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Mimicry of Native Peptide Antigens by the Corresponding Retro-Inverso Analogs Is Dependent on Their Intrinsic Structure and Interaction Propensities[1]

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Retro-inverso (ri) analogs of model T cell and B cell epitopes were predictively designed as mimics and then assayed for activity to understand the basis of functional ri-antigenic peptide mimicry. ri versions of two MHC class I binding peptide epitopes, one from a vesicular stomatitis virus glycoprotein (VSV$_p$) and another from OVA (OVAp), exhibit structural as well as functional mimicry of their native counterparts. The two ri peptides exhibit conformational plasticity and they bind to MHC class I (H-2K^b) similar to their native counterparts both in silico and in vivo. In fact, ri-OVAp is also presented to an OVAp-specific T cell line in a mode similar to native OVAp. In contrast, the ri version of an immunodominant B cell peptide epitope from a hepatitis B virus protein, PS1, exhibits no structural or functional correlation with its native counterpart. PS1 and its ri analog do not exhibit similar conformational propensities. PS1 is less flexible relative to its ri version. These observed structure-function relationships of the ri-peptide epitopes are consistent with the differences in recognition properties between peptide-MHC vs peptide-Ab binding where, while the recognition of the epitope by MHC is pattern based, the exquisitely specific recognition of Ag by Ab arises from the high complementarity between the Ag and the binding site of the Ab. It is evident that the correlation of conformational and interaction propensities of native L- peptides and their ri counterparts depends both on their inherent structural properties and on their mode of recognition. *The Journal of Immunology,* 2003, 170: 1362–1373.

T he reverse synthesis of a peptide using D-amino acids results in a peptide analog wherein the direction of the peptide bonds is reversed and the N- and the C-termini are interchanged. The retro-inverso (ri)[3] transformation produces a structural isomer that maintains the original stereochemical configuration at all the chiral centers but reverses the sequence. Native peptide and its ri analog on superimposition show that the carbonyls of the peptide bonds in the native molecule are present over the peptide bond amides in the ri molecule and vice versa. The superimposition also shows that the identical side chains overlap in the two versions, although the polarity of the peptides is exactly opposite. These transformations lead to a peptide showing a high degree of topochemical equivalence with the parent peptide. The synthesis of ri peptides has been widely used in peptidomimetic drug design to create molecules resistant to naturally occurring peptidases (1, 2). As the natural proteases are stereospecific, they are unable to cleave the peptide bond in retro-D analogs, leading to higher bioavailability and consequently removing the need for high doses. This topochemical mimicry of side chain residues by retro-inversion has been exploited to generate cross-reactive antipeptide Abs and to design various peptidomimetic drugs (3).

The structural features of molecular recognition involving different systems, although exploit similar noncovalent forces, are often unique and have distinct characteristics enabling diverse specificities. Recognition of T and B cell peptide epitopes provide an interesting example of the molecular recognition by two different proteins of the immune system: MHC and Abs (4). The recognition involving MHC molecules with T cell epitope peptides of diverse sequences is achieved by restricting the sequence specificity to a few anchoring residues. Peptides bind MHC in an extended conformation and the length of the peptides that can bind to MHC is nearly constant. In contrast, antipeptide Abs follow an entirely different strategy of Ag recognition. In many cases, the peptides derived from soluble proteins are recognized in conformations resembling those found in the original protein. Abs have perhaps the highest specificity, although not necessarily the highest affinity, where several individual side chains in the peptide Ag cannot be replaced without substantial loss in binding affinity. In peptide-Fab complexes, usually only 7–10 residues are visible in the Ab binding pocket. The exquisitely specific recognition of Ag by Ab stems from the high complementarity in size and shape between the Ag and Ab binding site, and by the formation of specific hydrogen bonds and charged interactions with the Ag.

Examples of both the failure and success of retro-D analogs of immune epitopes to exhibit antigenic mimicry are present in the literature (1). However, the exact parameters that influence the competence of a retro-D analog remain to be completely elucidated. To find a plausible solution for this structural puzzle, ri analogs of model T cell and B cell peptide epitopes were designed and assayed for activity. The T cell epitopes chosen were octam-

ers, [RGYVYQGL] (vesicular stomatitis virus glycoprotein peptide; VSVp) and [SIINFEKL] OVA; OVAp) (5), derived from a vesicular somatitis virus protein and the OVA protein, respectively. These antigenic peptides have been studied extensively and the structures of their complexes with the MHC class I (MHCI) molecule, H-2K^b, have been elucidated (6, 7). The B cell epitope used is PS1, a 15-mer peptide (HQLDPAFGANSTNPD) derived from the large surface Ag of hepatitis B virus. An exhaustive study of the murine immune response against PS1 has shown that the Abs in the secondary response are unanimous in their specificity for the four-residue stretch of PS1, DPAF (8). The structures of PS1 bound to three distinct cognate mAbs from the secondary response have been determined (9, 10). It is evident that all the three Abs recognize a common immunodominant epitope incorporating primarily DPAF stretch of the peptide Ag. The Abs show conformational convergence on binding, although they display dissimilar binding-site structures in the absence of the Ag. The epitope also exhibits conformational similarity when bound to each of these Abs although the peptide Ag was otherwise flexible. The observed conformational convergence in the epitope and the Ag-binding site was facilitated by the plasticity in the nature of interactions.

Due to the dichotomy associated with the structural features in antigenic recognition, a comparative analysis of structure and interaction preferences of the ri analogs of these T cell and B cell epitopes has been conducted and functionally assessed using biochemical and immunological assays. The correlation of conformational propensities of the native L peptides and their ri counterparts depends on the inherent structural properties of the individual peptides. The T cell epitopes VSVp and OVAp, which both adopt the extended conformation, exhibit similar conformational propensities in L and ri forms, and the ri analogs of VSVp and OVAp are functionally active through similar molecular interactions with their receptor. In contrast, the B cell epitope PS1 shows distinct conformational propensities in the ri version. Consequently, the ri analog of the B cell epitope was found to be unable to bind to any of the anti-PS1 Abs. Overall, it appears that the degree of correlation in conformational propensities—and hence functional equivalence—between native L peptides and their ri counterparts are influenced by the inherent structural properties of the individual peptides. Additionally, the degree of leeway permitted for successful recognition also appears to have a bearing on the appearance of successful antigenic mimicry by ri analogs.

Materials and Methods

Analysis of molecular stereochemistry

The Ramachandran plots were made using Procheck from the CCP4 suite of programs (Warrington, U.K.). The ϕ and φ angles for the native peptide were exchanged and the handedness of the amino acids was changed to get the ri versions of the three peptides. The Ramachandran plots were also computed for the ri analogs of the three peptides. The plots for native and ri versions of the same peptide were compared.

Molecular dynamics analysis

The native peptide conformations and their ri analogs were subjected to molecular dynamics simulation. The peptide was initially soaked in a water box of appropriate size with periodic boundary conditions and then subjected to molecular dynamics. All simulations were conducted at 300 K for 500 ps. The system was allowed to equilibrate for the first 50 ps and conformations were written out for the remaining 450 ps at an interval of 4.5 ps. The trapped conformations were compared with the starting conformation in terms of root mean square deviation (rmsd) in the position of C$^\alpha$ and all atoms. The frequency distributions of the calculated rmsds for both native and ri peptides were plotted and compared.

Docking

The ri analog was initially manually docked into the H-2K^b binding groove while monitoring intermolecular energies using the DOCKING module of

INSIGHTII (San Diego, CA). The conformation of the native peptide was used as a guide. Next, the complex of MHC peptide was subjected to conjugate gradient minimization until convergence using the DISCOVER module of INSIGHTII. No explicit hydration shell was defined as the peptide docking in the binding site was being optimized. Instead, distance-dependent dielectric constant was used, and all energy-based computations were done using a consistent valence force field. The hydrophobic surfaces of the MHCI-peptide complexes were computed and compared using INSIGHTII. The van der Waal interactions between peptide and the MHC molecule (distance cutoff 4 Å) were listed using CONTACT program of the CCP4 suite. The solvent accessible surface area, which gets buried on complexation, was calculated using HOMOLOGY module of INSIGHTII.

Peptide synthesis, purification, and characterization

VSVp, OVAp, PS1, and their ri analogs were synthesized by solid phase method using an automated peptide synthesizer model 431A (Applied Biosystems, Foster City, CA), using standard F-moc methodology. Although the wild-type MHCI-specific T cell epitopes had free N- and C-termini, ri peptides were amidated at C-terminal and acetylated at N-terminal unless explicitly mentioned. For ease of reference the residue numbers are identified by their positions in the native L peptide throughout. The peptides were cleaved from the resin by treatment with trifluoroacetic acid/thioanisole/phenol/water/1,2-ethanedithiol in ratio as recommended by Applied Biosystems. The crude peptides were purified using C-18 column (Deltapak-100 Å, 15 μ, spherical, 19 × 300 mm; Waters, Bedford, MA) and peptide purity was verified using C-18 analytical column (Deltapak-300 Å, 15 μ, spherical, 7.8 × 300 mm; Waters). The peptides were characterized by molecular mass measurement using single Quadruple mass analyzer (Fisons, Loughborough, U.K.).

MHCI-peptide binding and T cell stimulation

The H-2^b cell lines RMA and RMA-S were used for assaying the binding of peptides and their analogs to MHCI. RMA-S is deficient in the cytosol-to-ER peptide transporter TAP; therefore, empty MHCI molecules come to the surface and stay there for very short periods, leading to a reduction in the cell surface MHCI levels. Addition of an MHCI-binding peptide from outside stabilizes this cell surface MHCI on RMA-S cells and increases the net levels of MHCI. Thus, the binding of a given peptide to MHCI is indicated by its ability to increase the equilibrium levels of MHCI on the surface of RMA-S cells. Therefore, peptides were incubated for 24 h with RMA-S cells (1 × 10^6 cells/ml) at various concentrations as indicated, and H-2K^b levels on the cells were determined using the mAb Y-3, followed either by anti-mouse Ig fluorescein and flow cytometry (Elite EPS; Coulter, Hialeah, FL), or by anti-mouse Ig β-galactosidase and color development with chlorophenol red β-D-galactopyranoside (Roche, Nutley, NJ) read at 570 nm. Any increase in MHCI level on RMA-S observed at each peptide concentration was expressed as a percentile of the MHCI level on wild-type RMA cells.

The OVAp + H-2^b-specific T cell transfectoma B3 was used to examine cross-reactivity of the OVAp analogs at the TCR level. B3 T cells (1 × 10^5 cells/well) were stimulated with titrated concentrations of various peptides in the presence of C57BL/6 spleen cells (1 × 10^5 cells/well) as APCs in triplicate cultures in 200 μl DMEM with 10% FCS, antibiotics, L-glutamine, and 0.05 mM 2-ME in 96-well flat-bottom plates (Falcon, Franklin Lakes, NJ). IL-2 levels induced were estimated by bioassays on culture supernatants collected 24–36 h later by using them to stimulate the IL-2-dependent cell line CTLL-2 (1 × 10^4 cells/well) for 24 h, followed by pulsing of the plates with 0.5 μCi/well of [^{3}H]thymidine (NEN, Boston, MA) for 12–16 h to measure the proliferative response. Data are shown as mean cpm ± SEM in triplicate cultures.

ELISA

Plates were coated with the PS1CT3 peptide (8) (1 μg/well) in 100 μl of carbonate buffer (pH 9.6) at 4°C for overnight. Subsequently, wells were blocked with 300 μl of 1% gelatin in PBS (pH 7.2) at 37°C for 2 h. Then, 100 μl of the appropriate dilution of mouse monoclonal ascites was added and incubated at 37°C for 1.5 h. After washing, bound Ab was detected with HRP-labeled secondary Ab (37°C, 1 h) followed by color development with o-phenylenediamine as chromogen. Absorbance was measured at 490 nm. Initially, experiments were conducted to find out the working dilution of the monoclonal ascites for each Ab. Once the working dilution was established, the competition ELISAs were conducted. The plates were coated with PS1CT3 peptide. The diluted mouse monoclonal ascites were incubated with the ri-PS1 and native PS1 peptides before assaying for PS1 binding.

Results

ri versions of the T cell epitopes, VSVp and OVAp, exhibit conformational correlation with their native counterparts

It was expected that a ri-peptide analog corresponding to a given sequence should adopt a conformation similar to that of the corresponding L peptide (11). Therefore, we built the conformational models of ri analogs of VSVp and OVAp (called ri-VSVp and ri-OVAp). To generate the appropriate conformational model of the ri analog, initially, the sequence of the original L-peptide was reversed and the handedness of the residues was changed to D. According to the basic theory of ri mimicry, the topochemical equivalence is achieved because the side chains in the two versions have the overlapping conformations. To achieve such an overlap of the side chain for a particular residue, the N-C^{α} bond in native peptide should overlap with the C^{α}-C bond in the ri version and vice versa. For this to occur, the dihedral angles ϕ and φ in the native peptide would have to necessarily be equal to the dihedral angles φ and ϕ in the ri analog, respectively. Thus, to achieve conformational equivalence between the two versions, the backbone dihedral angle values in the L peptide have to be interchanged in the ri analog for corresponding residues. Also, to be consistent with change in handedness and to achieve the overlap between the side chains, the dihedral angle $\chi 1$ in the case of ri-peptide would have to be $\chi 1 + 180°$ with respect to the corresponding residue of L peptide. These transformations were conducted on the H-2K^{b}-bound conformations of the OVAp and VSVp peptides. The final models obtained from these transformations for the ri-OVAp and ri-VSVp peptides were superimposed onto the parent L peptides (Fig. 1). Most of the side chains of different residues of the native peptide overlap in space with the identical side chains on the ri version, but it is evident that the complete superimposition with the native peptide (as seen in the MHCI-bound conformation) is still not achievable.

The distribution of the backbone dihedral angles, ϕ and φ, for the native and ri versions were compared using the Ramachandran plot (12) in the case of the T cell epitopes. In both cases, the distribution of ϕ and φ angles for the native peptide and its ri analog are localized in the equivalent regions of the Ramachandran plot as can be seen from Fig. 2. The ϕ and φ angles in the case of the native peptides are present in the core regions for β-strands. In the case of the ri versions of the peptides, these dihedral angles are localized to the core and allowed regions corresponding to the β-strands. The plots show that when the ri peptide is made to adopt a conformation which overlaps that of the native peptide in its bound form, the backbone dihedral angles are all in the allowed regions and also localized in the same area on the map as that of the native peptide. Thus, it is energetically possible for the ri versions of the two T cell epitopes to assume a conformation that mimics that of the native peptide in its MHC-bound state. In other words, the ri analog of each T cell epitope can potentially act as the functional mimic of the native peptide.

The N- and C-termini of the native peptides VSVp and OVAp have been known to play a significant role in binding to MHCI molecule H-2K^{b} (13). Reversal of the termini—as happens in the ri-peptide—could preclude stabilization of these analogs in the MHC-binding groove due to change in the nature of the charge at the ends; therefore, it is necessary to account for the charge reversal. Earlier attempts to design ri mimics of T cell epitopes by chemically converting the carboxylate group into amido and the amino group into carboxylate had failed to give desired binding (14). Therefore, we made an attempt to remove the repulsive interactions that might arise due to the charge reversal by simply neutralizing the charges on both the end groups—through acety-
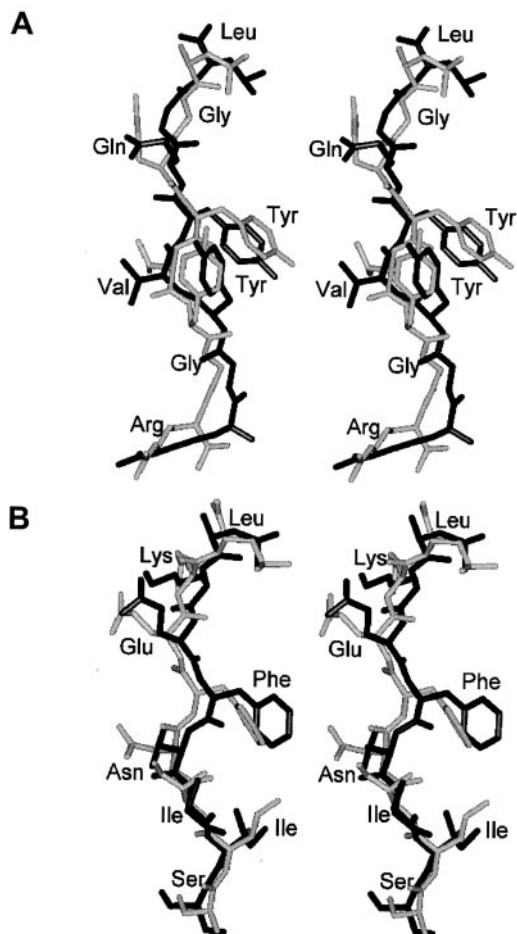

FIGURE 1. Structural comparisons of L and ri peptide analogs of the T cell epitopes. The structural alignment of the native and ri versions for VSVp (*A*) and OVAp (*B*) are shown. The native conformation is shown in black and the ri peptide in gray in stereo pairs.

lation of the amino group and the amidation of the carboxylate group.

It is anticipated that for an ri analog to functionally mimic the corresponding L peptide, it should exhibit the conformational propensities of the L peptide. To compare the propensities of the two peptides in solution, molecular dynamics simulation (15) was conducted in both the cases. The molecular dynamics analyses show that the distribution of the backbone rmsd values is comparable in the case of both native L and ri versions of each of the T cell epitopes (Fig. 3). The distribution is bimodal and the average of the rmsd values is also very close for the native and ri versions in the case of each of these peptides. The mode values for the rmsds, in case of OVAp and VSVp, were 1.92 and 1.72 Å, and for the corresponding ri versions were 2.02 and 2.07 Å, respectively. Therefore, the extent of change with respect to the starting conformation that the two versions undergo in solution is the same, indicating an inherent similarity in conformational flexibilities. The analyses of the Ramachandran plots have already shown that the conformations of the ri versions of the T cell epitopes that mimic the bound conformation of the native peptides are energetically possible. Thus, based on the similar conformational variability in solution as observed in molecular dynamics simulation and the accessible conformational space as inferred from the Ramachandran plot analyses, it is anticipated that the ri analogs should be able to adopt conformations similar to those of the corresponding native peptide, in case of the above two T cell epitopes.
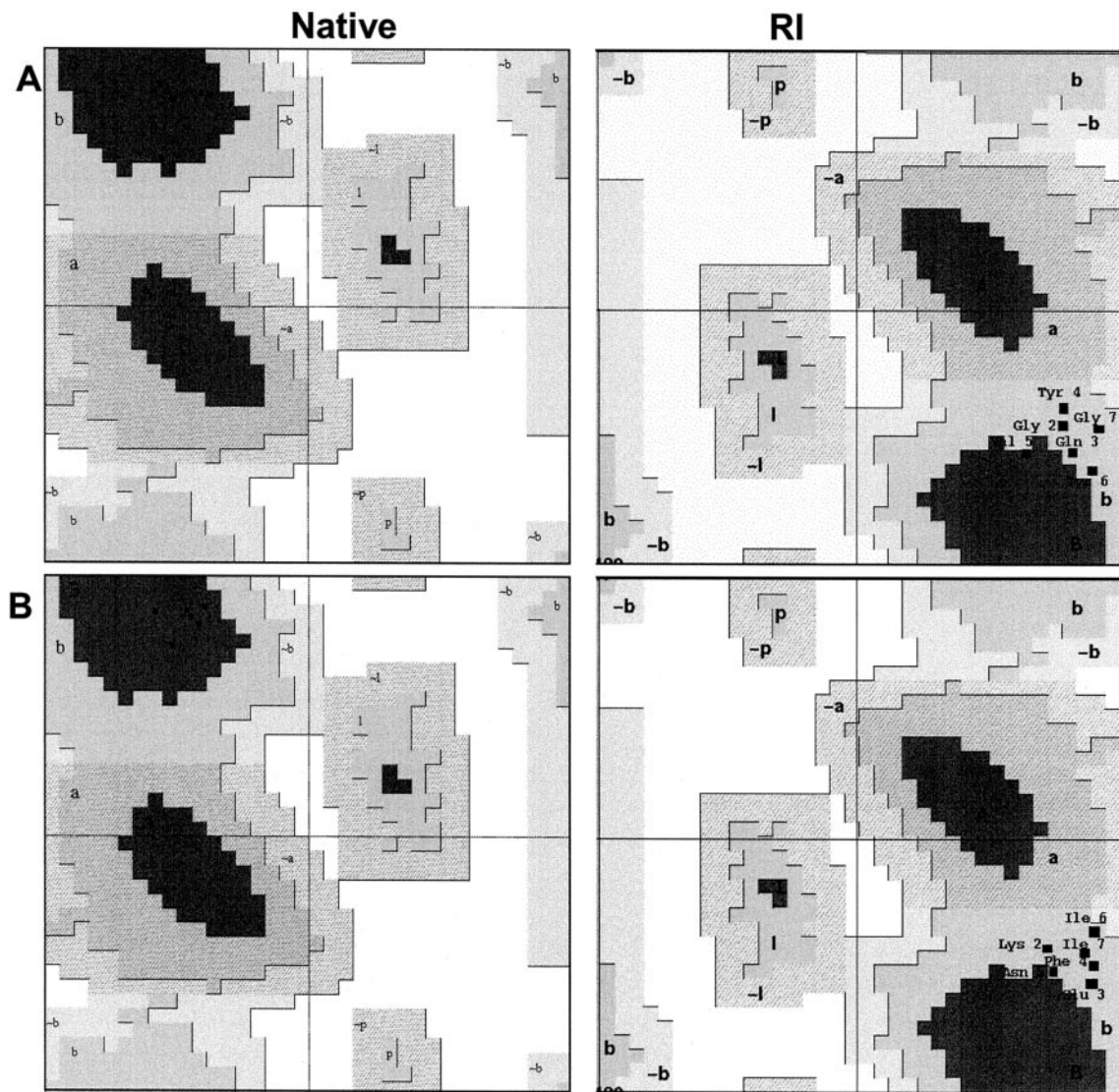
FIGURE 2. Conformational analyses of T cell epitopes in L and ri forms. The Ramachandran plot for VSVp and ri-VSVp (*A*) and OVAp and ri-OVAp (*B*) are shown. The core regions are shown in dark gray, allowed regions in gray, and generously allowed regions in light gray. The disallowed regions are shown in white.

In other words, in the same environment—such as the binding site groove of the cognate MHC molecule—the ri peptides can show conformational similarity with their parent native peptides. Consequently, the ri peptides might be able to functionally mimic the biological activity of their parent native L peptides.

Interactions of the two ri peptides (ri-VSVp and ri-OVAp) with MHCI (H-2K^b) resemble those of their native counterparts

Because the analyses of dihedral angles and conformational propensities showed that ri analogs could potentially mimic the native T cell epitope peptides, immunological assays were designed to test for this activity. The ri versions of OVAp and VSVp (ri-OVAp and ri-VSVp, respectively) with blocked N- and C-termini were synthesized and assayed for binding to the MHCI molecule H-2K^b. Both ri-OVAp and ri-VSVp, which had the N-terminal acetylated and the C-terminal amidated, bind with H-2K^b (Fig. 4). The dose-dependent binding of ri-OVAp is comparable to that of OVAp, while ri-VSVp binds with slightly more efficiency than VSVp. We had anticipated that the N- and the C-terminal unblocked versions of ri-OVAp and ri-VSVp should not be active as the changed polarity of the two ends might significantly affect binding. Indeed,

this is what was observed when the free peptides were tested for MHCI binding (Fig. 4).

Structural comparisons of MHCI molecules bound to cognate T cell epitopes have shown that the MHCI binding groove exhibits the presence of distinct structural pockets occupied by residues present at particular positions on the peptide epitope. To visualize whether the binding mode observed for native peptide in the crystal structures holds true for the ri analogs also, ri-OVAp and ri-VSVp were docked into the binding site of the H-2K^b molecule. The docking studies divulge that the mode of binding in the MHCI groove is similar for both native and ri versions of OVAp and VSVp peptides. Comparison of the computer-docked models of the ri-peptide-H-2K^b complexes and the crystal structures of the corresponding native L peptides complexed with H-2K^b reveal that the same peptide residues are present in the corresponding structural pockets (Fig. 5). Conformations of the peptide side chains overlap to a great extent and the backbones show an extended structure in the bound forms (Fig. 1). In the case of the MHCI molecule, the side chain and backbone conformations of the residues which form the groove also show considerable overlap.
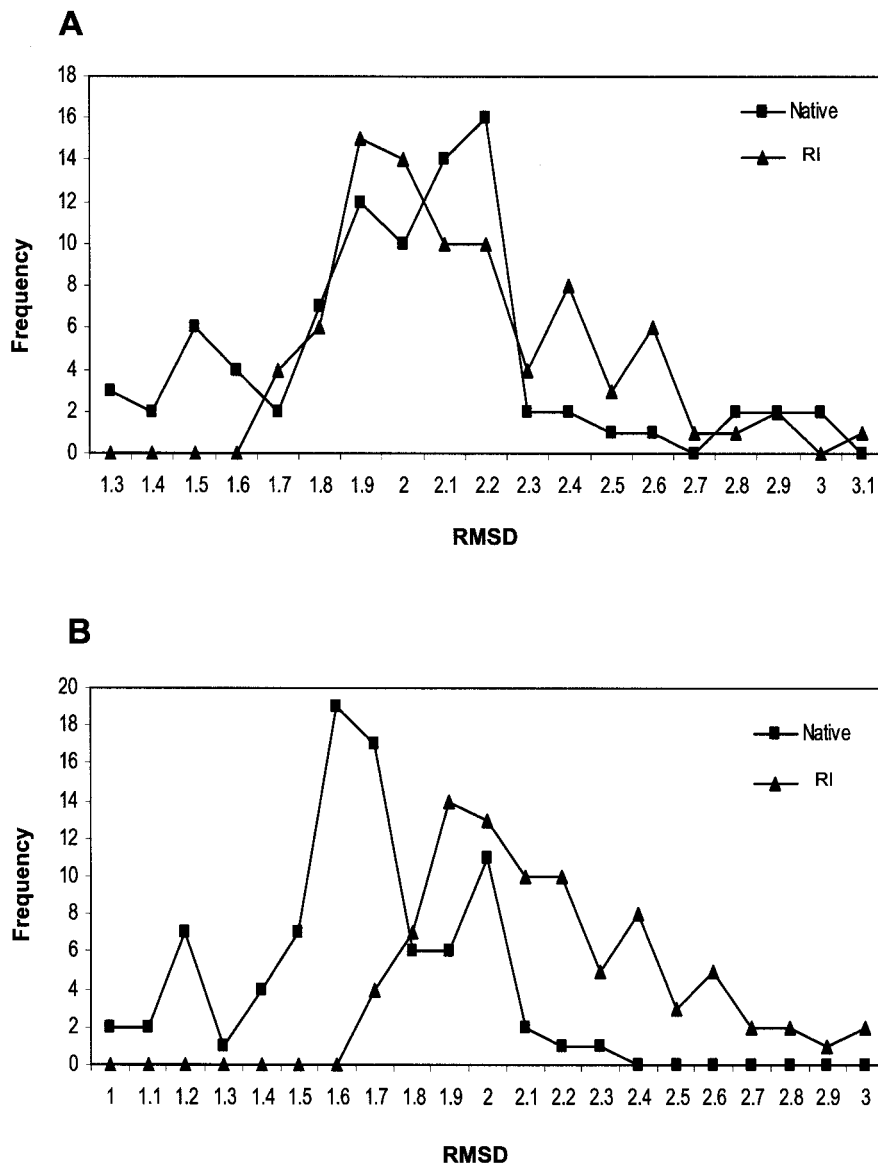
A

B

FIGURE 3. Conformational propensities of T cell epitopes in L and ri forms. The frequency distribution of rmsd values during a 500-ps molecular dynamics simulation run for VSVp and ri-VSVp (*A*) and OVAp and ri-OVAp (*B*) in an aqueous box.

The mimicry between the ri peptides and their corresponding native L counterparts is evident from the contacts between the MHCI molecule and the bound peptides. Apparently, common residues of the MHCI molecule interact with the corresponding residues on the two versions of the same peptide as seen in Tables I and II. In case of the VSVp:H-2K^b complex, a marginally higher number of contacts was observed compared with the case of the ri-VSVp:H-2K^b model. The number of contacts formed is substantially higher in case of the residues Arg1P, Gln6P, and Leu8P. The acetyl group present forms a significant number of interactions with the MHCI molecules. For the OVA peptide, it was observed that the number of contacts formed is nearly equal in case of the native and ri versions. The blocking acetyl group present forms many contacts raising the effective number of contacts in case of ri-OVAp slightly more than those of OVAp. Thus, the ri peptides and the corresponding native L peptides show similar contacts and the acetyl group of the N-terminal is closely interacting with the MHC molecule in both the cases.

The solvent accessible surface area buried on complexation is similar in the case of the two versions of the VSV peptides, VSVp and ri-VSVp, but shows some degree of difference in the case of

the OVA peptides, OVAp and ri-OVAp (Table III). The solvent accessible surface area buried in the case of the ri peptides is higher. One reason for this could be that the blocking acetyl and amide groups are not present in the case of the native peptide. The docking studies indicate that the ri versions of the VSVp and OVAp can bind the H-2K^b molecule as competently as the corresponding native peptides.

The in silico analyses suggested that the mode of binding of the ri versions of the two T cell epitopes resemble their L counterparts. To validate this experimentally, we designed two analogs of the ri-VSVp and tested for MHCI binding. As evident from the crystal structure of VSVp-H-2K^b complex (6), the residues of the peptide critical for H-2K^b binding are P3 (Tyr), P5 (Tyr), and P8 (Leu). These residues fit into the interior of the H-2K^b molecule with no appreciable surface exposure. In contrast, the side chains of P1 (Arg), P4 (Val), and P6 (Gln) are exposed on the surface of the complex. If the ri-VSVp interacts similar to the VSVp, then the D residues corresponding to the P3, P5, and P8 positions should be critical for H-2K^b binding and those corresponding to P1, P4, and P6 positions should not be particularly important for this binding. We designed two analogs of ri-VSVp with L residue substitutions

FIGURE 4. MHCI binding of the wild-type T cell epitopes OVAp and VSVp and of their ri analogs. H-2K^b levels on the cell surface were estimated for RMA cells and RMA-S cells incubated overnight with various concentrations of the indicated peptides. Results are shown as increase in MHCI levels on RMA-S cells as a percentage of the levels seen on RMA cells. Data are representative of five independent experiments. The ri-SIINFEKL(free) and RGYVYQGL(free) have free N- and C-termini. Wild-type L peptides also have free N- and C-termini. L amino acid is indicated by L in bracket preceding that residue.
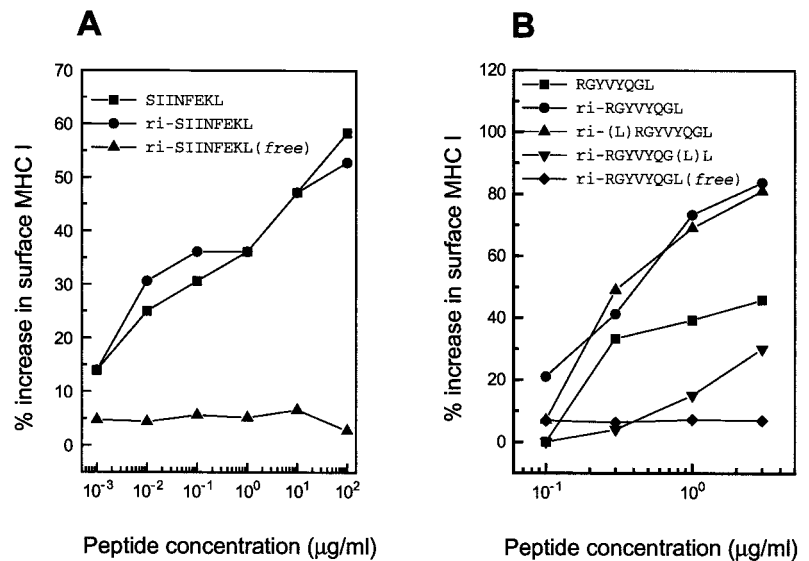
at positions P1 (L-Arg1-ri-VSVp) [ri-(L)RGYVYQGL] and P8 (L-Leu8-ri-VSVp) [ri-RGYVYQG(L)L]. As expected, the L-Arg1-ri-VSVp analog shows H-2K^b binding comparable to that of the ri-VSVp and the binding is almost completely abrogated in case of the L-Leu8-ri-VSVp analog in H-2K^b stabilization assays using RMA-S cells (Fig. 4). Thus, the experimental data of side chain interactions is consistent with the in silico interpretations.

The ri-OVAp and its rationally designed analogs functionally resemble their corresponding native L counterparts

The stereo drawings of the hydropathy features on the surfaces of the OVAp:H-2K^b and ri-OVAp:H-2K^b complexes as presented to the TCR are shown in Fig. 6. The almost precise correspondence of the surface features between the two cases is evident. The complexes of H-2K^b with native and ri versions of OVAp would thus present a similar surface for the cognate TCRs to recognize. The topological features and the localization of the regions of similar hydrophobicity are remarkably alike for the two versions of each peptide. Hence, it can be anticipated that the H-2K^b:OVAp and H-2K^b:ri-OVAp complexes will bind to the same TCR with comparable affinity. This is indeed the case, as seen from Fig. 7. It was evident that ri-OVAp and OVAp, the two versions of the peptide, possess similar abilities to stimulate an OVAp-specific T cell line.

The analysis of MHCI-peptide recognition has shown that residues at certain critical positions on the peptide make a major contribution to the free energy of binding. These positions, P3, P5/P6, and P8, are known as the anchor positions and the residues at these positions are called anchor residues. Mutational studies involving substitutions at these anchor positions are reported in the literature for OVAp (15). The functional consequences of these mutations, in terms of the nature of the mutation, have been extensively analyzed by T cell activation assays. To experimentally verify further that the mode of binding of ri-OVAp resembles that of the OVAp, the ri versions of some of these OVA peptide analogs were tested for biological activity using T cell activation assays, because recognition of peptide by peptide-specific T cells is dependent on both MHC binding and TCR recognition. These N- and the C-terminal protected ri-peptide analogs corresponding to the rationally designed changes at the critical positions were synthesized and assayed for T cell stimulation activity. It was observed unambiguously that for those L peptide analogs, which showed biological activity, their ri analogs were also active. In the

case of those L peptide analogs found inactive, the ri versions were also inactive. The substitution by similar residues in these anchor positions does not reduce the activity significantly, but substitution by completely different amino acid results in substantial loss in activity. For example, at P3 change of Ile to Tyr leads to loss of activity. At P5, Phe to Tyr results in not a significant loss, but Phe to Ala results in complete loss of activity. Similarly, at P8, Leu to Val results in small loss of activity, but the change Leu to Ala leads to complete loss of activity (Fig. 7). Also, the peptide having N- and C-terminal ends free, which provides exactly opposite charge in the ri version, does not show any activity at all. These observations are consistent with the activity profiles of the corresponding L analogs (16).

ri versions of the B cell epitope PS1 exhibits no conformational or functional correlation with its native counterpart

The 15-residue immunodominant peptide, called PS1, from hepatitis B virus surface Ag has been extensively characterized in terms of immune response and Ab recognition (8–10, 17, 18). To test whether the mimicry observed in case of the T cell epitopes also exists in case of the B cell epitopes, analogous computational and biochemical experiments were conducted using PS1. A 7-mer analog (QLDPAFG) of PS1 whose ends are blocked has been observed to completely inhibit the binding of PS1 to anti-PS1 Abs (data not shown). As far as Ab recognition is concerned, the 7-mer possesses all the functional properties of the 15-mer PS1, but would be less intensive to handle computationally. Therefore, all further computational and biochemical studies were conducted with this 7-mer peptide referred to as PS1 and the corresponding ri analog as ri-PS1.

Unlike the T cell epitopes, PS1 does not adopt an extended conformation when bound to any of the three independent mAbs with which it has been crystallized (9, 10). The stretch DPAF, which also represents an immunodominant epitope, adopts a β-turn conformation. In case of the native 7-mer analog, comparison of the Ramachandran plot shows that the distribution of ϕ and φ angles is localized to distinctly different regions for native and ri residues (Fig. 8A). Although the native peptide occupies the core and allowed regions corresponding to α-helix, some residues of ri analog occupy the allowed and generously allowed regions corresponding to the left-handed helices while others are found in the disallowed regions. Thus, in the case of the ri-PS1 peptide, the
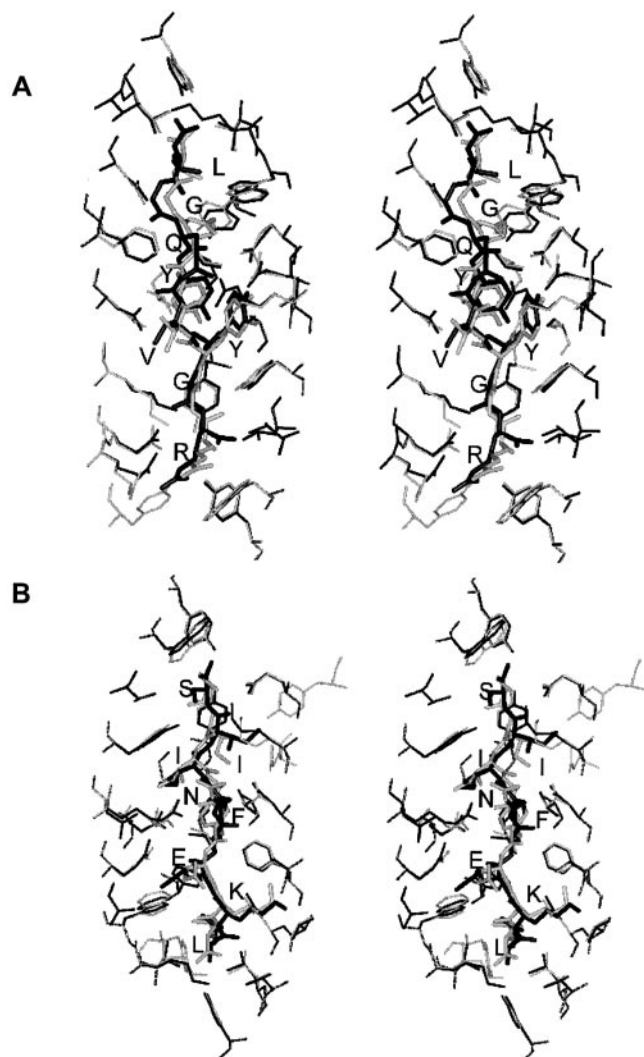
FIGURE 5. Comparison of MHCI-native and MHCI-ri peptide complexes. The residues of H-2K^b, which interact with VSVp and ri-VSVp (*A*) and with OVAp and ri-OVAp (*B*), are shown here. The MHCI-ri peptide complexes are shown in black and the MHCI-native peptide complexes are shown in gray in stereo pairs.

Table I. *Interaction table for native and ri versions of the VSV peptide with the H-2K^b molecule*

Peptide Residue	Interacting Residues With L Peptide	Interacting Residues With ri Peptide
Arg1P	Y159(4)	Y159(4)
	Y171(5)	Y171(6)
	K66(2)	K66(4)
	W167(7)	W167(5)
	R62(1)	R62(1)
	E163(6)	E63(3)
	L5(1)	Y59(4)
	T163(2)	
	Y7(8)	
Gly2P	Y159(1)	Y159(4)
	E63(3)	E63(3)
	Y7(3)	
	K66(4)	
Tyr3P	Y159(6)	Y159(7)
	L156(7)	L156(5)
	Q114(2)	Q114(1)
	R155(11)	R155(13)
	E152(4)	E152(5)
	N70(3)	
Val4P	N70(2)	N70(3)
	R155(3)	R155(2)
	K66(1)	K66(2)
Tyr5P	N70(6)	N70(7)
	S99(4)	S99(2)
	Y116(3)	Y116(3)
	F74(5)	F74(6)
	Q114(2)	Q114(3)
	V97(1)	V97(1)
	S73(2)	
Gln6P	E152(7)	E152(6)
	R155(2)	R155(2)
	D77(1)	
	Y116(1)	
	W147(3)	
	R155(1)	
	W147(2)	
Gly7P	D77(2)	D77(2)
	W147(2)	S73(3)
Leu8P	D77(6)	D77(10)
	T143(9)	T143(3)
	W147(1)	W147(3)
	L81(1)	L81(3)
	Y84(5)	Y116(1)
	K146(6)	
	Y116(1)	
Ace		Y84(5)T143(5)

conformation which would be a topochemical equivalent of the bound PS1 conformation is energetically unfavorable. Hence, the plots indicate that there might be a drastic difference between the functional properties of the native and ri versions of PS1.

To check this hypothesis molecular dynamics simulations of the peptides were conducted and it was seen that there is a considerable difference between the frequency distribution of backbone rmsds between native and ri analog (Fig. 8*B*). Additionally, the mode value for the native peptide was ~0.8 Å, whereas that for the ri analog was 1.3 Å. The ri analog exhibits a higher variation in its conformation than the native L peptide. Thus, the extent of conformational change that the two versions undergo in solution—compared with starting conformation—are considerably different indicating heterogeneous conformational flexibilities. The DPAF epitope in the native peptide adopts a β-turn conformation on Ab binding. It is stereochemically not possible for ri-PS1 to adopt a conformation similar to that of the bound PS1. This is evident from the Ramachandran plot analysis; the equivalent conformation for the ri analog lies in the disallowed region of the Ramachandran plot. Additionally, different extents of variability in the conformation, exhibited by the ri and the native peptides, imply heteroge-

neous conformational propensities for the two peptides. Differences in the conformational propensities might preclude the formation of this β-turn and consequently result in ri-PS1 not binding to anti-PS1 Abs. Hence, it is possible that the ri analog of PS1 might not be functionally active.

ri version of the B cell epitope PS1 does not bind to the anti-PS1 mAbs, consistent with their structural differences. The ri peptide analog was assayed by ELISA for its ability to compete with the coated native PS1 peptide to bind to the Abs. It was evident that the ri peptide shows negligible inhibition of binding to PS1 in case of all the three anti-PS1 Abs, PC283, PC282, and PC287 (Fig. 9). In the control experiments, the native 7-mer L peptide could show dose-dependent inhibition of the three anti-PS1 Abs binding to PS1. Thus, the ri version of the B cell epitope PS1 is not functionally active unlike the T cell epitopes VSVp and OVAp.

Discussion

It was anticipated that a ri peptide analog would adopt conformation similar to that of the corresponding L peptide. However, as is

Table II.   *Interaction table for native and ri versions of the OVA peptide with the H-2K^b molecule*

Peptide Residue	Interacting Residues with L Peptide	Interacting Residues with ri Peptide
Ser1P	W167(7)	W167(3)
	Y171(3)	Y171(4)
	E63(5)	E63(3)
	Y159(4)	Y159(3)
	K66(2)	T163(2)
Ile2P	Y159(2)	Y159(5)
	E63(1)	E63(5)
	K66(6)	K66(3)
	E24(5)	E24(4)
	Y45(1)	N70(4)
		K66(2)
Ile3P	Y159(11)	Y159(17)
	R155(2)	S99(2)
	L156(1)	Q114(3)
		L156(1)
Asn4P	N70(6)	N70(2)
	R155(6)	R155(6)
Phe5P	N70(7)	N70(7)
	Y116(3)	Y116(2)
	F74(3)	F74(5)
	Q114(3)	Q114(4)
	V97(1)	V97(1)
	S73(1)	
Glu6P	R155(2)	R155(2)
	E152(4)	E152(8)
	D77(1)	Y116(2)
		W147(4)
		A150(2)
Lys7P	D77(4)	D77(2)
	S73(5)	S73(1)
	W147(3)	
	K146(1)	
	V76(3)	
Leu8P	T80(1)	T80(1)
	D77(3)	D77(10)
	T143(9)	T143(2)
	K146(9)	L81(3)
	Y84(4)	W147(1)
	T143(7)	
Ace		Y84(3)
		K146(3)
		T80(1)
		Y84(6)
		T143(4)

Table III.   *Solvent accessible surface area buried in case of the native and ri- versions of the VSV and OVA peptide*

	Solvent Accessible Surface Area That Is Buried on Complex Formation	
Complex	Peptide	MHCI
VSV	1096	814
ri-VSV	1122	796
OVA	715	979
ri-OVA	1167	832

served, perhaps due to the stable intramolecular hydrogen bonding. In contrast, ri-PS1 showed significant conformational variability. Thus, the B cell epitope exhibits low structural plasticity and different conformational propensities for the native and the ri versions of the epitope.

Indeed, the functional data with regard to the immune epitopes analyzed in this study are consistent with the corresponding structural comparisons. Although ri-VSVp and ri-OVAp exhibit binding to H-2K^b, the MHCI that recognizes the two T cell epitopes, the ri version of the B cell epitope PS1 does not bind to the anti-PS1 mAbs. Because the conformational space of the T cell epitopes was found to be accessible to their corresponding ri analogs, it was expected that the ri analogs would adopt a conformation similar to that of their native counterparts while binding to MHCI. In that case, ri-VSVp and ri-OVAp ought to exhibit similar interactions while binding to MHCI molecule and while being presented to the TCR leading to functional mimicry. This is indeed the case, as was evident from the molecular docking analyses involving dominant conformations of the ri T cell epitopes (ri-VSVp and ri-OVAp) into the peptide binding site of H-2K^b and the T cell activation by ri-OVAp and its designed analogs. The binding interactions in the refined computer-docked models of the retro peptides (ri-OVAp and ri-VSVp) with H-2K^b substantially resemble the interactions of OVAp and VSVp with H-2K^b in the crystal structure. Also, the designed analogs of ri-OVAp resemble the corresponding native T cell epitope analogs in their T cell activation profile, implying that the functional role of different side chains of the ri peptide were equivalent to the corresponding L peptide. Thus, the ri mimics of the T cell epitopes used here exhibit pattern-based recognition involving a set of anchor residues similar to those defined for the corresponding native peptide while binding to MHCI molecule.

Even though the functional ri mimics could be designed for the T cell epitopes OVAp and VSVp, the same was not possible for the B cell epitope, PS1. One of the reasons for this could be the differences in the bound conformation of the peptide. The T cell epitopes and PS1 show an extended structure and a β-turn, respectively, when bound to their cognate receptors. This could imply that it would be easier for a ri analog to successfully mimic the extended conformation rather than a folded conformation with extensive intramolecular interactions. This is a plausible hypothesis considering that the extended conformation is more likely to be flexible and would provide substantial leeway in optimizing common interactions. However, there are two cases where it has been shown that the ri analog does possess turn-like structures similar to the native peptide (19, 20). In one of the cases, the presence of a number of glycine residues (three of nine) could be imparting flexibility to the two versions of the peptide allowing the conformational propensities to overlap (19). Overall, although the nature of the bound structure of the native peptide could be important in determining the success or failure of ri-antigenic mimicry, it would certainly not be the only criterion.
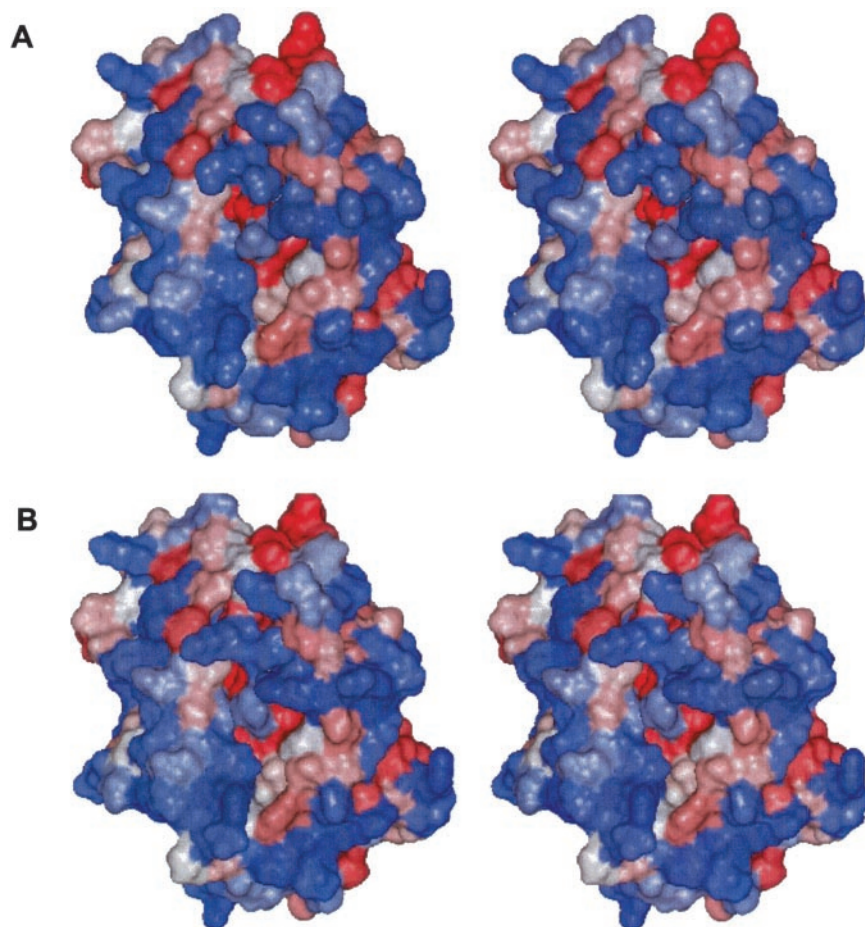
evident from our studies, based on the antigenic peptides, this need not be the case. Conformations of the ri peptide analogs—arrived at by the basic conformational transformations instrumental in ri mimicry—do not superimpose very well, as seen in case of both the T cell epitopes, OVAp and VSVp. Nevertheless, the φ-ψ angles of the native and the ri versions of these peptide Ags lay in the allowed regions of the Ramachandran plot. Additionally, both the native as well as the ri versions of these peptides exhibit substantial conformational plasticity. Therefore, it can be inferred that the conformational space of each of the native and ri peptides is mutually accessible, even though they do not have identical structures. In contrast, the ri version of the B cell epitope PS1 exhibits no significant conformational correlation with its native counterpart. The native conformation of PS1 is such that the corresponding equivalent conformation of the ri analog does not belong to the allowed region of the Ramachandran plot. It is also evident that while the native PS1 does not show any significant conformational plasticity, the ri peptide analog of PS1 exhibits substantial flexibility. No significant change with respect to the starting conformation in PS1 during molecular dynamics simulation was ob-

FIGURE 6. Comparison of surface features of H-2K^b-OVAp and H-2K^b-ri-OVAp complexes. The Connolly surface is shown in stereo, colored according to hydrophobicity for H-2K^b-OVAp (*A*) and H-2K^b-ri-OVAp (*B*). In the color spectrum used, blue indicates maximum hydrophilicity and red indicates maximum hydrophobicity.

Before our studies, many groups have reported design of ri mimics of immune epitopes. There are several instances of the ri version of a B cell epitope exhibiting similar immune response properties as those of the native peptide, while there are certain other instances where ri analogs did not show functional mimicry (21, 22). A critical aspect of designing ri analog of any peptide is the interchange of the N- and the C-termini of the peptide. In case of the MHCI molecule H-2K^b, for example, a significant role has been attributed to the N- and C-termini of the native peptides in binding to H-2K^b (13). The positive and negative charges at the N- and C-termini have been exploited to form stabilizing polar interactions with oppositely charged surfaces on the MHC surface. It was anticipated that the reversal of the termini could preclude stabilization of these analogs in the MHCI-binding groove due to repulsion between similarly charged surfaces. Therefore, it was considered necessary to compensate for this structural feature. Earlier attempts to design ri-T cell epitopes, involving VSVp, by chemically converting the carboxylate group into amide and the amino group into carboxylate through a major modification at D-Leu residue—replacing it with isobutyl malonic acid—had failed to give desired binding (14), perhaps due to the formation of racemic mixture during modification. In the present study, this issue has been addressed by simply neutralizing the charges on both the end groups acetylation of the amino group and the amidation of the carboxylate group. It was expected that any chemical modification of the termini would abolish some stabilizing hydrogen bonds made with the class I molecules but it would prevent repulsive polar interactions. There is one example of a naturally occurring T cell epitope with a chemical modification neutralizing the charge of the α-amino group. The MHCI molecule, H-2 M3, has been

shown to specifically bind to *N*-formylated mitochondrial and bacterial peptides (23). The fact that the blocked peptides ri-OVAp and ri-VSVp could successfully bind H-2K^b reveal that this is a simple and elegant solution to the problem of end modification.
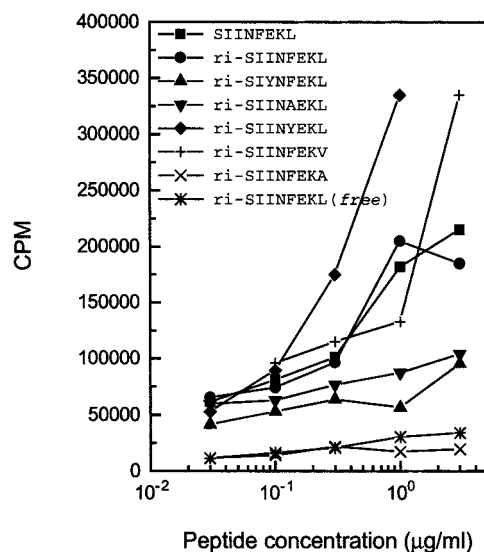

FIGURE 7. T cell activation by the T cell epitope OVAp, its ri counterpart, and its rationally designed ri analogs. The responses of the OVA-specific MHCI-restricted T cell line B3 to C57BL/6 (H-2-b) macrophages are shown in titrating doses of various peptides indicated. The data are representative of three independent experiments. The native SIINFEKL and ri-SIINFEKL(free) have free N- and C-termini.
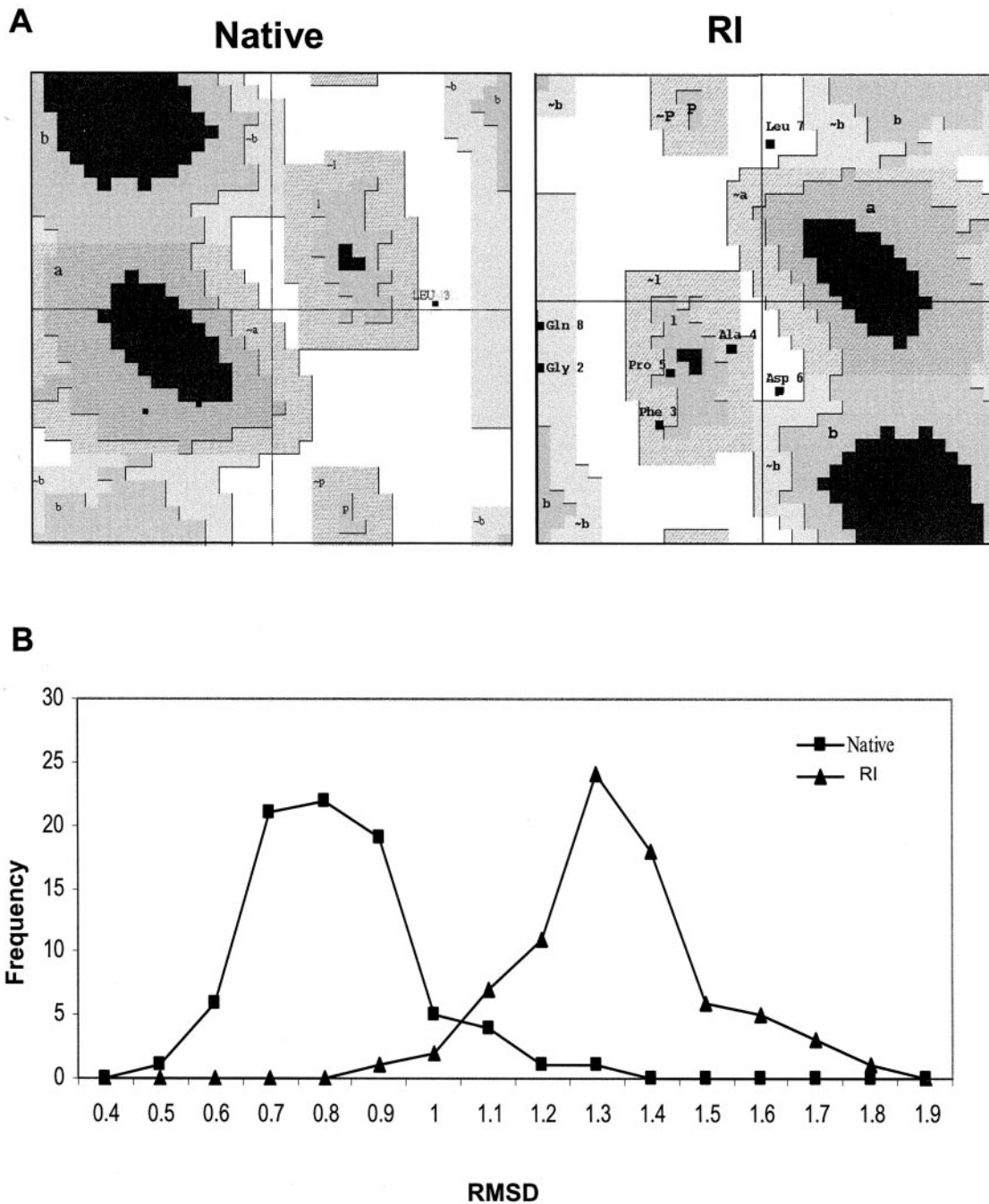
FIGURE 8. Conformational propensities of PS1 in L and ri forms. *A*, Ramachandran plot analysis for B cell epitopes. The Ramachandran plot for PS1 and ri-PS1 are shown here. The core regions are shown in dark gray, allowed regions in gray, and generously allowed regions in light gray. The disallowed regions are shown in white. *B*, Molecular dynamics simulation analysis. The frequency distribution of rmsds during a 500-ps molecular dynamics simulation run for PS1 and ri-PS1 in an aqueous box is shown here.

The manner in which B cell and the T cell epitopes are recognized by the immune system could also be relevant in the design of the immune epitope mimics. MHCI molecule recognizes peptides of diverse sequence with the sequence specificity being restricted to a few anchoring residues (24). The T cell epitope peptides bind MHCI in an extended conformation with a very specific set of critical residues that act as anchors suggesting that the side chain interactions are more crucial for MHCI-peptide recognition and the presence of appropriate residues at the anchor positions, rather than the backbone interactions, defines the binding specificity. In other words, both the MHCI molecule which recognizes the peptide epitope and the TCR to

which the MHCI peptide complex is presented exhibit a degree of degeneracy in peptide recognition. The higher level of conformational flexibility associated with T cell epitope described above combined with the pattern-based recognition property in MHCI binding, and presentation to TCR makes it possible for the ri peptide to adopt a functionally competent conformation and thus successfully bind to the MHCI molecule as well as activate the TCR.

Plasticity in recognition of T cell epitopes by the immune system appears to be instrumental in the successful antigenic mimicry exhibited by the ri analogs. In contrast, the recognition of the peptides by antipeptide Abs appears to follow a different strategy. The
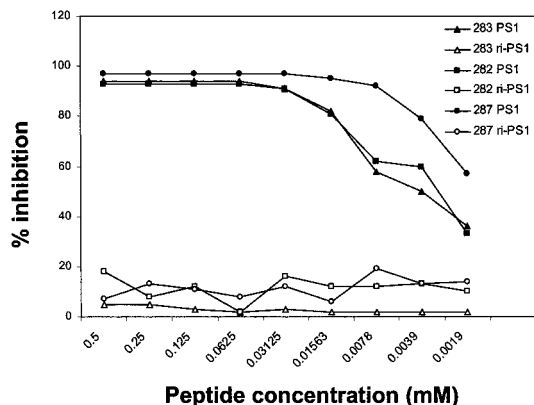
FIGURE 9. Ab binding of PS1 and its ri counterpart. The graph shows competitive inhibition of PS1 binding to PC283, PC282, and PC287 by the cognate B cell epitope PS1 and its ri analog using ELISA.

recognition is based on complementarity between Ag and the matured Ab as the binding interactions are optimized during the Ab response. No specific pattern of interactions exists in this case. The exquisitely specific recognition of Ag by Ab arises from the high complementarity in shape and charge between the Ag and the Ab-binding site. Thus, in the case of the peptide epitope-Ab interaction, optimization of complementarity between the peptide and the Ab appears to be a key feature. During maturation, Ab optimizes the paratope through somatic mutations, which are driven by a template of the paratope-stabilized conformation of the peptide epitope. In that sense, although a small number of residues are involved, it is hard to define any universal anchoring pattern. For the B cell epitope PS1 it must be remembered that the mAbs tested were part of a highly specific immune response focused on the immunodominant DPAF epitope. Because stepwise Ab maturation could lead to the presence of Abs of diverse levels of specificity, it is possible that in some cases ri mimicry can be observed. However, it must be said that the low level of plasticity in the epitope conformation and epitope-paratope interactions can significantly lower the possibility of functional B cell epitope mimicry.

Although our data provide clear evidence for the functional ability of ri peptide analogs of MHCI-binding epitopes, it is as yet unclear how complete the L and ri peptide equivalence would be in vivo. Thus, the next question for us would be whether our in vivo oligoclonal SIINFEKL-specific CD8 T cell response is efficiently recalled and/or triggered with full functional equivalence by ri-SIINFEKL. If this indeed were the case, it would lead to possible usage of long-lived ri peptides in the design of antiviral vaccines. However, our present data also indicate that the rate of success of using such ri peptides for B cell epitope mimicry may not be very high. Furthermore, the issue of MHC class II (MHCII)-peptide interactions also becomes interesting in light of our observations. It appears that the interaction of the MHCII-specific T cell epitopes with MHCII molecules depends substantially more on the peptide backbone and less on the side chains unlike in case of MHCI (26). Although the MHCI as well MHCII-specific T cell epitopes show similar conformational preferences, the above observation, in conjunction with our data, would suggest that ri-peptide analogs of MHCII-specific peptides may not be able to function as well as they can for MHCI. However, this would require experimental validation.

To summarize, while the T cell epitopes to MHCI could be mimicked by a ri analog, the highly specific binding of PS1 through a β-turn conformation could not be mimicked by a similarly designed ri analog. This is not surprising as the peptides interact with their corresponding protein receptors in a variety of different modes (25, 27), and that ought to be taken into account while designing peptidomimetics. Even though the postulated tenets for ri mimicry appear to be satisfied by B cell epitopes in earlier reports (28), the low level of plasticity in the epitope conformation and epitope-paratope interactions may have been the reason for not achieving the functional mimicry. In contrast, the inherent conformational flexibility in the peptide and the plasticity of MHCI-peptide interactions facilitated the functional mimicry in case of the two T cell epitopes. The observations made in this study highlight the differences in the mode of recognition for T cell and B cell epitopes consistent with the perception that while the key features of T cell epitope are pattern and plasticity, those of the B cell epitope are specificity and complementarity.

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