direct ELISA for binding to cDn (n = 1, 2, 3, and 4) peptides and their corresponding linear precursors. Controls with inactivated virus were also included in the experiment. Microtiter plates (Costar polystyrene, high binding, Corning, NY) were coated overnight with peptide (250 ng in 50 μl PBS) or virus (0.5 pmol in 50 μl PBS) at 4°C and then blocked with 100 μl 2% BSA in PBS for 2 hr at 25°C. Diluted antibodies, 50 μl in 1% BSA in PBS, were incubated for 1 hr at 25°C; plates were washed three times with 100 μl 0.1% Tween, 0.1% BSA in PBS, goat peroxidase-labeled anti-mouse IgG (Sigma) was then added at 1/2500 dilution in 0.1% BSA in PBS, and incubation was carried out for 1 hr, followed by several washing steps and treatment with H_2O_2/o-phenylenediamine. After 30 min, the reaction was stopped by the addition of 50 μl 4 M H_2SO_4, and absorbance was measured at 490 nm.

Guinea Pig Immunization and Challenge

Groups of guinea pigs (male Dunkin Hartley Hsd Poc; DH) were inoculated subcutaneously with each cDn peptide (0.5 mg) in PBS (100 μl) and complete Freund's adjuvant (100 μl) and boosted intradermally with the same amount of immunogen in PBS and incomplete Freund's adjuvant (100 μl each) on days 21 and 42 preimmune (p.i.). Blood samples were taken at days 0 p.i. and 63 p.i., stored at 4°C overnight, and centrifuged at 10,000 rpm and 4°C to obtain the clarified sera. Two animals injected with PBS only were used as negative controls. Animals were challenged by intradermal injection in the metatarsal pad of the hind left foot with 10^6 PFU of VpC-2C/3A, a guinea pig-adapted type C FMDV [15].

Plaque Reduction in Cell Culture

A plaque reduction assay was carried out on BHK cell monolayers. Serum serial dilutions were preincubated in duplicate with 150 PFU of FMDV for 90 min at 25°C. Aliquots (200 μl) of each sample were added to p60 Petri plates and incubated for 60 min at 37°C. Cell monolayers were washed with Dulbecco's modified Eagle's medium (DMEM), and agar medium (DMEM, 0.5% agar, 0.1 mg/ml DEAE-dextran, 1% fetal bovine serum) was added. After 24 hr, cells were fixed with 10% formaldehyde and stained with crystal violet. Plaque reduction levels were determined relative to a positive control (~150 PFU of FMDV, no sera) and corrected for background signals (plaque reduction of p.i. sera).

STD-NMR

NMR samples were prepared by dissolving 1.25 mg cD2 peptide (1.38 mM) and when applicable, 2.1 mg of polyclonal antibody mixture (46 μM, Ab/cD2 = 1/30), in 300 μl of phosphate-buffered saline solution (12 mM phosphate [pH 6.10], 137 mM NaCl, 3 mM KCl, 10% D_2O, 0.02% NaN_3).

Peptide assignment experiments (1D, TOCSY, and NOESY) were performed on a Bruker DRX-600 spectrometer at 278 K. A 1D spectrum was acquired with 64 scans. TOCSY was acquired using 80 ms mixing time, with 48 scans and 256 increments. NOESY was acquired with 300 ms mixing time, 128 scans, and 256 increments.

STD-NMR spectra were acquired at 500 MHz and 278 K on a Varian Inova VXR-500. Saturation frequencies were set at −1 ppm (on-resonance) and −15 ppm (off-resonance) using a series of Gaussian pulses of excitation width 50 Hz, with 5 ms delay between pulses, for a total saturation time of 2.5 s. Water suppression was achieved by WATERGATE. Protein was eliminated with a 20 ms spin lock. Subtraction of saturated spectra (on-resonance) from reference spectra (off-resonance) was performed by phase cycling after every scan. A total of 8K scans was acquired for sensitivity in the difference experiment. Subtraction control was performed accumulating 8K scans, with equal saturation frequencies on- and off-resonance at δ −1 ppm.

Supplemental Data

Supplemental Data including cyclization conditions for cD peptides (Table S1), antibody titers of peptide-immunized guinea pigs (Table S2), and NMR assignment of cD2 peptide (Table S3) may be found at http://www.chembiol.com/cgi/content/full/13/8/815/DC1/.

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