Conformationally Constrained Peptide Mimetics: The Use of a Small Lactam Ring as an HIV-1 Antigen Constraint

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Received August 7, 1997

In recent years, small peptide fragments have become increasingly popular as antigens for eliciting immune responses to protein epitopes.[1] While a number of notable successes have been reported, the overall approach suffers from the fact that the peptide antigens are conformationally flexible and cause a wider range of antibodies to be raised against the peptide than would be generated to the same epitope in a protein. This leads to a very inefficient immune response. Satterthwait and co-workers reported the use of a conformationally constrained peptide fragment to alleviate this problem.[2] In that work, two asparagine side chains were linked with an ethane bridge to form an 18-membered ring. The constrained peptide fragment was used to elicit an improved immune response to the malaria virus. It is tempting to suggest that small lactam rings might also make effective constraints for fixing small peptide antigens into specific conformations.[3] Such an approach would further reduce the flexibility of the peptide fragment and further bias the subsequent immune response toward conformations found in the native protein. In addition, new constrained antigens could be designed by simply replacing spatially close hydrogens in a conformational epitope with carbon bridges.[4] However, will the addition of extra carbon bridges to a peptide fragment interfere with its ability to serve as a viable antigen? We report herein an immunological response to a lactam ring containing antigen that was not altered by the presence of an additional ethane bridge.

As an initial target, a portion of the hypervariable V3 loop of the gp120 ENV protein of HIV-1 was selected. The crown of the disulfide-bridged V3 loop contains a highly conserved sequence (GPGR) that forms part of the PND (principal neutralizing determinant) of HIV-1[5] and has been the target of many immunological studies.[6] This region contains a type II reverse β-turn that has been well characterized by X-ray[7] and NMR.[6g,8] The conformation of the GPGR region taken from the X-ray crystal structure of an antibody bound to the V3 loop is illustrated in Scheme 1.[7]

Scheme 1

1. V3 - GPG region **2**

Three potential locations for the placement of bridges (and the addition of conformational constraints) into the GPGR region of the V3 loop are indicated by the arrows on structure **1**.[9] To determine if carbon bridges can be added to these locations without interfering with a subsequent immune response, one bridge is being placed in at a time and the resulting immune response is then examined.

Analog **2** (Scheme 2) was selected as the first constrained antigen for study because the spirocyclic building block needed for its construction was already known.[10,11] Accordingly, the spirocyclic lactam **3** was synthesized using the procedure of Johnson and co-workers[11,12] and then coupled into a peptide fragment to complete the desired IGPGRAF sequence.

For these initial studies, the I, A, and F amino acids were included in the synthetic antigen in order to create a larger epitope. The peptide fragment was then coupled to BSA (bovine serum albumin) as a carrier protein using the known literature

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(12) We found it useful to allow the osmylation reaction in this sequence to run for 24 h. In this case, the intermediate aldehyde cyclizes *in situ* to the diastereomeric spirocyclic alcohol in a 70% yield. The *N*-α-hydroxylamide can then be quantitatively reduced to **3** by treatment with $NaBH_4$ in neat trifluoroacetic acid (TFA).

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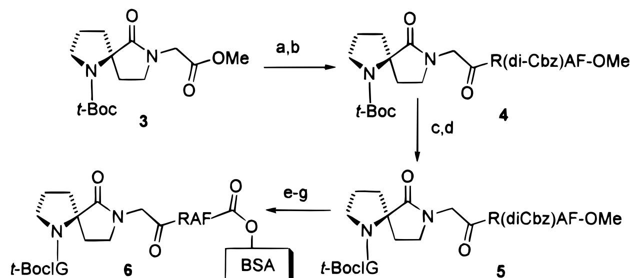
Scheme 2[a]



[a] Reagents: (a) i. 1 N NaOH, MeOH, pH = 13; ii. 1 N HCl, pH = 6; (b) H_2NR(di-Cbz)AF-OMe, EDCI, HOBt, Et_3N, CH_2Cl_2, 44% over two steps; (c) TFA; (d) *t*-BocIG-OH, EDCI HOBt, Et_3N, CH_2Cl_2, 89% over two steps; (e) H_2, Pd/C, MeOH, 80%. (f) i. 1 N NaOH, MeOH, pH = 13; ii. 1 N HCl, pH = 6, carried forward without purification; (g) BSA (1 mmol:13 mmol of peptide), 30 equiv of EDCI, H_2O.

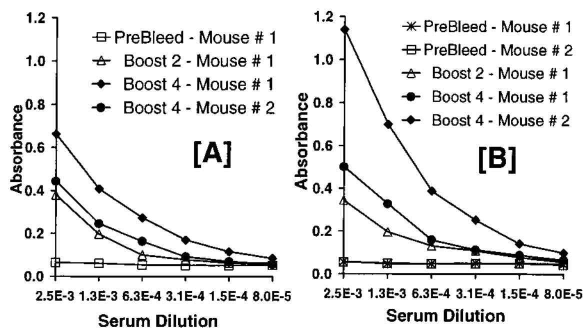


Figure 1. Noncompetitive assays of serum from mice immunized with unconstrained [A] and constrained (Boc-IGPGRAF)$_x$-BSA [B]. Plate antigen is gp120$_{MN}$ coated at 0.1 ug/mL.

procedure.[13] A second antigen was prepared by coupling the equivalent unconstrained peptide (without the bridge) to BSA. Mice were immunized with four boosts of the two bioconjugates according to typical protocols.[14]

The resulting sera were tested for immunological response by ELISA.[15] The results of noncompetitive binding to the native protein (gp120$_{MN}$) are shown in Figure 1. Both sets of sera showed a positive and roughly equivalent immune response to the peptide antigens. To ensure that binding was specific for the native protein, a competition ELISA was performed using gp120$_{MN}$ as both the solid- and solution-phase antigens.[16] The results indicated that our conformationally constrained antigen gave a specific immune response that was at least as good as that obtained with a typical unconstrained carrier bound short peptide antigen.

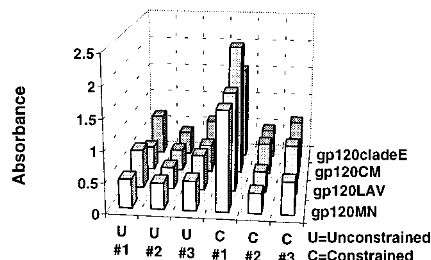


Figure 2. Noncompetitive assay of sera at 1:1000 dilution against various gp120 plate antigens to assess breadth of immune response. Plate antigens are coated at 0.1 ug/mL.

The effect of the constrained antigen on the ability of the immune response to recognize variant gp120 proteins was evaluated with a noncompetitive ELISA using four variants of gp120[17] bound to the solid phase (Figure 2).[18] The results showed that both sets of sera were able to recognize gp120 proteins with variant amino acid sequences in the hypervariable regions flanking the target GPGR region, presumably by focusing the immune response on the conserved GPGR region. Specific binding to gp120 was confirmed in this assay by competition with gp120$_{MN}$.[16]

In conclusion, we have found that the placement of a carbon bridge in the middle of the V3 loop conserved region of HIV-1 did not interfere with the ability of the peptide to serve as an antigen and that antibodies raised against this constrained molecule bound the gp120 ENV protein in a similar fashion to antibodies raised against the antigen without the bridge. The ability to place a small lactam ring in the middle of a key epitope region without interfering with the resulting immunological response suggests that it may be possible to use lactam rings as constraints for building probes for examining the 3-D conformational requirements of antibody−antigen interactions, as well as subunit vaccines for targeting small conformational epitopes of proteins.

Acknowledgment. We thank the National Institutes of Health and the National Science Foundation for their generous financial support of our programs. We also gratefully acknowledge the Department of Education GAANN program for providing a graduate fellowship for R.D.L. Finally, we thank the Washington University High Resolution NMR Facility, partially supported by NIH grants RR02004, RR05018, and RR07155, and the Washington University Mass Spectrometry Resource Center, partially supported by NIH RR00954, for their assistance.

Supporting Information Available: Spectral data for all new compounds, a graph summarizing the results of the competition ELISA study, and the raw data for all of the ELISA studies (19 pages). See any current masthead page for ordering and Internet access instructions.

JA972758X

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(14) Eight-week-old BALB/c mice were treated with antigen (1 mg/mL in phosphate-buffered saline (PBS)) and Freund's adjuvant's (complete and incomplete) as part of a typical immunization protocol administered by the Washington University Animal Care Facility.

(15) This data is included in the Supporting Information.

(16) ELISA testing was performed on CoStar EIA plates (catalog no. 3590), antigens were bound to plates using Carbonate-Bicarbonate buffer (Sigma C-3041) overnight at room temperature (rt), plates were blocked for 1 h with 1% BSA (Sigma A-7906) in PBS (Sigma P-4417) at rt, after 2 washes with PBS solution, sera diluted with 0.25% BSA in PBS was added to the plates (with or without competing antigen) and equilibrated at r.t. for 2 h, after washing 4 times with PBS, Anti-mouse IgG-HRP conjugate diluted 1:5000 in 0.25% BSA in PBS was added and equilibrated for 1 h, finally the plate was washed 6 times with PBS containing 0.05% Tween 20 (Sigma P-7949) then pre-mixed TMB (tetramethylbenzidine) solution (Sigma T-8540) was added. After 30 min optical densities were read at 650 nm on an ELISA plate reader.

(17) The gp120 proteins were obtained through the NIH AIDS Research and Reference Reagent program, DAIDS, NIAID, NIH. gp120$_{MN}$ (V3 tip sequence = IHIGPGRAFYTT), gp120$_{LAV}$ (IRIQRGPGRAFVTI), and gp120$_{CM}$ (ITIGPGQVFYRT) were donated by MicroGeneSys, Inc. gp120$_{cladeE}$ (VRIGPGQVFYRT) was donated by S. Showalter and M Garcia-Moll (BioMolecular Technology).

(18) The higher values obtained using "constrained mouse #1" are most likely not significant and a result of error in the concentration of the serum.