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Structural and Immunological Reactivity of the Principal Neutralizing Determinant V3 of Glycoprotein gp120 of HIV-1

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Abstract: The variable domain V3 in the outer glycoprotein gp120 of HIV-1 is a highly important region with respect to immune response during the course of viral infection. Neutralizing antibodies are produced against this domain; in addition, it has been shown to be a functionally active epitope for T helper and cytotoxic T cells. The high degree of amino acid variability in individual HIV-isolates, however, limits the use of the V3-domain in approaches to vaccine development. In order to characterize the residues important for antibody interaction and binding to MHC class I proteins, we constructed a consensus sequence of the V3-domain with broad reactivity [1] and used synthetic peptides derived from this consensus sequence with individual residues altered to alanine. These peptides were used as antigens in ELISA tests to define the amino acids which are important for binding to human and rabbit/anti-peptide immunoglobulins. In addition, we used these alanine-derived peptides in interaction studies with human HLA-A2.1 and mouse H-2D^d by testing their capacity to stabilize the respective MHC class I protein complexes on the surface of mutant cell lines T2 and RMA-S transfected with D^d gene. The experimental tests allowed us to define individual residues involved in antibody and MHC–protein interaction, respectively. In a further approach, we used those results to design interaction models with HLA-A2.1 and H-2D^d. Therefore, a structural model for H-2D^d was built that exhibits an overall similar conformation to the parental crystal structure of HLA-A2.1. The resulting interaction models show V3-peptide bound in an extended β-conformation with a bulge in its centre for both H-2D^d and HLA-A2.1 complexes. The N- and C-termini of V3 peptide reside in conserved pockets within both MHC-proteins. Anchoring residues could be determined that are crucial for the binding of the respective MHC class I haplotype. The cross-reactivity of V3-peptide in enhancing the expression of two different MHC class I molecules (H-2D^d and HLA-A2.1) is shown to be based on similar peptide binding that induces an almost identical peptide conformation.

Keywords: Human immunodeficiency virus; V3-domain; antibody reactivity; MHC–peptide interaction; molecular dynamic simulation

Abbreviations

CPU, central processing unit; HIV, human immunodefficiency virus; MD, molecular dynamics; MHC, major histocompatibility complex; r.m.s., root mean square; TcR, T-cell receptor

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INTRODUCTION

The V3-region of gp120/HIV-1 is immunologically and functionally one of the most important protein domains involved in the pathobiological course of virus infection and development of the acquired immune efficiency syndrome (AIDS) [2]. Neutralizing antibodies are mainly directed against the V3-domain [3] and progression of the disease in AIDS patients is correlated with V3-specific antibody titres [4]. The V3-region ranges from position 300 to 340 of gp120 and locates the conserved GPGR-motif in its

centre which is flanked by highly polymorphic positions on each side [5]. These highly variable sites seem to be critical for specific antibody binding, virus type-specific neutralization, and inhibition of syncytia formation. The N- and C-terminal ends of the V3-region locate two conserved cysteines that were shown to be covalently linked by a disulfide bridge and are thought to be important for the correct folding of this domain [6]. To date, little is known about the actual mechanism of how V3-specific antibodies trigger neutralization of HIV-1 infections. The most common hypothesis focuses on a sterical hindrance by antibodies bound to a proteolytic cleavage site within the V3-domain [7]. Cell surface-exposed CD26 molecules and serine proteases were recently shown to recognize and bind the conserved GPGR-motif [8, 9]. Cleavage of this region is thought either to stabilize the interaction of gp120/conserved region 4 with the CD4 receptor or to facilitate gp41-mediated membrane fusion [10, 11]. Binding of antibodies to the V3-domain would therefore inhibit structural rearrangements induced by proteases and protect the cell from being infected. In addition, the V3-region is known to serve as murine and human cytotoxic and T helper cell epitope, H-2D^d and HLA-A2.1 restriction could be documented [12, 13]. Furthermore, the V3-domain functions as target region for ADCC-response that can be included into a concept to eliminate HIV-infected cells [14]. In order to overcome the high sequence variability of the V3-protein domain to include it into a rational vaccine design, we synthesized a 36-mer consensus sequence oligopeptide (V3-C36) which was shown to react in a group-specific manner with isolate-specific antibodies and to exhibit neutralizing abilities [1]. Two-dimensional NMR analyses of V3-C36 resulted in an overall random coil structure, with a regular β-turn within the conserved GPGR-motif [15]. In order to study the functional reactivities involved in antibody binding, we synthesized a panel of 12-mer peptide derivatives spanning the central GPGR-motif with the original consensus sequence altered at each position to an alanine residue. These peptides were tested for their ability to bind monospecific antipeptide and patient's antibodies. The alanine-altered peptide derivates were further used to characterize the amino acid residues involved in binding to human and murine MHC class I proteins. This interaction is a prerequisite for the recognition of the self/foreign complexes at the surface of infected cells by cytotoxic T cells [16]. Mutant murine RMA-S [17] and human T2 [18] cell lines were used to identify residues within the antigenic V3-peptides responsible for binding the appropriate MHC class I protein (agretope). These cell lines harbour mutations in their peptide transporter, which renders them unable to form stable complexes between MHC proteins and endogenously processed antigens. Exogenously added peptides with affinity to a given MHC class I specificity can bind and thereby stabilize MHC complexes that can be detected as an enhanced expression of MHC proteins at their surface [17], thus being quantitatively detectable by fluorescence [19]. The data obtained from these experiments were combined with molecular dynamic simulations to study the structural properties that determine the antigenic quality of the V3-peptides. Here, several observations were included in our model-building. Crystal structures that were resolved for several different MHC class I proteins contain the antigen binding cleft with specific pockets for interaction with amino acid residues of an antigenic peptide [20–23]. Studies of biochemically isolated peptides reveal an optimal length of eight or nine amino acids [24]. Alignments of different naturally processed peptides indicate allele-specific binding motifs of antigenic/self peptides [25]. Recent crystallographic and NMR data show that peptide antigens are bound in an extended conformation in association with MHC class I [23, 26] and MHC class II antigens [27, 28].

In this work we successfully combined structural and model building data with experimental approaches to characterize the highly immunogenic V3-domain. These results should therefore allow the construction of an optimal V3-loop sequence which can be included into a straightforward design of vaccines against HIV.

MATERIAL AND METHODS

Peptide Synthesis

A series of 12-mer peptides were synthesized including the conserved GPGR-motif with the natural residues altered to alanine at each position (sequences of the peptides are listed in Table I). Additionally, the 9-mer RIGPGRAFV (V3-C9), the 10-mer RIGPGRAFVT (V3-C10) and the 16-mer RIRIGPGRAFVTIGKI (V3-C16) peptides were synthesized to map the minimal binding site for antibodies directed against the V3-consensus peptide (V3-C36). The peptide synthesis was performed with a 9050 PepSynthesizer (Milligen, Eschborn, FRG) using Fmoc (9-fluorenylmethyloxycarbonyl)-protected amino acids, as described earlier [29]. The peptides were purified by reversed phase high-performance liquid chromatography (HPLC) using C2/C18 copolymer

column (PepS, Pharmacia, Freiburg, FRG) and a gradient of 0–70% acetonitrile in 0.1% trifluoracetic acid. The fractions containing purified peptides were lyophilized and characterized by amino acid sequencing (Applied Biosystems, Weiterstadt, FRG). The synthesis of the V3-consensus peptide (V3-C36) was described earlier and contains the sequence QCTRP-NNNTR-KRIRI-GPGRA-FVTIG-KIGNM-RQA-HC-N [1].

ELISA Tests

In order to test the immunoglobulin binding capacities, the V3-peptides were used as antigens in ELISA assays. 200 ng of the respective peptides were coupled overnight in 0.2 M sodium carbonate buffer (pH 9.5) to 96-well microtitre plates (Maxisorb, Nunc GmbH, Mainz, FRG). Free protein-binding sites were saturated by 2 h incubation with 5 mg/ml gelatine solution (Sigma Chemicals, Munich, FRG). Before and after addition of the respective serum dilutions in PBS (0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4, 0.15 M NaCl; pH 7.2) for 2 h at 37 °C, the plates were washed several times with PBS containing 0.5% Tween 20.

The monospecific antipeptide sera were produced by inoculation of the V3-C36 consensus peptide in rabbits [1]. Human sera derived from asymptomatic HIV-infected individuals were kindly provided by Prof. Dr L. Gürtler from the diagnostic department of the Max von Pettenkofer-Institut, Munich, FRG. All sera were diluted in PBS. Human immunoglobulins were detected by rabbit anti-human IgG, diluted 1:1000 in PBS, rabbit immunoglobulins by swine anti-rabbit IgG (both were purchased from Dako, Hamburg, FRG). For staining, 100 µl of sodium phosphate buffer at pH 6.0 containing 1.0 mg/ml of *o*-phenylenediamin and 0.1% H_2O_2 were added for 10 min; the staining reaction was stopped with 100 µl of 1 M H_2SO_4. The optical density was determined at 492 nm.

Table 1 Alanine-substituted V3-peptides Tested for their Reactivities to Antibodies Raised Against V3-C36 and Their Effects on Expression of MHC Class I Proteins with Cell Lines .174/T2 and RMA-S (D^d transfectant)

Peptide	Sequence	Antibody reactivity (titre)	MHC class I expression HLA-A21 FI[a]	HLA-A21 %[b]	H2Dd FI	H2Dd %
V3-C12-1	**A**IGPGRAFVTTG	3.6×10^{-3}	1.4	−18	2.4	−64
V3-C12-2	R**A**GPGRAFVTIG	2.1×10^{-3E}	1.2	−29	3.5	−48
V3-C12-3	RI**A**GPRAFVTIG	1.2×10^{-3}	1.0	−41	2.5	−63
V3-C12-4	RIG**A**GRAFVTIG	1.2×10^{-3}	1.2	−29	4.2	−37
V3-C12-5	RIGP**A**RAFVTIG	1.6×10^{-3}	1.5	−12	6.3	−6
V3-C12-6	RIGPG**A**AFVTIG	1.2×10^{-3}	1.2	−29	3.4	−49
V3-C12-7	RIGPGR**A**FVTIG[c]	1.6×10^{-3}	1.7	0	6.7	0
V3-C12-8	RIGPGRA**A**VTIG	–	1.6	−6	6.2	−8
V3-C12-9	RIGPGRAF**A**TIG	2.6×10^{-2}	2.0	+18	5.9	−12
V3-C12-10	RIGPGRAFV**A**TG	–[d]	1.6	−6	4.5	−33
V3-C12-11	RIGPGRAFVT**A**G	8.5×10^{-2}	1.5	−12	3.1	−54
V3-C12-12	RIGPGRAFVTI**A**	–[d]	2.1	−24	3.5	−48
V3-C9	RIGPGRAFV	–[d]	0.8	−60	3.4	−43
V3-C10	RIGPGRAFVT	–[d]	0.9	−53	5.6	−16
V3-C16	RIRIGPGRAFVTIGKI	3.2×10^{-3}	1.5	−12	5.9	−12
V3-C36		2.6×10^{-4}	not tested		not tested	

The underlined tetramer sequence represnets the location of the GPGR-motif. Bold letters mark the site of substitution within the respective peptide sequence by an alanine residue.

[a] The results were calculated on the basis of fluorescence intensity (FI): FI_{sample}: $FI_{control}$, where 'sample' means peptide-treated cells and 'control' means cells without prptide.

[b] The increase or decrease of MHC class I protein expression has been calculated in comparison to 12-mer V3-consensus peptide (V3-C12-7), which was set to zero.

[c] 12-mer V3-consensus peptide.

[d] No reactivity found with sera diluted 1:10 (1×10^{-1}), undiluted sera showed unspecific reactions.

Cell Lines and Indirect Immunofluorescence Assay for MHC–Peptide Interaction

The two mutant cell lines RMA-S [30] and .174/T2 [18] were used for binding experiments with 12-mer alanine-derivated V3-peptides. For studying murine MHC class I response to V3-peptides, the H-2D^d gene was transfected into RMA-S cells, as described earlier [19]. Aliquots of 2.5×10^5 RMA-S or .174/T2 cells were incubated with peptide in 200 µl RPMI 1640 for 24 h at 37 °C. The final peptide concentration was 100 µM. MHC class I protein levels were detected by indirect immunofluorescence. The results were calculated on the basis of fluorescence intensity (FI), $FI_{sample}:FI_{control}$, where 'sample' means peptide-treated cells and 'control' means cells without peptide. Living cells were stained for MHC class I antigens by indirect immunofluorescence. The first layer was the 34-5-8S anti H-2D^d, mouse $IgG_{2a}K$ [31] or the BB7.2 anti HLA-A2.1 α1 domain, mouse IgG_{2b} [32]. All reagents were culture supernatants of hybridomas obtained from the American Type Culture Collection (Rockville, MD, USA), used at 10–15 µl/ml. The second layer was a fluorescein isothiocyanate (FITC)-labelled affinity purified rabbit anti-mouse antibody (Sigma Chemicals) at 1:20 dilution. Samples were analysed by cytofluorimetry using a fluorescence-activated cell sorter (FACS IV, Becton Dickinson).

Conformational Search

The conformational search for the analysis of alanine-derivatized peptides to explain their different immunological reactivities in ELISA tests, were performed according to the protocol of Mackay *et al.* [33]. The main idea was to search the conformational space for different 12-mer mutants to find preferred peptide conformers. Four selected alanine-altered peptides were built with graphical inspection in an extended conformation with the *append* option of the graphics program INSIGHT II 2.0.0 (Biosym Technologies, San Diego, USA), and subjected to high-temperature dynamics at 900 K. A 100 ps trajectory was recorded. The calculations were performed *in vacuo* and electrostatic charges were set to zero. After each picosecond (1000 steps), the resulting structure was cooled to 300 K followed by energy minimization with a convergence criterion of 0.002 kcal $mol^{-1}A^{-2}$. In order to avoid *trans* → *cis* transitions of the peptide bond, a force constant of 5 kcal $mol^{-1}rad^{-2}$ was applied during the high-temperature dynamics. The resulting 100 refined dynamical structures were saved and analysed in a cluster graph which compares every structure with each other, with less than 3.0 Å r.m.s. regarding their backbone coordinates (N, C^α, C and O). Structural families can be observed that are clustered along the diagonal of the graph. When individual conformers of each family are superimposed, conformational preferences of a peptide can be studied.

Generation of H-2D^d Model

Since no structural data were available from a single murine MHC class I molecule, we decided to build a model for H-2D^d on the basis of HLA-A2.1 crystal structure at 2.6 Å resolution [34]. The atomic coordinates of HLA-A2.1 structure were extracted from the protein file *3hla* of the Brookhaven Data Bank [35] and refined to remove crystal-packing forces. Using the UWGCG program ALIGN, we defined homologous regions between the two protein sequences. INSIGHT II 2.0.0 was used to interactively replace the amino acid residues within the energy-minimized crystal structure of HLA-A2.1 for positions different from those in H-2D^d. In order to eliminate steric clashes caused by the replacement operation, energy minimization was performed in water, keeping the Cα coordinates at their fixed positions. The refined H-2D^d model was subjected to docking experiments with 12-mer V3-consensus peptide.

MD simulation of Antigenic Complex

MD simulations were performed with the Consistent Valence Forcefield (CVFF) parameters from DISCOVER 27 (Biosym Technologies, San Diego, USA). Refined coordinates of HLA-A2.1 crystal structure (3hla.pdb) and H-2D^d model were used as starting conformations. The complex of 12-mer V3-consensus peptide with its respective MHC class I protein was solvated within a range of 5Å DISCOVER water molecules. The crystal water molecules Wat944, Wat946 and Wat947 located within the antigen binding cleft of HLA-A2.1 were included in the calculation. Others, outside this range were discarded for HLA-A2.1. All simulations were done under periodic boundary conditions to avoid artefacts at the protein–solvent interphase. The temperature during the MD run was kept at 300 K and the system was coupled to a heat bath with a relaxation time of t=0.5 ps. The overall time of the calculations covered 30 ps and took 317 CPU hours on a 4D/50 GT Silicon Graphics workstation. The first 15 ps were used to equilibrate the complex, which was monitored by defining the values for the total energy and

root-mean-square (r.m.s) for the backbone atoms (N, C^{α}, C and O) of the system. The last 15 ps were used for analysis. Coordinates, energies and velocities were recorded every 1000 steps (1 ps). The 12-mer V3-consensus peptide was initially built in an extended conformation with the *append* option of INSIGHT II 2.0.0.

RESULTS

Reactivities of Truncated and Alanine-mutated V3-peptide Derivates

In order to define the minimal binding site for antibodies directed against V3-C36, the truncated peptides V3-C10, V3-C12-7 and V3-C16 were used for mapping. Their reactivities were tested with monospecific antipeptide sera raised in rabbits against V3-C36, as described above. The tests (Table 1) showed that similar serum dilutions recognize the subfragments V3-C12-7 (1.6×10^{-3}) and V3-C16 (3.2×10^{-3}) in slightly reduced titres as the full length V3-C36 peptide (2.6×10^{-4}). Further truncation of the C-terminal positions I11 and G12 from V3-C12-7 to V3-C10 largely reduced reactivity. Therefore, the sequence RIGPGRAFVTIG was defined as the core sequence within V3-C36 to be recognized by antibodies. To further determine the amino acid positions of the core sequence involved in binding antibodies, ELISA tests using the alanine-derived 12-mer peptides including the GPGR-motif as antigens were performed. Table 1 shows the results of those tests. Alterations of the original amino acid sequence to alanine occurring in six positions in the N-terminal half of the 12-mer peptides did not influence the binding of antibodies with respect to V3-2-7 peptide. Neither an exchange at position P4 (V3-C12-4) to GAGR, nor mutations of the two positively charged arginines at positions R1 (V3-C12-1) and R6 (V3-C12-6) reduced antibody reactivity. Even a slight increase in antibody recognition could be seen when R1 (V3-C12-1) was altered to an uncharged alanine. Differences in antibody reactivity could be seen using peptides altered in the carboxy-terminal positions adjacent to the GPGR-motif. Thus, positions F8 (V3-C12-8), T10 (V3-C12-10) and G12 (V3-C12-12), respectively, strongly reduced binding of antipeptide immunoglobulins, bound antibodies could not be detected in ELISA-assays. These amino acids were therefore determined to be crucial for recognition by monospecific antibodies which are directed against the original V3-C36 consensus peptide. Substitutions at positions V9 (V3-C12-9) and I11 (V3-C12-11) reduced the reactivity only slightly.

Conformational Search Studies on Selected V3-mutations

For correlation of the different immunological reactivities of the alanine-derived peptides in the ELISA experiments with preferential peptide conformations the individual alterations may induce, conformational search analyses of selected alanine exchanges at positions P4, (V3-C12-4), F8 (V3-C12-8), T10 (V3-C12-10) and G1 (V3-C12-12) were performed. Fig. 1A shows the cluster analysis of the amino acid alteration of P4–shown to be of minor influence on antibody reactivity–which exhibited four structural families in a time course of a 100 ps trajectory. Interestingly, three of them (families I, III and IV) showed a β-turn in the GAGR-motif. Family II exerted a loop structure rather than a β-turn in the GAGR sequence and is generally more distorted than the other families. The flanking regions around the β-turn of families I, III and IV are oriented in an extended β-conformation. The stability of the β-turn even after a long MD simulation is documented with families III and IV that still exhibited a regular β-turn after 23 and 60 ps MD simulation. Therefore the exchange of P4 induced a similar β-turn structure as found in the original V3-consensus peptide [15, 36] which may explain the rather similar behaviour of both peptides in antibody binding.

In contrast to the P4/alanine exchange, cluster analyses of mutations at positions F8, T10 and G12 which had shown drastic loss in capacity to bind to antipeptide immunoglobulins, either destabilized the GPGR β-turn and/or formed additional loop structures in their carboxy-terminal region (Fig. 1B, C and D). Representative are families I, III and VI of F8/alanine; families III, IV and V for T10/alanine, and families II and IV for exchange G12/alanine. Here, the alternate loops were formed between the residues F8, V9, T10 and I11 which mainly represent highly hydrophobic residues. In addition, other families either can be observed to be completely disordered (family V for alteration F8/alanine; family II for T10/alanine) or showed a regular antiparallel β-sheet (families I of G12/alanine and T10/alanine).

V3-peptides Affecting the Amount of HLA-A2.1 and H-2D^{d} on .1741T2 and RMA-S Cell Lines

Alanine-derivatized peptides (V3-C12-1 to V-C12-12) were assayed for their ability to enhance the expression of HLA-A2.1 on .174/T2 and H-2D^{d} on RMA-S (D^{d} transfectant) cell lines. The enhancement of expression can be analysed as an indicator for the binding affinity of the particular peptide to its

respective MHC class I protein [19]. The quantified cytofluorimetric results (Table 1) indicated that V3-peptides are generally stronger bound by D^d molecules than by A2.1 proteins, e.g. V3-C12-7 elevated expression of H-$2D^d$ by a factor of 3.9 compared with the results with .174/T2. Furthermore, it could be observed that V3-C12-7 bound strongest among all other alterations in experiments with D^d protein, whereas (assayed with A2.1) a slight increase of binding could be observed when V9 (V3-C12-9; FI = 2.0) and G12 (V3-C12-12; FI = 2.1) were exchanged. Further shortening of the 12-mer peptide to V-C10 abolished complex formation to HLA-A2.1. V-C12-10 still interacted with H-$2D^d$ in contrast to V3-C9. Generally, no alteration of the original sequence to alanine abolished binding completely to either MHC class I molecule. In order to compare the binding properties of each variation with each other, the percentage of increase or decrease of H-$2D^d$ or HLA-A2.1 expression in both panels has been calculated in comparison with V3-C12-7. Therefore, the values obtained for V3-C12-7 were set to zero. Binding studies performed with .174/T2 (Table 1) indicated that V3-C12-7 peptide can be divided into two parts regarding its binding properties. Any exchange introduced in the N-terminal region and especially at positions R1 (V3-C12-1), I2 (V3-C12-2), G3 (V3-C12-3), P4 (V3-C12-4) and R6 (V3-C12-6) resulted in a loss of binding capacity up to 41% (V3-C12-3). In the C-terminal part of the peptide, a different situation could be observed. Exchanges at position F8 (V3-C12-8) and T10 (V3-C12-10) hardly reduced and at positions V9 (V3-C12-9) and G12 (V3-C12-12) even increased binding in comparison to V3-C12-7. Exchange at I11 (V3-C12-11) showed the greatest loss of binding in C-terminal part of the

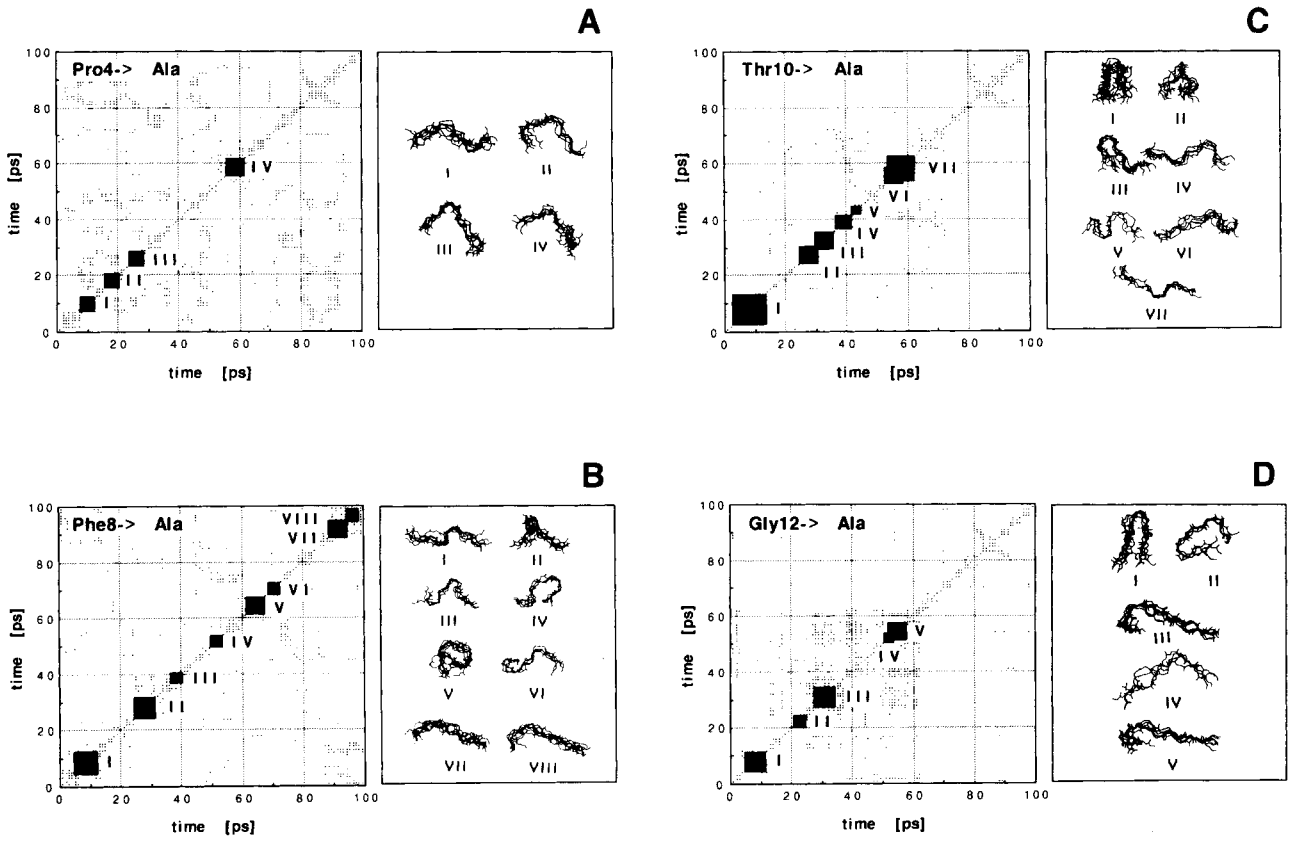

Fig. 1 Cluster graphs of selected mutations at positions of 12-mer alanine-substituted V3-peptides: A, proline 4 to alanine (V3-C12-4); B, phenylalanine 8 to alanine (V3-C12-8); C, threonine 10 to alanine (V3-C12-10); D, glycine 12 to alanine (V3-2-12). A trajectory for 100 ps at 900 K was recorded for each alteration. The graph compares the topologies of the obtained dynamical, refined and minimized structures in the range up to 3.0 concerning their backbone (N, C^{α}, C, O) root-mean-square (r.m.s.) values. Filled boxed regions within the graph represent structural families of a particular alteration. The individual structures of each family are extracted, superimposed on each other, and are shown adjacent to the graph. Labelling of structural families is according to the boxed area within the graph. The structures are oriented from left to right in N- to C-terminal direction.

peptide. In contrast to the situation with alanine-altered peptides interacting with .174/T2, every individual substitution reduced binding when assayed with the D^d transfectant of RMA-S cell line (Table 1). The N- and C-terminal residues showed the greatest loss in binding to H-$2D^d$, in particular positions R1 (−64%), I2 (−48%), G3 (−63%), P4 (−37%), I11 (−54%) and G12 (−48%). In the central region of the peptide, position R6 (V3-C12-6) reduced binding up to 49% when exchanged to alanine. Regarding both sets of experiments it could be observed that alterations introduced in R1, I2, G3, P4 and R6 and at residue I11 of the 12-mer consensus peptide largely reduced binding to both HLA-A2.1 and H-$2D^d$, although the extent was generally stronger when assayed with H-$2D^d$.

Model for Murine H-$2D^d$ Class I Protein

Model building of H-$2D^d$ was based on the observation that proteins of identical biological functionalities and high degrees in sequence similarity are also highly homologous and similar in structure [37]. This is well documented by various crystal structures of human [20–22] and, recently, murine [23] MHC class I proteins. Therefore, the model of H-$2D^d$ was built on the basis of sequence homology to the structurally known HLA-A2.1. Both sequences were aligned with each other from position 1 to 180 covering the entire antigen binding cleft consisting of the intramolecular $\alpha1/\alpha2$ domains each about 90 amino acids long [34]. Both proteins exhibit 67% sequence identity and 89% sequence similarity (Figure 2). No insertions or deletions between the two sequences can be observed. Note the presence of two cysteines at positions 101 and 164 within the H-$2D^d$ sequence which are highly conserved amongst all MHC class I proteins [20]. Both cysteines form a covalent disulfide bridge that connects $\alpha2$ helix with the bottomed, β-pleated sheet in HLA-A2.1 and is essential for the architecture of the binding cleft.

Molecular replacement and model refinement was performed, as described in 'Material and Methods'. The only *N*-glycosylated residue at position 86 within the $\alpha1$ and $\alpha2$ domain also present in all MHC class I molecules was replaced by a regular asparagine, since earlier studies indicated that this carbohydrate moiety is not essential for folding or function [38]. The resulting model of H-$2D^d$ had several exchanges in comparison to HLA-A2.1. These substitutions either occurred in the antigen binding cleft (L5M, V9F, Y22P, E24A, Y45M, P66K, A67V, N70H, S73T, E74H, I80T, A81L, W97R, A99Y and W114H), point upward from the helices (R62G, R79G, R83G, Q149A, G151H, E163T and Q169R), or are located at the 'interdomain' region to $\alpha3$ domain (D172L, K173E, N176K, A177E and L180Q). In addition, several exchanges can be observed outside the binding cleft within loops that connect two β-sheets (F17R, E41A, N42S, P43Q, A89E, G90A, E104G, G107W, L109F, C121K, K131R and T132S). After calculating a Connolly surface with a probe radius of 1.4 [39], we graphically examined the van der Waals surface of the H-$2D^d$ structure. Here, several depressions and pockets within the antigen binding cleft could be visualized that were basically similar to those found within HLA-A2.1, and given the same denotation (nomenclature of pockets according to [34]). Two pockets located at each end of the binding cleft could be observed in H-$2D^d$ that correspond to pockets A and of within HLA-A2.1. Pocket A is formed by the residues Y7, Y59, E63, P66, Y159, E163 and W167. In comparison to HLA-A2.1, this pocket is more negatively charged in H-$2D^d$ due to a substitution of T163 to E which can also be observed in other mouse haplotypes like H-$2D^b$ and H-$2K^d$. Pocket F consists of positions D77, I80, A81, Y84, F116, Y123, T143, K146, W147, and only three conservative substitutions can be found compared to HLA-A2.1 (T80 by I, L81 by A and Y116 by F). The conserved positively charged K146 is located on the top of this pocket and is surface-exposed in both proteins. Adjacent to pocket A, another subsite (pocket B) is formed by Y7, E63, P66, N70 and A99 that build the outer rim, and A67, V9 and Y45 that make up the inner wall. One major change that occurs in pocket B is the exchange from K66 (in A2.1) to a proline that leads to the disruption of a salt link between E63 and K66 at the top of this cavity. In general, pocket B is larger in H-$2D^d$ because bulky residues are substituted by smaller ones (F9 by V, V67 by A and H by N). Pocket C is built by V9, N70, S73, E74 and W97. This pocket is known to be highly polymorphic within MHC class I molecules which explains that every position with H-$2D^d$ is substituted in comparison to HLA-A2.1. Next to pocket C another cavity is formed by residues A99, W114, Y155, Y156, Y159 and L160 (pocket D). The main substitution occurring in H-$2D^d$ is the exchange of the positively charged histidine at position 114 (in A2.1) to a tryptophan which is located at the bottom of this pocket and points up the binding cleft. Position 114 is known to be polymorphic and is flanked by the conserved Q115 that points down and was shown to interact with W90 of the membrane proximal $\beta2$-microglobulin domain with-

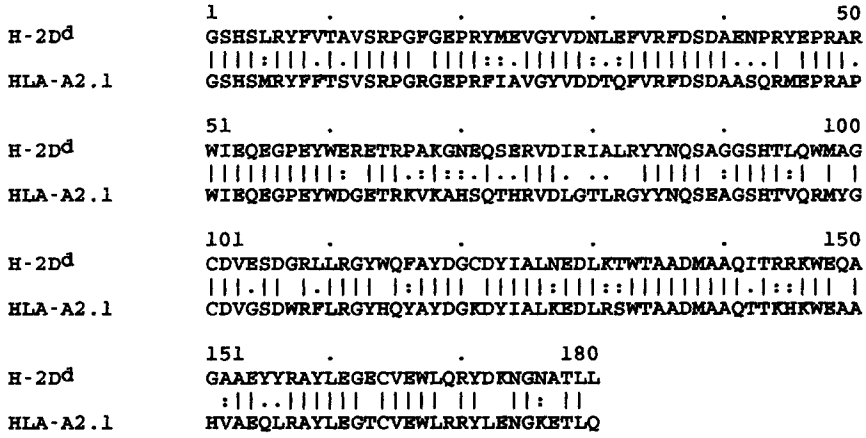

Fig. 2 Sequence alignment of murine H-2D^d with human HLA-A2. 1 in the region from 1 to 180 that defines the $\alpha 1/\alpha 2$ domain of MHC class I proteins. (I) marks identical residues, (:) indicates very similar exchanges and (.) represents weak homologies between residues. A gap defines no homology.

in the crystal structure of HLA-A2.1 [34]. A further shallow depression can be observed between pockets D and F that is formed by W97, W114, W133, W147 and A152 (pocket E). This pocket is entirely hydrophobic and consists of four tryptophanes of which W133 and W147 are conserved. The exchanges to tryptophan dramatically reduced the size of this subsite within H-2D^d.

MD Simulation of 12-mer V3-consensus Peptide Complexed with H-2D^d and HLA-A2.1

The immunological data obtained from the experiments using the mutant cell lines .174/T2 and RMA-S D^d transfectant identified either positions R1, I2, G3, P4, R6, I11 (with both cell lines), T10 and G12 (additionally for H-2D^d) to be important for binding to the respective MHC protein. The remaining residues G5, A7, F8 and V9 were regarded as spacer residues because they did not influence the interaction with either MHC protein. Docking experiments started with modelling the N-terminus into pocket A and the C-terminal end of peptide V3-C12-7 into pocket F of both MHC class I proteins. Additionally, I2 was fitted into pocket B of both MHC proteins shown to be large enough to accommodate a leucine or isoleucine residue in HLA-A2.1 and H-2D^d. Furthermore, pocket D was selected to harbour R6 in both complexes. The rest of the peptide chain was oriented accordingly to Ramachandran geometries concerning its main chain atoms (N, C$^\alpha$, C and O [40]). The MD simulations were performed accordingly to the protocol outlined above. The status after a 30 ps MD simulation is shown in Table II that lists the hydrogen-bond interactions found for both complexes; Fig. 3 shows models of the peptide–MHC complexes. Peptide V3-C12-7 complexed with HLA-A2.1 showed a total of 32 interactions. Seven interactions represented hydrogen bonds between HLA-A2.1 side chains and the peptide's main-chain atoms, four are salt bridges, and 19 contacts are made to the solvent. Two hydrogen bonds were formed to the crystal water molecule Wat 944 which was further hydrogen-bonded to D77 (OD1) of HLA-A2.1. This resulted in hydrogen-bond formation of peptide residues V9 and T10 with D77 of HLA-A2.1 mediated by Wat 944. In comparison to HLA-A2.1, the H-2D^d–peptide complex exhibited a total of 29 interactions where five contacts were made to main-chain atoms, four salt bridges and 14 contacts to solvent molecules could be observed. Comparing the hydrogen-bond pattern found within both complexes, the first and last two residues of V3-C12-7 could be shown to form similar contacts to the respective MHC molecule. These contacts were made to conserved residues (Y7, Y116, T143, K146, W147, Y159 and Y171) where the aminoterminal R1 (HN3) is salt-linked to E63 (OE1) and the carboxy-terminal group G12 (OXT) to K146 (NZ).

The rest of the peptide chain was mainly hydrogen-bonded to solvent molecules that account for the majority of contacts. A major difference between the two complexes was the formation of an intramolecular hydrogen bond between residues G3 and G5 of V3-C12-7 peptide that occurred with

H-2D^d. This resulted in the stabilization of the peptide itself and probably of the whole complex. The resulting structures of V3-C12-7 in complex with HLA-A2.1 and H-2D^d are shown in Fig. 3A and B. It can be seen that both peptide structures exhibit an extended β-conformation including a kink at positions G3, P4 and G5. The kink is surface-exposed and therefore solvent-accessible. Residue R1 is located in pocket A and its side-chain points upwards to form a salt bridge to E58. Positions I2 and R6 point into the antigen-binding cleft and reside in pockets B and D which anchors the peptide into its respective MHC protein. Additionally, I11 reached into pocket F within both complexes. Residues A7 (for both complexes), V9 (for H-2D^d) and F8 (additionally for HLA-A2.1) are oriented up the V3-peptide/MHC complex. Residues T10 and F8 (for H-2D^d) form intermediate topological positions. The overall length measured between the N- and C-terminal ends of bound V3-C12-7 was 25.3Å for HLA-A2.1, and 25.2Å for H-2D^d complex.

DISCUSSION

HIV-1 vaccine strategies to date have either been based on recombinant gp160/120 proteins or inactivated virus preparations. These approaches so far failed to induce group-specific immunity against HIV. In order to avoid adverse side effects such as antibody enhancement mediated by gp160 [41], we focused on immunologically well-defined epitopes

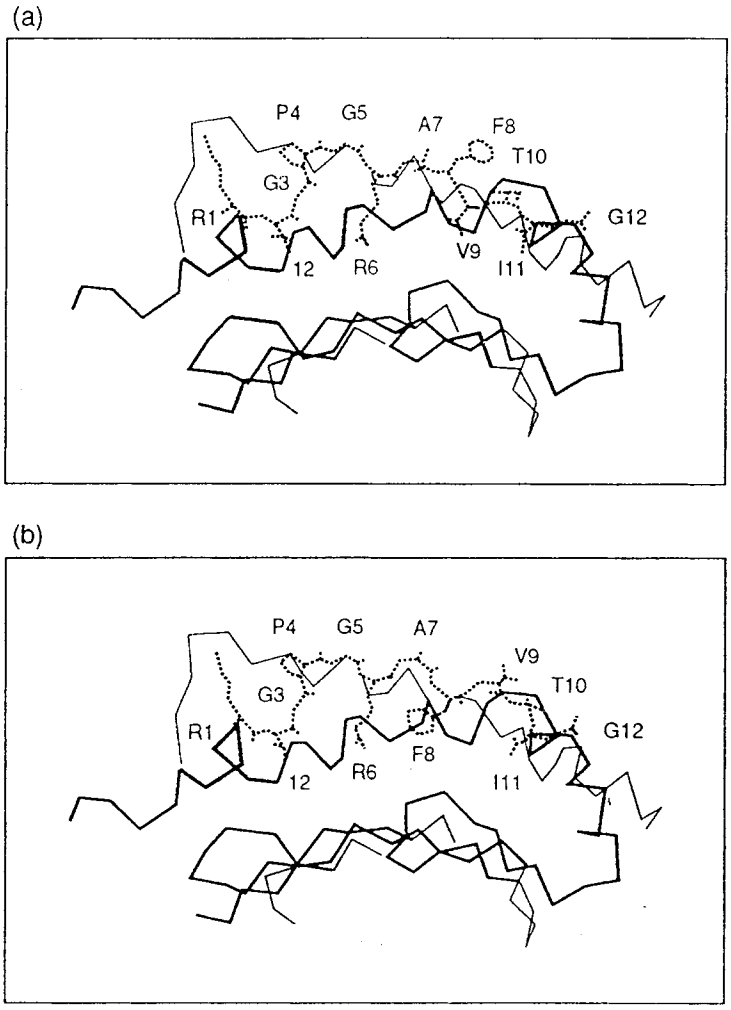

Fig. 3 Interaction model of V3-C12-7 peptide complexed with HLA-A2.1 (A) and H-2D^d (B). After 30 ps recorded trajectory at 300 K in water. Side views into the $\alpha 1/\alpha 2$ antigen binding cleft of both complexes. The C^{α} trace of the MHC protein (bold) and the heavy atom representation of the complexed V3C12-7 peptide (- - - -) is shown. The NH2-terminus of the V3-C12-7 peptide in each complex is located on the far left and the individual residues are labelled respectively.

Table 2 Peptide–MHC–Solvent Hydrogen-bond Network (<3.2 Å) of V3-2-7 Peptide complexed with HLA-A2.1 and H-2D^d after a 30 ps trajectory recorded in water at 300 K

V3-2-7 Positions		Interactions with HLA-A2.1/solvent	Interactions with H-2D^d/solvent
R1	–HN3	Y171 (OH); E63 (OE1)	Y171 (OH); E63 (OE1)
	–O	Y159 (OH)	Y150 (OH)
	–HN1	WTR 1043[a]	
	–HN2	WTR 933	
	–HE		WTR 170
	–HH11	WTR 1024	WTR 170
	–HH12	WTR 583	WTR 273
	–HH21	E58 (OE1)	WTR 273
	–HH22	WTR 991	E58 (OE1)
I2	–HN	Y7 (OH)	
	–O	WTR 1018	Y7 (OH)
G3	–O		WTR 189; G5 (HN)[b]
P4	–O		WTR 159
G5	–O	WTR 1021; WTR 777	
	–HN		G3 (O)[b]
R6	–O		WTR 790
	–HN	WTR 753	
	–HH11	Y155 (O)	
	–HH12	WTR 1090	
	–HH21	Q155 (O)	
	–HH22	WTR 1021	
A7	–HN		WTR 292
F8	–HN		WTR 778
	–O	WTR 663	WTR 315
V9	–HN	WTR 767	
	–O	Wat 944[c]	WTR 778
T10	–HN	WTR 745	D77 (OD1; OD2)
	–O	WTR 791	WTR 412
	–OG1	WTR 712; Wat 944[b]	
I11	–HN		WTR 512
	–O	W147 (HE1)	W147 (HE1)
G12	–HN	D77 (OD1)	D77 (OD1)
	–O	R97 (HH12); Y116 (OH) D77 (OD1)	Y84 (OH)
	–OXT	Y116 (OH); T143 (HG1); K146 (NZ)	Y84 (OH), T142 (HG1) K146 (NZ)

[a] WTR denotes DISCOVER water molecules.
[b] Intramolecular hydrogen bond between G3 and G5 of V3-C12-7 peptide.
[c] Crystal water molecule within HLA-A2.1.

such as the V3-region of gp120/HIV-1. Based on sequential alignments of different virus isolates, a 36-mer V3-consensus peptide (V3-C36) was synthesized that was shown to induce a broad virus type-specific immune reaction and promote neutralization [1]. The peptides V3-C10, V3-C12-7 and V3-C16 were used for mapping the specific recognition site for antibodies directed against V3-C36. ELISA tests determined the sequence RIGPGRAFVTIG to be the V3-core region for humoral recognition within V3-C36. V3-C12-7 and V3-C16 reacted with monospecific antipeptide sera in high titres, which were, however, reduced with respect to V3-C36, whereas V3-C10 showed drastically reduced recognition due to the truncation of the C-terminal residues I11 and G12. Another interesting aspect that emerges from these experiments questions the importance of the conserved disulfide bridge within the V3-domain that is thought to be important for correct folding and immunogenicity [42]. We showed V3-C12-7 and V3-

C16 to exhibit similar reactivities reacting with serum dilution higher than 1:1000 which indicates that the functional and structural properties within the core region are to a certain degree sufficient for antibody recognition (Table 1). These findings are further documented by NMR studies on the V3-C36 consensus [15] and similar peptides [36]. These data show, that β-turn structures can be found in shortened peptides spanning the GPGR-motif but do not include the terminal cysteines. Peptide V3-C36, however, containing the conserved cysteine residues reacted about 20 times better indicating that additional epitopes may be formed by the loop.

In order to study the structure–function relationship of the conserved GPGR-motif and its adjacent positions involved in specific antibody interaction, the core sequence was altered by alanine residues at each position. Alanine was chosen because it resembles the mildest means of removing an interaction beyond the β-carbon position without introducing disruptive or productive interactions. ELISA tests showed that alterations at the six positions in the aminoterminal half (R1, I2, G3, P4, G5 and R6) did not affect binding, whereas all other substitutions resulted in a huge loss in antibody recognition. Conformational search experiments were performed to find an explanation for this. Therefore, selected mutations were analysed to determine whether they induce preferential conformations that might be correlated with their different immunological activities. Alanine substitution at position P4 within the GPGR-motif showed a high population of a regular β-turn conformations, whereas substitutions at positions F8, T10 and G12 induced alternate loop structures formed by a cluster of hydrophobic amino acids. Our NMR data of the core sequence further show hydrogen bonding between the two glycines of the GPGR-motif and every second position in the carboxy-terminal half of the core region. These hydrogen bonds are either involved in stabilizing the β-turn and/or the adjacent residues F8, T10 and G12. One possible explanation is that substitutions with the potential to induce alternate loop structures destabilize the entire V3-peptide conformation and therefore reduce or inhibit antibody interaction. In contrast to the diverse capacities for interaction with monospecific V3-C36 antipeptide sera to alanine-altered peptide-derivates (Table 1), anti-V3 immunoglobulins in sera of human HIV-infected individuals showed a rather homogeneous reaction with the respective peptide derivatives (data not shown). This may be due to the fact that the V3-domain is continuously mutated in individual isolates during HIV-infection in the patient [43], thus exposing a high degree of various V3-sequences accompanied by minimal structure alterations that induce the formation of antibodies with slightly different reactivities. In comparison, monospecific antipeptide sera produced in animal systems recognize only one specific sequence since variations cannot occur.

Antibodies directed against the V3-region might preferentially recognize the β-turn conformation of the GPGR-motif and induce structural alterations in the adjacent amino acids. This would lead to the more general hypothesis that immunogenic peptides contain a relatively small 'core' region that is crucial in determining the specificity of antibody binding. Additionally, an outer area is needed to be structurally compatible with binding. This hypothesis is consistent with numerous experimental observations. First, the minimum length of immunizing peptides was shown to be only six residues long and replacement studies showed that in some cases as few as three amino acids are critical for raising antibodies against a particular peptide [44, 45]. Second, studies with antibodies raised against short peptides were shown to cross-react with their folded counterpart sequence in the native protein, which could also be demonstrated for the V3-region [46]. Third, spectroscopic NMR analyses showed that preferential conformations for ordered or nascent secondary structures like β-turns within short peptide antigens are indeed crucial for inducing antipeptide antibodies [47, 48]. Although it is still believed that peptides generally do not show ordered conformations, these findings strongly suggest that at least some immunogenic peptides indeed exhibit structural preferences. A more detailed understanding about the structural properties of V3-peptides might evolve from recent X-ray data [49], and therefore divulge valuable information for designing peptide vaccines inducing antiviral antibodies with group-specific reactivity and neutralizing efficiency.

In addition to its functional activity as B-cell epitope for the elicitation of HIV-neutralizing antibodies, the V3-region has been shown to serve as a human epitope for cytotoxic T cells in combination with HLA-A2.1 [13]. The recognition of the V3/HLA-A2.1 complex on the surface of HIV-infected cells may lead to their elimination by cytotoxic T-cells and thus reduce the virus load in the patients. This system has also been extensively studied in the mouse, since peptides derived from the V3-region were shown to interact with the mouse-specific MHC class I molecule H-2D^d [12]. This epitope is also

recognized by specific mouse CTLs when the sequence is inserted in the context of HIV-specific gag-proteins [50]. These hybrid proteins may serve as a potential vaccine, the possibility to test the reactivities in an experimental system renders the capacity of V3-peptides to bind to mouse H-2D^d to an important model for HIV vaccine development, also including cytotoxic T-cell response. In order to characterize the V3-domain as CTL epitope, 'peptide feeding' experiments and MD simulations were combined to determine how V3-peptide is bound in different complexes with murine H-2D^d and human HLA-A2.1. Our resulting models showed an extended peptide conformation in both cases. The first and last two residues are bound in a very similar manner as indicated by their hydrogen-bond contacts with the respective MHC protein. The side chain of arginine at position 1 points upward the antigen binding cleft and forms a salt bridge with E58 conserved in both MHC proteins. An exchange made at peptide position 1 by alanine would disrupt the salt link and therefore reduce binding of V3-peptide, which indeed corresponds to our immunological data (Table 1). Accordingly to our model, positions G3, P4, G5 and A7 are also shown to point their side chains up the antigen binding groove in both complexes. In contrast to R1, these residues do not make contacts to the respective MHC-proteins but instead several hydrogen bonds to the solvent can be observed. This seems to be contradictory to the observation that alterations, especially at positions G3 and P4, showed a huge loss in the binding assays. One possible explanation may be that residues like glycine or proline function as spacer residues and are responsible for introducing kinks or bulges into a peptide chain as described earlier [23, 26, 51]. Measurements of V3-C12-7 built in an extended conformation showed an overall length of 40.5 Å, whereas in the MHC-interaction complex it is reduced to 25.2 Å (with H-2D^d) and 25.3 Å (with HLA-A2.1). Therefore, any exchanges at positions G3 and P4 that do not promote kinks result in an elongated peptide conformation that prevents its residues from fitting adequate binding pockets within the MHC-protein, although containing the same number of residues.

This disputes studies indicating that the optimal length of T-cell epitopes is eight or nine residues [25]. That the optimal length for V3-C12-7 bound to H-2D^d is a 12-mer instead of a 9-mer peptide could be demonstrated in binding assays using peptides RIGPGRAFV and RIGPGRAFVT which reduce the amount of H-2D^d to about 50 (FI = 3.4) in comparison with V3-C12-7 peptide. According to our models, this find may be due to the inability to form a salt bridge between the C-terminal end of the truncated peptides with K146. That peptides longer than nine residues are also naturally presented has recently been shown by peptide eluation from T2 cells [52, 53] and crystallographic data [54]. Our models furthermore determine residues I2, R6 and I11 as anchoring residues within both complexes. Here I2 is located in pocket B, R6 in pocket D, and I11 reaches into pocket F. Any exchange or truncation made at these positions should introduce a most significant loss in binding. First, the salt bridge established between R6 and position 155 within the respective MHC molecule would be disrupted. Second, a reduction of van der Waals contacts would result by exchanging the strongly hydrophobic side chain if isoleucine at position 11 when exchanged to alanine. When both residues (R6 and I11) are altered to alanine we could observe a high reduction of MHC/peptide complex formation (Table I) not regarding exchanges of G3 and P4 that contribute to kink formation and R1 forming a salt bridge to E58. In addition, cytolytic assays using V3-C10, V3-C12-7 and V3-16 showed that residues I11 and G12 are essential for recognition of binary complex of the peptides with H-2D^d by CTLs [50]. Since binding of peptides to MHC proteins is the initial step for their recognition by the T-cell receptor, these observations support the importance of I11 and G12 in the V3-peptide region to act as specific agretop residues necessary for the correct orientation of the peptide chain.

Comparing our results to the proposed HLA-A2.1 restricted peptide motif that contains leucine/isoleucine at position 2 and valine at position 9 of a nonamer peptide [25] a similar motif can be observed in V3-C12-7. Here residues I2 and I11 have the same distance to each other as positions 2 and 9 in other nonamer peptides due to the proposed formation of the kink induced by G3 and P4. Additionally we propose a third anchoring position (R6) located in pocket D.

H-2D^d-specific peptide motifs have not been published until now. Our results indicate that hydrophobic residues at both ends (I2 and I11), and additionally a positively charged residue in the centre (R6) of a respective peptide are needed for specific binding to H-2D^d haplotype. Such an anchoring mechanism that induces a similar peptide conformation within HLA-A2.1 and H-2D^d complex might explain the cross-reactivity in elevating two different MHC class I proteins by V3-peptide. These findings might lead to the more general assumption that

cross-reactivity is correlated with a similar peptide conformation bound to a particular MHC protein. The inverse phenomenon where two different T-cell epitopes are bound in an almost identical mode in association with the same MHC class I molecule has been described [23]. For vaccine development these findings could represent the first straightforward step for a rational design of cross-reactive immunogens. Here, general features (N-terminus resides in pocket A and C-terminus in pocket F of a respective MHC protein) as well as the selection of specific anchoring positions for antigen presentations have to be included. Further studies still have to be done to characterize these complex biological and physiochemical reactions. This combinational approach of immunological binding assays coupled with model building may facilitate further characterization of the highly immunogenic V3-domain. The obtained results in analysing the V3-consensus peptide helped to define its role in antibody recognition, neutralization and cell-mediated response. Its broad virus type-specific and cross-reactive properties make it important that it be included in a potential vaccine for protection against a variety of HIV-isolates and therefore the development of AIDS.

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