A Rationally Designed Synthetic Peptide Mimic of a Discontinuous Viral Antigenic Site Elicits Neutralizing Antibodies

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Synthetic peptides have a successful record of immunological applications as models of continuous epitopes or immunogens capable of eliciting antibodies of predetermined specificity.[1] The peptide approach has been crucial for the molecular dissection of protein antigens and has also resulted in useful applications, including peptide-based vaccines.[2] The success of the methodology, however, tends to obscure the fact that most antigenic sites in proteins are *discontinuous*, that is, they involve residues remote from each other in the sequence, or even belonging to independent protein chains, which are brought into spatial proximity upon folding of the antigen.

In contrast to continuous antigenic sites, which in general can be adequately reproduced by linear peptides, discontinuous protein antigenic sites pose serious challenges to peptide chemists, since attempts to design and reproduce them synthetically must not only incorporate into a single molecule different sequences but do it so that the antigenically relevant areas are displayed in the proper orientation for antibody recognition.[3] In principle, a rational approach to this problem requires at least two types of information: (i) the three-dimensional structure of the antigen, and (ii) the identity of the residues involved in antigenic recognition.

Foot-and-mouth disease virus (FMDV) is a good candidate to test this approach, since its crystal structure is known[4] and it has one such discontinuous site (site D), involving three residues from envelope protein VP2 and one each from VP1 and VP3.[5] These five amino acids cluster within a discrete area on the interface between VP1, VP2, and VP3,[4] and four of them are located on highly exposed loops (Figure 1). Site D is involved in FMDV (serotype O) binding to heparan sulfate units on the host cell surface and thus plays an important role in the mechanisms of cell entry and viral infectivity.[6] We were therefore interested in designing a peptide that could act as a mimic of the antigenic site, capable of eliciting virus-reactive, ideally neutralizing, antibodies.

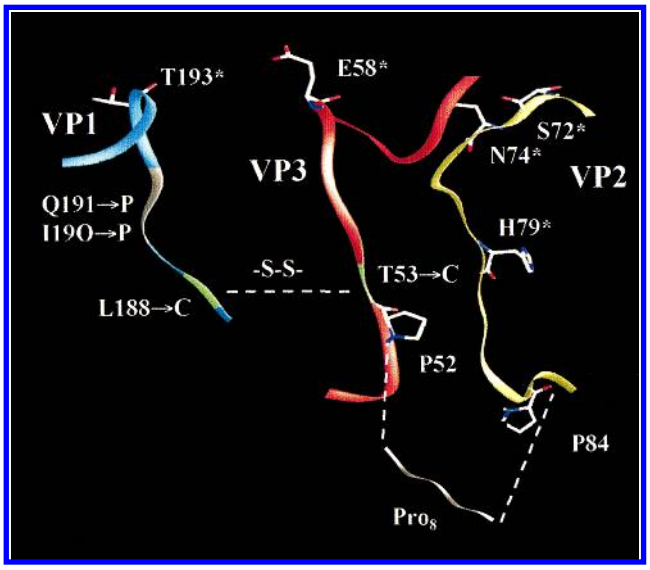

Figure 1. Antigenic site D of FMDV, isolate C-S8c1, involves one loop each from capsid protein VP1 (blue), VP2 (yellow), and VP3 (red). The side chains of the five antigenically relevant residues (labeled with an asterisk) are displayed. The VP2 and VP3 segments, antiparallel to each other, have been joined by an 8-residue poly-Pro helix connecting the carboxyl of Pro84 (VP2) and the amino group of Pro52 (VP3). Residues Ile190 and Gln191 of VP1 (in gray) have been mutated to Pro to reinforce the incipient poly-Pro conformation of this region. Residues Thr53 (VP3) and Leu188 (VP1) (in green) have been mutated to Cys to allow linkage of the VP2−VP3 and VP1 segments through a disulfide.

The goal of our design was to link the three VP1−VP3 loops in such a way that the five antigenically critical amino acids and neighbors with interfering van der Waals spheres would be displayed "surface-exposed" on the same side of the construction and in native-like orientation. The five residues define distances in the 10−30 Å range, well beyond the reach of conventional organic scaffolds. We therefore chose to join the antiparallel VP2−VP3 segments into a single sequence by means of a poly-Pro[7] helix, expected to provide some needed rigidity at the "inside" part of the construction (Figure 1). The VP2−VP3 unit was then linked to the VP1 segment by means of a disulfide bridge.

The structural fitness of the design was evaluated by molecular dynamics (MD).[8] The most stable structures resulting from 100 cycles of 3-ps unrestricted MD at 750 K were found to be relatively consistent with the native structure, particularly at the five residues involved in antigenic recognition, whose Cα−Cα distances did not deviate irreversibly from the native structure but rather fluctuated around it within reasonable intervals (5−15

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(4) Lea, S.; Hernández, J.; Blakemore, W.; Brocchi, E.; Curry, S.; Domingo, E.; Fry, E.; Abu-Ghazaleh, R.; King, A.; Newman, J.; Stuart, D.; Mateu, M. G. *Structure* **1994**, *2*, 123.

(5) Site D has been defined by mutational experiments under immune pressure.[4] When FMDV is grown in the presence of neutralizing monoclonal antibodies, escape mutants are selected which consistently display changes at only five positions: Thr193 of VP1, Ser72, Asn74 and His79 of VP2, and Glu58 of VP3. The fact that these five repeatedly substituted amino acids allow antibody escape argues strongly for their involvement in the recognition event.

(6) Fry, E. E.; Lea, S. M.; Jackson, T.; Newman, J. W. I.; Ellard, F. M.; Blakemore, W. E.; Abu-Ghazaleh, R.; King, A. M. Q.; Stuart, D. I. *EMBO J.* **1999**, *18*, 543.

(7) Rabanal, F.; Ludevid, M. D.; Pons, M.; Giralt, E. *Biopolymers* **1993**, *33*, 1019−1028.

(8) Molecular modeling was performed using the INSIGHTII/Discover software package, with the Cvff force field and $\epsilon = 4r_{ij}$. The starting extended conformation was first minimized by restricting the dihedral angles and Cα−Cα distances among the five relevant residues to native values. The molecule was then submitted to 100 cycles of unrestricted MD, consisting each of a 100 fs equilibration step followed by 3 ps at 750 K. Structures sampled every 100 fs were analyzed after minimization with no restrictions.

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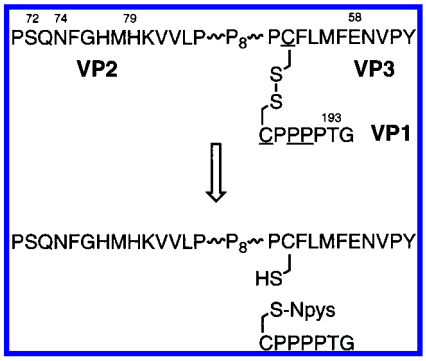

Figure 2. Retrosynthetic analysis of the peptide model of site D of FMDV. The five antigenically critical amino acids are numbered. Residues mutated from the native structure are underlined. Regioselective heterodisulfide formation is favored by the use of the Cys-protecting−activating group Npys on one of the fragments.

Å). This analysis provided sufficient grounds for the synthesis of the peptide (Figure 2).

The synthetic approach[9] relied on the regioselective ligation of 33-mer I [VP2(71−84)-Pro_8-[Cys^{53}]VP3(52−62)] and 7-mer II, [Cys^{188},$Pro^{190,191}$]VP1(188−194), by disulfide formation between their free and Npys-protected[10] Cys residues, respectively.[11] The resulting 40-residue heterodimeric peptide was satisfactorily characterized by HPLC, amino acid analysis, and MALDI-TOF mass spectroscopy after reverse phase purification. CD analysis[12] showed low levels of poly-Pro II conformation.[7]

Validation of this synthetic peptide as a model of the antigenic site D of FMDV C-S8c1 was achieved by immunization of guinea pigs.[13] Antisera raised against the peptide reacted specifically with plate-bound FMDV in ELISA.[14] Of higher significance was the fact that the antisera competed in ELISA[15] for the virus with the same monoclonal antibodies used to define site D (Figure 3), proving that the peptide was able to induce an immune response targeted roughly at the same region than the virus. Further

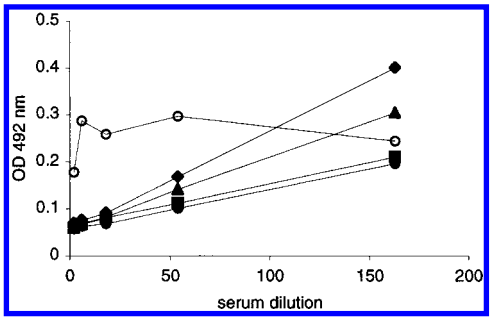

Figure 3. ELISA of the competition between antipeptide sera and peroxidase-labeled, site D-specific monoclonal antibody 5C4[4] for plate-bound FMDV. ◆, ●, ■, and ▲ points correspond to antisera from animals 1−4, respectively. ○ points correspond to a negative control experiment between antipeptide sera from animal 2 and SD6, a noncompeting monoclonal antibody mapping at an antigenic site other than D.

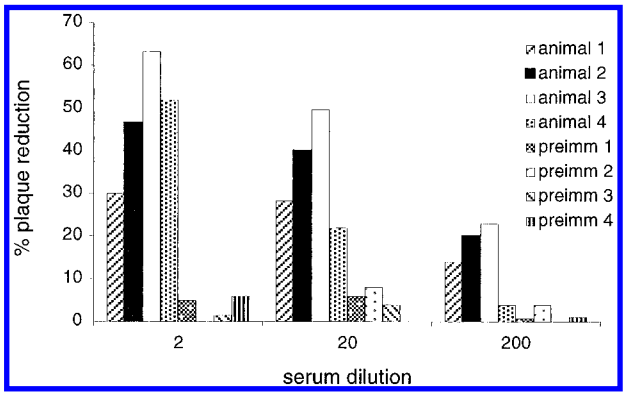

Figure 4. Neutralization titers of antipeptide and preimmune sera in a plaque reduction assay.

confirmation of the antigenic mimicry attained with the peptide came from neutralization experiments (Figure 4), which showed that peptide antibodies could achieve modest but unequivocal levels of reduction in FMDV infectivity.[16] Taken together, these results demonstrate a substantial degree of reproduction of discontinuous antigenic site D of FMDV and therefore support the validity of a structure-guided approach to this type of problem. The presence of a modular poly-Pro unit is particularly fit for structure−activity studies in which the number, configuration, and replaceability of Pro residues can be investigated, with a view to further refine the immunological performance of the corresponding peptides. This work is presently under realization.

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Supporting Information Available: Molecular dynamics data, chemical characterization of monomers I and II and heterodimer, CD spectra, ELISA of antipeptide sera against FMDV and a control (nonsite D) plate antigen, and competition ELISA with two additional monoclonal antibodies mapping at site D (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) Peptide I was synthesized in C-terminal carboxamide form on a PAL linker (Albericio, F., Barany, G. *Int. J. Pept. Protein Res.* **1987**, 30, 206−216) attached to a PEG−polystyrene resin. Fmoc chemistry protocols were used, with *tert*-butyl (Glu, Ser, Tyr), trityl (Asn, Cys, Gln, His), and Boc (Lys) protecting groups. Couplings were mediated by TBTU [2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate] in the presence of DIEA (4 equiv each) in DMF. Final deprotection and cleavage were done with trifluoroacetic acid−thioanisole−ethanedithiol−anisole (90:5:3:2), 2 h, rt. Peptide II was synthesized by Boc chemistry on *p*-MBHA resin, using benzyl and 3-nitro-2-pyridylsulfenyl protections for Thr and Cys, respectively. The peptide−resin was deprotected in HF-*p*-cresol (95: 5, 0 °C, 1 h). Both crude peptides were purified to homogeneity by reverse phase HPLC and were satisfactorily characterized by amino acid analysis and MALDI-TOF MS.

(10) Matsueda; R., Kimura, T., Kaiser, E. T., Matsueda, G. R. *Chem. Lett.* **1981**, 737−740.

(11) Peptides I and II (15 and 5 mg, respectively) were reacted at pH 4.5, 1 h, rt, as described (Albericio, F., Andreu, D., Giralt, E., Navalpotro, C., Pedroso, E., Ponsati, B., Ruiz-Gayo, M. *Int. J. Pept. Protein Res.* **1989**, 34, 124). The reaction was stopped by acidification with HOAc and the heterodimer was purified by reverse phase HPLC.

(12) CD spectra were acquired from 25 μM peptide solutions in 1 mM phosphate at pH 3, 5, and 7.3. The content in poly-Pro II conformation[7] was maximal at low pH and temperature.

(13) Guinea pigs were inoculated with 0.5 mg peptide in 100 μL PBS and 100 μL complete Freund adjuvant and boosted with the same amount in 100 μL PBS and 100 μL incomplete Freund adjuvant on days 21 and 42. Preimmune, 21-, 42-, and 63-day sera were processed.

(14) Serial dilutions of 63-day sera were added to ELISA plates coated overnight with 1 pmol virus at 4 °C and then blocked with 5% BSA. After 1 h, the plates were washed, and bound antibody was quantitated after incubation with peroxidase-labeled goat anti-guinea pig IgG for 1 h and washes, by reaction with *o*-phenylenediamine. Absorbance was read at 492 nm and corrected for background (wells with no virus).

(15) Serial dilutions of 63-day sera in PBS were incubated for 90 min, rt, with a nonsaturating amount of peroxidase-labeled monoclonal antibodies 5C4, 2A12, and 2E5,[4] then added to virus-coated ELISA plates previously blocked with 5% BSA. After 1 h incubation at room temperature, the plates were washed, and bound monoclonal antibody was quantitated as above.

(16) Neutralization of infectivity was carried out in vitro by a plaque reduction assay on BHK cell monolayers. FMDV was incubated with dilutions of either antisera or preimmune controls, following described procedures (Habel, K. In *Fundamental techniques in virology*; Habel, K., Salzman, N. P., Eds.; Academic Press: New York, 1969; pp 288−296). For comparative purposes, sera from FMDV vaccinations give 50% plaque reduction when diluted 100−1000-fold.